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Quantitative Analysis of Multiple Charged Large Molecules in Human or Rat Plasma Using Liquid Chromatography Tandem Mass Spectrometry

Matthew Halquist
Virginia Commonwealth University

QUANTITATIVE ANALYSIS OF MULTIPLY CHARGED LARGE MOLECULES IN HUMAN OR RAT PLASMA USING LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

By

Matthew Sean Halquist
Bachelor of Science, Biology Track, Virginia Commonwealth University
Richmond, VA, 1998
Minors in Forensics Science and Chemistry, Virginia Commonwealth University

Director: H. Thomas Karnes, Ph.D.
Professor, Department of Pharmaceutics

Virginia Commonwealth University
Richmond, Virginia
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This work is dedicated to my wife and children for their unconditional love, patience, and support.

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ABBREVIATIONS

2D	two-dimensional
2D-LC	two-dimensional liquid chromatography
3-NBA	3-nitrobenzyl alcohol
Å	angstrom
Ab	antibody
ACN	acetonitrile
ANOVA	analysis of variance
APCI	atmospheric pressure chemical ionization
BSA	bovine serum albumin
CA	California
CAD	collision activated dissociation
CE	collision energy
CEM	channel electron multiplier
CXP	collision exit potential
Da	Dalton(s)
DIGE	Difference in gel electrophoresis
DRAD	denaturation, reduction, alkylation, and digestion
DP	declustering potential
EP	entrance potential
ESI^+	positive electrospray
FA	formic acid
FDA	Food and Drug Administration
GS1	gas one

GS2	gas two
H_2O	water
HCl	hydrochloric acid
HILIC	hydrophilic interaction chromatography
HLB	hydrophilic lipophilic balanced
HPLC	high performance liquid chromatography
HQC	high quality control
HRP	horse radish peroxidase
IA	Iowa
IF	infusion
IGF-1	insulin growth factor
IPA	isopropanol
IT	intratracheal instillation
IV	intravenous
kDa	kilodalton
LC-MS/MS	liquid chromatography tandem mass spectrometry
LC-MS	liquid chromatography mass spectrometry
LLOQ	lower limit of quantification
LQC	low quality control
MA	Massachusetts
MACsz	most abundant charge state
MAX	mixed mode anion exchange
MCX	mixed mode cation exchange

MeOH	methanol
MI	Michigan
mM	millimolar
MO	Missouri
MP A	mobile phase A
MP B	mobile phase B
MP C	mobile phase C
MQC	medium quality control
MRM	multiple reaction monitoring
MWCO	molecular weight cut-off
NPIF	no product ion formed
OXM	oxyntomodulin
PC-IDMS	protein cleavage isotope dilution mass spectrometry
pI	isoelectric point
PPE	protein precipitation extraction
PTH	parathyroid hormone
PYY_{1-36}	Polypeptide tyrosine tyrosine 1-36
PYY_{3-36}	Polypeptide tyrosine tyrosine 3-36
PTM	post-translational modification
Q1	first quadrupole
Q2	second quadrupole
Q3	third quadrupole
SI	subcutaneous injection

SPE	solid phase extraction
SRM	single reaction monitoring
STD	standard (calibration)
TFA	trifluoroacetic acid
TMB	3,3′,5,5′-tetramethylbenzidine
UPLC	ultra pressure liquid chromatography
WCX	weak cation exchange

ABSTRACT

QUANTITATIVE ANALYSIS OF MULTIPLY CHARGED LARGE MOLECULES IN HUMAN OR RAT PLASMA USING LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

By Matthew Sean Halquist, B.S.

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Major Director: H. Thomas Karnes, Ph.D.
Professor
Department of Pharmaceutics, School of Pharmacy

Immunoassays have traditionally been employed for the determination of plasma concentration-time profiles for pharmacokinetic studies of therapeutic proteins and peptides. These ligand binding assays have high sensitivity but require significant time for antibody generation (1 to 2 years) for assay development. Despite high sensitivity, these assays suffer from cross-reactivity that can lead to inaccurate results. As an alternative to immunoassays, this dissertation was focused on the development and validation of assays that can be used for quantitative analysis of peptides or proteins in plasma using liquid chromatography tandem mass spectrometry (LC-MS/MS).

Two approaches were considered for measurement of proteins and peptides fortified in plasma. The first approach involved employing signature peptides as quantitative surrogates of a target protein. This approach is a multistep process that includes: computer simulated (*in silico*) peptide predictions, protein purification, proteolytic digestion, peptide purification, and ultimately mass spectrometry. Signature peptides were determined through *in silico* peptide predictions and iterative tuning processes to represent Amevive® (Alefacept), a therapeutic for psoriasis, for quantification in human plasma. Horse heart myoglobin was chosen as a protein analogue internal standard to compensate for errors associated with matrix effects and to track recovery throughout the entire sample pretreatment process. Samples were prepared for analysis by selective precipitation of the target proteins with optimized pH and heat conditions followed by enzymatic digestion, dilution, and filtration. Combining selective precipitation and protein analogue internal standard lead to a method validated according to current FDA guidelines and achieved a linear range (250-10,000 ng/mL) suitable for monitoring the therapeutic levels of Alefacept (500 -6000 ng/mL) without the use of antibodies.

A second approach exploited the mass spectrometric behavior of intact polypeptides. A polypeptide can exist in multiple charge states separated by mass to charge ratio (m/z). Herein, the charge state distribution and the formation of product ions to form selected reaction monitoring (SRM) transitions for intact polypeptide quantitative analysis was evaluated in plasma. Oxyntomodulin, a 37 amino acid anorectic peptide (4449 Da), was employed as a model for analysis in rat plasma. The +7 charge state form of OXM was used to form an SRM for quantitative analysis. Two-dimensional reversed phase ion pair chromatography, a modified solid phase extraction, and a multiply charged SRM of oxyntomodulin enabled a lower limit of

quantification of 1 ng/mL. Following development of the LC-MS/MS method, a validation of this approach was performed according to FDA guidelines.

Finally, to show further utility of LC-MS/MS, the validated oxyntomodulin method was used in a pharmacokinetic study with sprague-dawley rats. Rats were dosed with oxyntomodulin through intravenous or intratracheal instillation routes of administration. Plasma concentration-time profiles were determined. Using these profiles, noncompartmental parameters were determined for each dose and routes of administration.

CHAPTER 1

INTRODUCTION: QUANTITATIVE LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY ANALYSIS OF MACROMOLECULES USING SIGNATURE PEPTIDES IN BIOLOGICAL FLUIDS

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1.A INTRODUCTION

The last two decades of proteomics investigations have fueled a need to transform qualitative research into quantitative analysis of macromolecules. Exploration of the human proteome has provided information about potential biomarkers and protein therapeutics and we are now in need of translation to quantitative measurement for these important proteins. Approximately 130 peptide or protein therapeutics have been approved by the Food and Drug Administration (FDA) (Leader et al., 2008). Continuing this trend will require quantitative methodologies that have been proven to be sensitive, selective, and reliable. Historically, immunoassays such as enzyme-linked immunosorbent assays (ELISA) have been employed for the quantitative determination of peptides and proteins in biological matrices. Unfortunately, the obstacles associated with this technique are significant. Method development time can exceed 1 year for antibody screening and production, and costs range from $100,000 to $2,000,000 (Whiteaker et al., 2007). Furthermore, the inability to produce multiple antibodies for selective recognition in sandwich immunoassays can result in falsely elevated results due to cross reactivity. This is particularly a problem in drug development due to drug metabolites which may

or may not be active but that resemble the target protein or peptide very closely structurally. The use of liquid chromatography tandem mass spectrometry (LC-MS/MS) has been established as the gold standard for small molecules and is gradually developing into an attractive alternative for large molecule quantification. The limited range of a triple quadrupole mass spectrometer typically requires proteolytic digestion of the target protein to be carried out followed by quantification of selected signature peptides. These signature peptides are employed as surrogates for the protein of interest for quantification purposes. Since Gerber et al coined the absolute quantification (AQUA) strategy in 2003(Gerber et al., 2003), employing signature peptides for protein quantification has grown significantly. A key advantage with this methodology over immunoassays, in addition to the selectivity advantage mentioned earlier, is the ability to more easily quantify multiple proteins, and therefore provide simultaneous biomarker screening. This technique uses the selectivity provided by a mass spectrometer through scanning multiple precursor ions and their product ions concurrently, which is known as multiple reaction monitoring (MRM). Figure 1 represents the overall process of multiple reaction monitoring of signature peptides in a triple quadrupole mass spectrometer using a horse heart myoglobin signature peptide as an example (peptide transition sequence: LFTGHPETLEK → ETLEK [+2/y6] or 636→716).

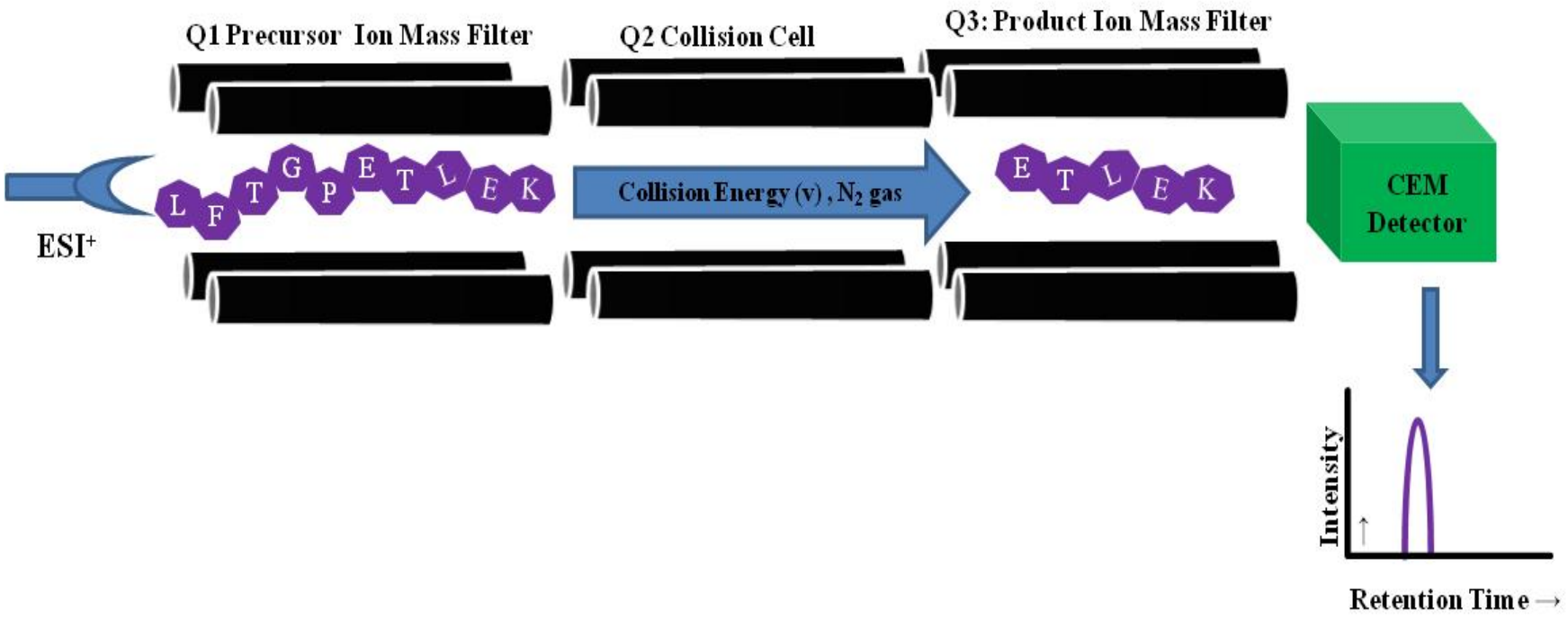

Figure 1.1 Example of an MRM transition (LFTGHPETLEK → ETLEK) of horse heart myoglobin signature peptide in a triple quadrupole mass spectrometer. Note: ESI^+ is positive electrospray ionization, CEM = channel electron multiplier.

In silico processes can predict the peptide sequence, charge state of the signature peptide, and product ions, which will be discussed in more detail later. Following optimization of tuning parameters for the ionization source and mass analyzer, samples are injected into the sample inlet to undergo ionization typically using positive electrospray ionization (ESI^+). Following ionization, the myoglobin signature peptide will be mass filtered by scanning only for the precursor and product ions. The first quadrupole (Q1) mass filters the myoglobin precursor ion (LFTGHPETLEK, or 636). The second quadrupole (Q2), also known as the collision cell, focuses and transmits the ions while introducing a collision gas and energy (v) and therefore causing fragmentation of the precursor ion. The third quadrupole (Q3) serves to analyze the fragment ions generated in (Q2). Finally the ions which have been simultaneously scanned reach the detector, normally a channel electron multiplier, and computer data are generated.

Multiple reaction monitoring based measurements of proteins has emerged as a promising technology for biomarker validation and pharmacokinetic studies for biologics (protein therapeutics) in biological fluids. Most of these studies are performed with blood plasma as the matrix of choice because majority of proteins exist in plasma, and nearly all cells in the body communicate chemically through plasma (Anderson et al., 2004). The complexity of plasma, however, which contains many proteins with a broad concentration range of approximately ten orders of magnitude (Anderson et al., 2004), requires extensive purification. In order to achieve maximum sensitivity and selectivity, purification of the target protein and /or its signature peptides should be considered. Selection of signature peptides is the most critical process in order to obtain a unique peptide(s) to represent the target protein. Integration of these processes results in hyphenated techniques needed to ultimately quantify target proteins. There are actually seven critical steps associated with signature peptide based quantification of

proteins. These seven steps as illustrated in Figure 1.2 are: 1) *In Silico* signature peptide prediction and modeling 2) *In silico* MRM peptide transition modeling coupled with real mass spectra 3) protein purification 4) enzymatic digestion 5) signature peptide purification 6) incorporation of an isotope labeled internal standard peptide or protein 7) quantitative LC-MS-MS using MRM transitions for signature peptide(s) and internal standard(s).

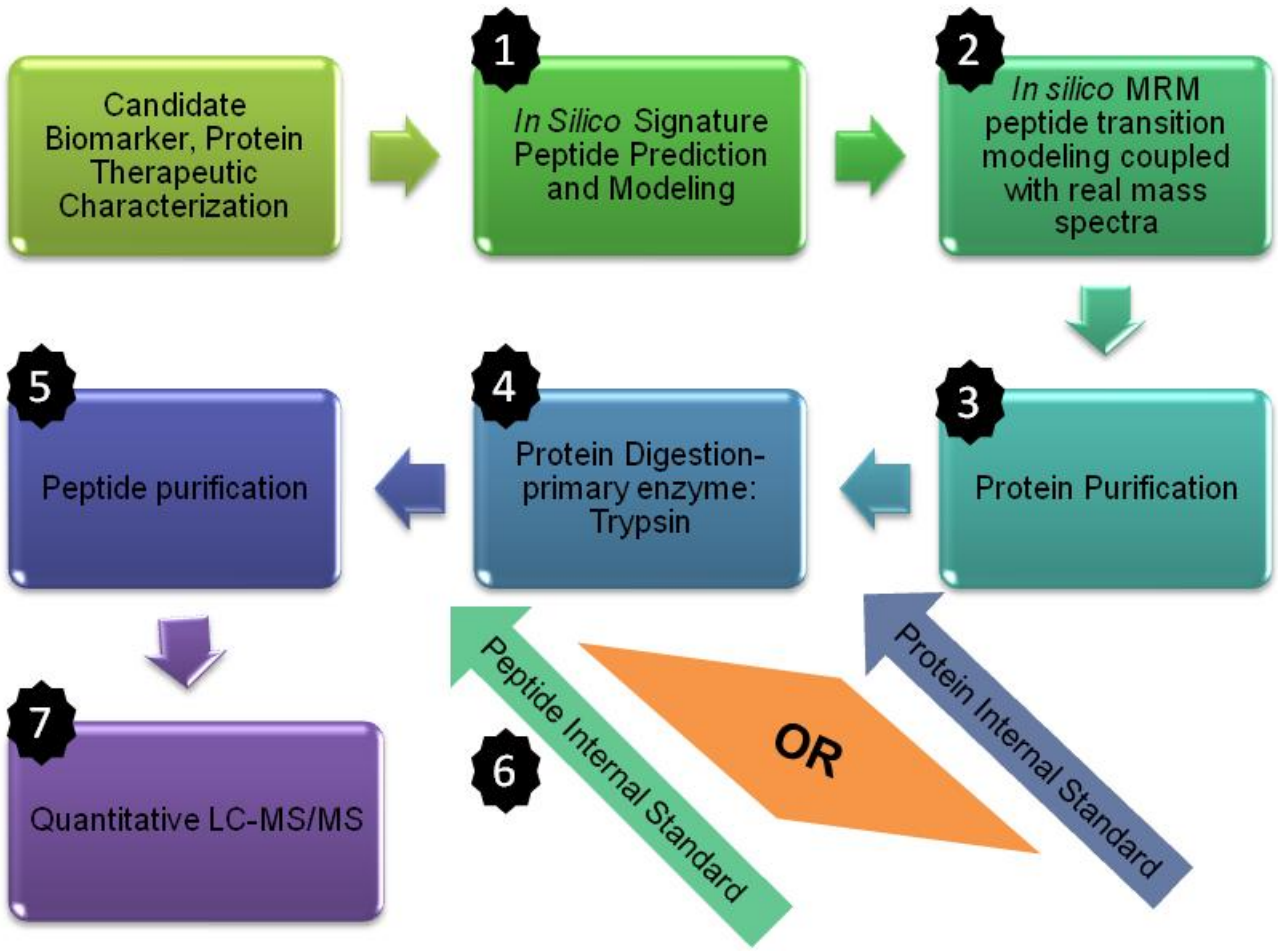

Figure 1.2. Hyphenated techniques are illustrated as a seven step process: 1) *In Silico* signature peptide prediction and modeling 2) *In silico* MRM peptide transition modeling coupled with real mass spectra 3) protein purification 4) enzymatic digestion 5) signature peptide purification 6) incorporation of an isotope labeled internal standard peptide or protein 7) quantitative LC-MS/MS using MRM transitions for signature peptide(s) and internal standard(s).

1.A.1 SCOPE

The scope of this introduction will be related to current strategies to achieve maximum sensitivity and selectivity for the quantification of macromolecules using a variety of sample preparation techniques coupled to triple quadrupole mass spectrometers. Additionally, approaches such as protein cleavage isotope dilution mass spectrometry (PC-IDMS), immunoaffinity purification of proteins, and immunoaffinity purification of peptides will be discussed and representative applications that contain quantitative analysis will be shown.

1.B SIGNATURE PEPTIDE SELECTION

Selection of signature peptides has been thoroughly discussed previously (Kirkpatrick et al., 2005; Mallick et al., 2007; Picotti et al., 2008; Sherman et al., 2009a; 2009b) .Nonetheless, it is the most critical of the steps in quantitative analysis of target proteins and it is necessary to provide an overview of the process. Multiple steps are necessary in order to use surrogate peptides for quantitative analysis of proteins. Initially, the selections of surrogate peptides are predicted through modeling and proteomic experimental data. Algorithmic steps produce theoretical cleaved peptides following an in silico digest. The serine endopeptidase trypsin is normally selected for enzymatic digestion due to its selective hydrolysis of peptide bonds only following the positively charged amino acid residues lysine or arginine (Evnin et al., 1990; Olsen et al., 2004). Signature peptides produced from trypsin digestion, undergo iterative processes to achieve a unique surrogate. The protein sequence or unique identifier for a sequence (accession number) is entered into software that simulates theoretical cleavage of the protein with trypsin. User defined criteria are considered prior to the in silico digestion. Tryptic peptides containing amino acid residues with potential post-translational modification (PTM) sites are avoided due to

a potential change in peptide mass that would affect reproducible quantification. Cysteine, methionine, and tryptophan are amino acid residues where oxidation can take place; therefore, reproducibly measuring peptides containing these amino acids may be difficult (Anderson, Anderson et al., 2004). Peptide Cutter is characterization software that predicts cleavage probability by proteases or chemicals for a given protein sequence. The sequences are entered into this site (http://www.expasy.org/peptide_cutter/) to help determine yield of signature peptides following enzymatic digestion. UniProt (http://www.uniprot.org/) and Ensembl genome browser (http:www.ensemble.org/) are databases that provide unique searching tools and data for polymorphisms and PTM's for several proteins that assist in selection of signature peptides. Exploration of databases may also include sequence similarity searches in databases such as the Basic Local Alignment search Tool (BLAST- http://blast.ncbi.nlm.nih.gov/) which offers theoretical predictions of homologous sequences that prove to be common and not sufficient for a signature peptide. Obtaining unique peptides for quantification even with database results may still require more examination. For example, a signature peptide may misrepresent a protein containing isobaric masses leucine and isoleucine in a peptide sequence. The sequence evaluation results in BLAST may indicate a unique peptide; however, the mass-to-charge of the peptide(s) could be the same (Sherman et al., 2009a). Tandem mass spectrometry would compensate for this similarity in the precursor ion by having a different product ion, which will be a different mass-to-charge. In silico investigation should ideally produce at least one candidate signature peptide after careful investigations using amino acid residue criteria, PTM criteria, and sequence similarity searches. Sherman et al. used computer simulations to achieve a unique ion signature in two proteomes, which revealed detection of at least one peptide in >99 and >96% of the E. coli and human proteomes, respectively (Sherman et al., 2009b)

Subsequent to the in silico process, mass spectra are evaluated in order to maximize selectivity of MRM's, optimize sensitivity, and avoid false peptide assignments. When tuning for signature peptides from a protein digest, this data will disclose the frequently missed cleavage sites from trypsin digestion (Picotti et al., 2008), but when present in spectra, the ion intensity is normally lower than the model predicted signature peptides. Therefore, mass spectrometer parameters are slowly ramped (i.e. declustering potential) to locate and avoid loss of peptides. Acquisition of signature peptides requires a significant amount of computational simulations coupled with analysis of actual mass spectra. Selection of MRM transitions is the next step in this process. The predecessor to MRM, single reaction monitoring (SRM), may not be compatible with signature peptide quantification given the high probability of homologous precursor ions and a lack of diagnostic fragment b and y ions (Sherman et al., 2009a) for an individual signature peptide. Fragmentation patterns of a peptide are based on primary sequence, internal energy, and charge state. The nomenclature originated by Roepstorff and Fohlman identified positively charged product ions on the N-terminus and C-terminus of the sequence as a, b, or c and x, y, or z ions, respectively (Roepstorff et al., 1984). These ions must carry at least one charge, and nomenclature depends upon the location of the fragment of the peptide sequence. Although, the most abundant product ions of signature peptides primarily determines MRM selections, exploration and monitoring of more than one transition is needed to optimize selectivity for quantification. A recent multi-laboratory evaluation of signature peptide quantification of target proteins using MRM-based measurements has been published (Addona et al., 2009). In this study, Addona et al. advocate monitoring peptides at least three MRM transitions per analyte (target protein) to ensure assay specificity and observe any discrepancies related to interferences and matrix effects from digested plasma. Producing more than one MRM

transition, either from the same precursor or multiple precursor ions, is a significant challenge for quantitative analysis of macromolecules using signature peptides in biological fluids. Employing empirical processes in silico and multiple investigations of selectivity, matrix effects, and recovery will improve the probability of achieving a useful method.

1.C PROTEIN PURIFICATION AND TRYPSIN DIGESTION

Target protein purification prior to enzymatic digestion will likely be necessary to achieve sufficient quantification limits for biomarker validation and pharmacokinetic studies of therapeutic proteins in complex matrices such as plasma. Various purification strategies have been explored prior to enzymatic digestion but much of the current literature does not employ purification procedures for target proteins prior to digestion in biological fluids. Most studies employ a direct assay approach known as protein cleavage isotope dilution mass spectrometry (PC-IDMS). This methodology involves quantification based on the ratio of an isotope labeled peptide internal standard and the "natural" or un-labeled signature peptide(s) analyzed (Deleenheer et al., 1992), which will be discussed in detail with internal standard selection below. This direct assay approach produces signature peptides through DRAD (denaturation, reduction, alkylation, and digestion). Initially, target proteins fortified in biological fluids will undergo denaturation (i.e., chaotrope such as 6 M urea), followed by reduction with dithiothreitol (DTT) to prevent intramolecular and intermolecular disulfide bonds from forming between cysteine residues of proteins, alkylation with iodoacetic acid for modifying sulfhydryl group (SH-groups or thiols) to prevent the re-formation of disulfide bonds and finally a trypsin digestion before any purification is performed. Table 1.1 (Non-Antibody Based Quantitative Applications) offers a comprehensive list of these methods from the literature. Table 1.2 (Protein

ImmunoPurification (Antibody-Based) Applications) provides a list of methods that have specifically used molecular recognition (antibody-based methods) for purification of target proteins which will be discussed later in this section.

Table 1.1 Non-Antibody Based Quantitative Applications

Target Protein(s)/ quantitative description	~Molecular Weight (kDa)	Limit of Quantification (LLOQ) (ng/mL or as reported)	Sample preparation/comments	Reference
Human growth hormone	22.1	State 1 fmol/L (0.022 ng/mL), but data only shown for 16 ng/mL	Direct assay approach without multi-dimensional purification in human plasma (spike 16 µg/µL of HgH into plasma).	(Wu et al., 2002)
C-reactive protein (CRP)	25.0	0.025 ng/mL*	1 mL serum: Selective removal of human serum albumin, immunoglobulin, haptoglobin using tandem affinity columns, followed by reduction and alkylation. Then samples underwent fractionation of serum by SEC, trypsin digestion, addition of synthetic internal standard. * Endogenous CRP is approximately 1 µg/mL; this LLOQ was determined based on an internal standard.	(Kuhn et al., 2004)

Table 1.1 Continued

Target Protein(s)/quantitative description	~Molecular Weight (kDa)	Limit of Quantification (LLOQ) (ng/mL or as reported)	Sample preparation/comments	Reference
Somatropin	22.0	500 ng/mL	10 µL plasma: Following DRAD, two-dimensional solid phase extraction using reversed phase (SPEC C18) and strong cation exchange (Waters Oasis MCX) SPE was perfomed for samples.	(Yang et al., 2007)
Growth hormones IGF-1 and IGFBP-3	17.0 31.6	4000 ng/mL 2000 ng/mL	100 µL serum: Direct assay approach without multi-dimensional purification in human serum.	(Kirsch et al., 2007)
alcohol dehydrogenase ADH1C1	39.0	~2.3 ng*	550 µL human liver sample: Direct assay approach without multi-dimensional purification. *Quantification was based on the heavy labeled ADH1CQ peak area versus concentration, not protein standardization.	(Janecki et al., 2007)

Table 1.1 Continued

Target Protein(s)/quantitative description	~Molecular Weight (kDa)	Limit of Quantification (LLOQ) (ng/mL or as reported)	Sample preparation/comments	Reference
Tenecteplase	58.7	2700 ng/mL	25 µL rat plasma: Direct assay approach without multi-dimensional purification.	(Buscher et al., 2007)
ceruloplasmin	122	200,000 ng/mL	3-mm punch dried blood spots: Following DRAD, digests were precipitated using cold acetonitrile and concentrated in 0.1% formic acid.	(Dewilde et al., 2008)
Therapeutic monoclonal antibody	~125	5000 ng/mL	50 µL serum: Following DRAD, digests were cleaned up using strong cation exchange (Waters Oasis MCX) SPE. Pharmacokinetic and correlation data to enzyme linked immunosorbent assay (ELISA) are presented.	(Heudi et al., 2008)

Table 1.1 Continued

Target Protein(s)/quantitative description	~Molecular Weight (kDa)	Limit of Quantification (LLOQ) (ng/mL or as reported)	Sample preparation/comments	Reference
45 proteins in human plasma	varies	15 to 25,919 amol/L given for signature peptides*	5 µL plasma. Following DRAD, signature peptide internal standards were added, and digests were cleaned up using Waters Oasis HLB reversed phase SPE*LLOQ and linearity was not based on proteins spiked into plasma. Trypsin digestion was performed first, then standardized for each protein.	(Kuzyk et al., 2009)
Urinary albumin	~65.0	3130 ng/mL (based on external calibration curve)	Purified human serum albumin (HSA) was added to charcoal stripped urine for calibration. ^{15}N-labeled HSA was added to calibrators, quality controls, and samples using 40 µL urine. Direct assay approach without multi-dimensional purification.	(Seegmiller et al., 2009)

Table 1.1 Continued

Target Protein(s)/quantitative description	~Molecular Weight (kDa)	Limit of Quantification (LLOQ) (ng/mL or as reported)	Sample preparation/comments	Reference
Prostate specific antigen	28.7	4.5 ng/mL	100 µL serum: Following DRAD, samples were spiked with peptide internal standard and digested samples were cleaned up using Waters Oasis HLB reversed phase SPE. Employment of MRM cubed (MRM^3) was used to improve sensitivity on a quadrupole linear ion trap QTRAP 5500. MRM^3 involves the signature peptide is first scanned in Q1, and then fragmented in the collision cell (Q2). Fragment ions are then trapped in the linear ion trap, followed by excitation to perform a second fragmentation. These secondary fragment ions are scanned to the detector.	(Fortin, et al., 2009)

Table 1.1 Continued

Target Protein(s)/ quantitative description	~Molecular Weight (kDa)	Limit of Quantification (LLOQ) (ng/mL or as reported)	Sample preparation/comments	Reference
Pegylated-interferon-α_{2a}	~60	3.6 ng/mL	200 µL serum: Following denaturation, reduction, and alkylation, samples were acidified and loaded onto SPEC C_{18} to perform SPE. After digestion, samples were cleaned-up using Waters Oasis MCX strong cation exchange SPE.	(Yang et al., 2009)
12 proteins evaluated multi-site reproducibility	varies	N/A-not intended for sensitivity evaluation	Study 3 revealed an acceptable quantification procedure by spiking target proteins directly into plasma. Following DRAD, signature peptide stable isotope internal standards were added. Samples then underwent SPE prior to LC-MS/MS.	(Addona et al., 2009)
C-reactive protein	25.0	1000 ng/mL	Direct assay approach without multi-dimensional purification. However, column trapping on a custom built C_{12} column was used prior to switching to analytical column.	(Williams et al., 2009)

Table 1.1 Continued

Target Protein(s)/quantitative description	**~Molecular Weight (kDa)**	**Limit of Quantification (LLOQ) (ng/mL or as reported)**	**Sample preparation/comments**	**Reference**
Recombinant growth hormone (rHGH)	22.0	1.70 ng/mL*	500 µL serum (*external calibration used). Following DRAD, 2DLC was performed on digested samples (serum or calibration sample) using a reversed phase LC column in the first dimension, trapping, and then loading onto a strong cation exchange column in the second dimension.	(Arsene et al., 2010)
C-reactive protein	25.0	1000 ng/mL	Direct assay approach without multi-dimensional purification. However, column trapping on a custom built C_{12} column was used prior to switching to analytical column.	(Williams et al., 2009)

Table 1.1 Continued

Target Protein(s)/quantitative description	~Molecular Weight (kDa)	Limit of Quantification (LLOQ) (ng/mL or as reported)	Sample preparation/comments	Reference
Insulin-like growth factor (IGF-1)	7.6	100 ng/mL (SPE method) 125 ng/mL (PPE method)	50 µL plasma.Bond Elut Plexa mixed mode SPE cartridges were employed to clean-up samples prior to DRAD or samples underwent a protein precipitation using acetonitrile, followed by LC-MS/MS	(Barton et al., 2010)
Recombinant growth hormone (rHGH)	22.0	1.70 ng/mL*	500 µL serum (*external calibration used). Following DRAD, 2DLC was performed on digested samples (serum or calibration sample) using a reversed phase LC column in the first dimension, trapping, and then loading onto a strong cation exchange column in the second dimension.	(Arsene et al., 2010)

Table 1.1 Continued

Target Protein(s)/quantitative description	~Molecular Weight (kDa)	Limit of Quantification (LLOQ) (ng/mL or as reported)	Sample preparation/comments	Reference
Therapeutic protein	~70	10 ng/mL	0.050 mL human plasma sample digested with lys-C overnight, then a strong cation exchange SPE was performed, followed by evaporation, reconstitution, and injection onto the UPLC-MS	(Plumb et al., 2012)
Pegylated Proteins (I, II, III, IV-propietary)	11-52 +40kDA PEG portion	10 ng/mL	A 0.050 mL monkey plasma aliquot was precipitated with 0.1% formic acid in IPA, the mixture was evaporated, and reconstituted with digestion buffer. Overnight trypsin digestion was performed, formic acid was added to stop the reaction, then sample was injected into the LC-MS/MS.	(Wu et al., 2011)

The intrinsic properties of proteins have been exploited for conventional purification prior to digestion with methods such as solid phase extraction (SPE), protein precipitation (PPE), size exclusion (SEC), ultrafiltration (UF), and liquid liquid extraction (LLE). All of these purification methods have some limitation such as throughput, selectivity, and ability to work with different molecular weights. Properties such as isoelectric point (pI), hydrophobicity, net charge, molecular recognition, ionic strength, and molecular size have been examined, of which molecular recognition indicates the most potential because of its ability to quantify low nanogram per milliliter concentrations in plasma or serum according to examples in Table 1.2. Traditionally in proteomics investigations, proteins have been separated by two-dimensional (2D) gel electrophoresis prior to excision of a spot from the gel for enzymatic digestion. This process involves a pH gradient for separation based on isoelectric points, followed by a second dimension for separation of proteins based on molecular size. This approach has been successful in resolving at least six thousand proteins (Geng et al., 2000). Difference in gel electrophoresis (DIGE) is a more recent version of this technique which has expanded the dynamic range by using fluorescent dyes (Unlu et al., 1997; Van Den Bergh et al., 2004); however, this approach is employed for relative quantification. Protein samples are prelabeled with different fluorescent dyes, run on the same gel, and detected by fluoresecence imaging (Unlu et al., 1997). Difficulties exist for these methodologies involving: manipulations of samples being slow and challenging, and poor quantitative reproducibility. Separation of certain proteins can also be difficult, including those that are in low abundance, acidic, basic, hydrophobic, very large, or very small (Fey et al., 2001; Geng et al., 2000). Additionally, dissolution of the excised spot is necessary to be compatible with electrospray ionization sources.

Table 1.2 Protein ImmunoPurification (Antibody-Based) Applications

Target Protein(s)/ quantitative description	~Molecular Weight (kDa)	Limit of Quantification (LLOQ) (ng/mL or as reported)	Sample preparation/comments	Reference
Coagulation factor V Adiponectin C-reactive protein Thyroxine binding globulin	251.7 26.4 25.0 46.3	2316 ng/mL 2904 ng/mL 3015 ng/mL 11,389 ng/mL	Immunodepletion of 6 abundant proteins (MARS-Aglient technologies). Followed by denaturation, reduction. Samples were then ultrafiltered with 5 kDa cut-off ultrafiltration and reversed phase SPE was used to clean-up samples prior to LC-MS/MS.	(Lin et al., 2006)
Bovine serum albumin (BSA)	~66	280 nmol/L	50 µL urine was injected into an immunoaffinity column with immobilized polyclonal antibodies against BSA. Samples were collected, digested, and cleaned using C_{18} SPE.	(Hoos et al., 2006)

Table 1.2 Continued

Target Protein(s)/ quantitative description	~Molecular Weight (kDa)	Limit of Quantification (LLOQ) (ng/mL or as reported)	Sample preparation/comments	Reference
Aprotinin Leptin Myoglobin Myelin basic protein PSA	6.4 10 17 18 30 48	33.4, 61.25 ng/mL 23.8, 65.0 ng/mL 31.7, 77.9 ng/mL 35.1, 114.5 ng/mL 46.1, 124.3 ng/mL 17.2, 56.7 ng/mL	Human IgG and chicken IgY immunodepletion (MARS hu7 and IgY-12) columns were compared for quantitative analysis. LOQ data (first number in LOQ column is from IgY-12) was evaluated and only the smallest coefficient of variance (%CV) LLOQ's are presented for each protein in the LOQ column of this table for the different depletion columns used. Proteins were spiked into a 100 µL of sample immunodepleted of abundant proteins using two different columns. Following depletion and DRAD, samples were further processed with Waters Oasis HLB reversed phase SPE.	(Keshishian et al., 2007)

Table 1.2 Continued

Target Protein(s)/ quantitative description	~Molecular Weight (kDa)	Limit of Quantification (LLOQ) (ng/mL or as reported)	Sample preparation/comments	Reference
insulin	~5.7	0.050 ng/mL	Immunoprecipitation was performed on 3 mL equine plasma, which was added to antibody coated magnetic beads (Dynabeads M-280 tosyl-activated-Invitrogen). The mixture was incubated overnight, beads were magnetically separated and supernatant discarded. Several washes, filtrations followed before elution of target proteins injected onto LC-MS/MS.	(Ho et al., 2008)
Human monoclonal antibody (HmAb)	~150	2000 ng/mL	50 µL serum and internal standard were combined prior to albumin depletion using ProteoExtract albumin kit. Following depletion and DRAD, samples were loaded onto LC-MS/MS. Samples were retained on column during the first 10 minutes directly to waste prior to elution gradient.	(Hagman et al., 2008)

Table 1.2 Continued

Target Protein(s)/ quantitative description	~Molecular Weight (kDa)	Limit of Quantification (LLOQ) (ng/mL or as reported)	Sample preparation/comments	Reference
N-terminal proBrain natriuretic peptide (NTproBNP)	8.6	0.10 ng/mL	Immunoprecipitation was performed on 100 µL sample and internal standard, which were added to Handee spin column (Pierce), diluted, and protein-A gel containing antibodies was added. Samples were washed, digested, and separated with spin columns prior to LC-MS/MS.	(Berna et al., 2008)
Erbitux	145.7	20 ng/mL	500 µL serum was immunoprecipitated using (Dynabeads M-280 tosyl-activated-Invitrogen). The mixture was incubated , beads were magnetically separated and supernatant was denatured and digested followed by LC-MS/MS.	(Dubois et al., 2008)

Table 1.2 Continued

Target Protein(s)/quantitative description	~Molecular Weight (kDa)	Limit of Quantification (LLOQ) (ng/mL or as reported)	Sample preparation/comments	Reference
Multiple proteins evaluated	11 to 84	2- 15 ng/mL	0.8-1.2 mL plasma samples were immunodepleted using LC10 column, which removes 12 high abundance proteins by capturing with IgY antibodies. Following DRAD, samples were fraction collected with strong cation exchange, and desalted with Waters HLB SPE prior to LC-MS/MS.	(Keshishian et al., 2007)
PSA	28.7	4 ng/mL	100 µL serum was immunodepleted of albumin followed by DRAD, and samples were subsequently cleaned with a mixed cation exchange extraction prior to LC-MS/MS. Additionally, sample data was correlated with ELISA for the first time for PSA patient serum.	(Fortin, Salvador, Charrier, Lenz, Lacoux et al., 2009)

Table 1.2 Continued

Target Protein(s)/quantitative description	~Molecular Weight (kDa)	Limit of Quantification (LLOQ) (ng/mL or as reported)	Sample preparation/comments	Reference
C-reactive protein (CRP)	25.0	1-point standard addition at 3000 ng	CRP in a 500 µL reaction volume was affinity purified using CRP monoclonal antibodies conjugated to polystyrene magnetic beads for capture of target antigen. Eluted CRP samples were evaporated, denatured, and digested, then injected into LC-MS/MS.	(Kilpatrick et al., 2009)
proGRP	~13	0.20 ng/mL	125 µL serum was immunopurified using a 96-well plate coated with ProGRP mAb. Samples underwent DRAD, and then were subsequently cleaned using C18 SPE tips prior to injection into LC-MS/MS.	(Winther et al., 2009)

Table 1.2 Continued

Target Protein(s)/ quantitative description	~Molecular Weight (kDa)	Limit of Quantification (LLOQ) (ng/mL or as reported)	Sample preparation/comments	Reference
PEGylated peptide (MK-2662)	47.5	2000 pmol/L (PPE) 1000 pmol/L (IAP)	PPE: a 0.2 mL aliquot was precipitated with 0.5 mL of 90:10 acetonitrile/methanol v/v, followed by evaporation, reconstitution, and enzymatic digestion with trypsin. IAP: 0.2 mL aliquot was combined with biotinylated antibodies bound to streptavidin coated magnetic beads in a suspension. The sample mixture was shaken for 2 h for complex to form, followed by washing beads, on-bead trypsin digestion, addition of formic acid to supernatant to quench reaction, and injection onto LC-MS/MS	(Xu et al., 2010)

Table 1.2 Continued

Target Protein(s)/ quantitative description	~Molecular Weight (kDa)	Limit of Quantification (LLOQ) (ng/mL or as reported)	Sample preparation/comments	Reference
Carbonic Anhydrase II	29.2	External calibration (27.3 pmol/mL^{-1} measured in patients)	Immunoprecipitation was performed with 20 µL serum using antibodies immobilized on Protein G coated magnetic beads. Following bead washing, samples were denatured and digested prior to column trapping online LC-MS/MS.	(Callipo et al., 2010)
Parathyroid hormone (PTH)	9.4	0.0309 ng/mL	PTH from 1 mL (charcoal-stripped) serum was immunocaptured using polystyrene beads coated with murine monoclonal antibodies (Anti- 44-84 recognition) in a 96-well plate. Following incubation and washing, samples were injected onto LC-MS/MS.	(Kumar et al., 2010)

Table 1.2 Continued

Target Protein(s)/ quantitative description	**~Molecular Weight (kDa)**	**Limit of Quantification (LLOQ) (ng/mL or as reported)**	**Sample preparation/comments**	**Reference**
Therapeutic Monoclonal antibodies 4 IgG_1 and 4IgG_2 mAb)	~150	100 ng/mL	A 0.200 mL sample was combined with magnetic streptavidin coated beads, which had an anti-human crystallizable fragment (anti-Fc) biotinylated antibodies bound to them. The sample mixture was then washed with surfactant in PBS, and eluted with 0.2 mL of 50% methanol and 3% formic acid. The eluate was dried, reduced with DTT, alkylated with iodoacetamide, digested with trypsin, reaction was stopped with formic acid, and 0.010 mL was injected into LC-MS/MS	(Li et al., 2012)

Precipitation of the target protein using organic solvents and/or salting out with ammonium sulfate has been explored (Jiang et al., 2004). Jiang et al. found that that using trichloroacetic acid, acetone, or ultrafiltration provided efficient desalting and sample concentration prior to 2D electrophoresis. This investigation was qualitative however and further evaluation is needed to determine the effectiveness of these techniques for quantitative applications. The potential exists to apply multiple fractionation steps involving precipitation and 2D electrophoresis purification prior to digestion, but time and lack of efficiency could be the limiting factors. Precipitation of major serum proteins was also evaluated using an acetonitrile-depletion strategy (Kay et al., 2008). In this study, the authors were able to characterize the presence of insulin-like growth factor (IGF-1) at 125 ng/mL in serum depleted of major background proteins after a protein precipitation with acetonitrile. However, more quantitative results are necessary to determine if this purification procedure can be useful for low abundance biomarkers or therapeutics. Precipitation with acetonitrile or alternative solvents is an efficient way for removal of proteins less than 15 kDa (Ackermann et al., 2007).

Molecular sieving has been exploited through the use of size exclusion chromatography (SEC) and ultrafiltration. In this approach, a gel stationary phase such as polyacrylamide, dextran or agarose is used and the sample is filtered under low pressure for size exclusion. Lecchi et al. concluded that size exclusion chromatography provides a practical approach for multi-dimensional separation of proteins and should not be overlooked (Lecchi et al., 2003). However, protein extracts obtained from bacterial cultures grown in stable isotope enriched media were used for this evaluation, and the complexity of plasma samples may limit usefulness of these stationary phases, as biomarkers or therapeutics may be lost in partitioning. Over 3,000 proteins have been successfully separated using a multi-dimensional approach including SEC in

human plasma (Pieper et al., 2003), but applying this technique to quantitative analysis was not presented. Ultrafiltration is another molecular size-based pre-treatment step in purification of proteins in which hydrostatic pressure forces a liquid against a semipermeable membrane. Various membrane pore sizes are available to retain molecules 1 to 1,000 kDa, while water and low molecular weight solutes pass through the membrane. Aresta et al. employed a 30 kDa molecular weight cut-off filter as a pre-treatment prior to reverse phase solid phase extraction (Aresta et al., 2008) for the extraction of serum from breast cancer patient samples. Additionally, Greening et al. evaluated ultrafiltration for isolation of low molecular weight (< 25 kDa) components of the human plasma proteome (Greening et al., 2009). The two studies advocate ultrafiltration as a protein purification step, in spite of the lack of throughput. The optimal conditions in the Greening et al. study were 35 minutes of centrifugation for only 100 µL of sample.

The hydrophobic and ionic properties of proteins have been exploited using solid phase extractions. Proteins have ionic and hydrophobic sites internally and on the surface. The ionic sites are provided by charged amino acids and by covalently attached modifying groups (i.e. carbohydrates and phosphate) (Simpson, 2004). The net charge of a protein is determined by the free α-amino group of the N-terminal residue, the free α -carboxyl group of the C-terminal residue, side chain R-groups capable of ionization, and modifications that may be attached to the protein. Additionally, non-polar amino acids contribute to the hydrophobicity of the protein. These are predominantly on the interior folds of the protein and denaturing may be required to expose them. These intrinsic properties of proteins offer potential selective interactions with hydrophobic and ionic stationary phases for sample purification to reduce interferences and matrix effects prior to enzymatic digestion. Barton et al. compared an acetonitrile precipitation

method (Kay et al., 2008) to mixed-mode solid phase extraction (SPE) for protein purification prior to enzymatic digestion and quantitative analysis of insulin-like growth factor (Barton et al., 2010) The SPE method showed pH-dependency to effectively remove abundant background proteins, whereas the acetonitrile precipitation removed albumin, but failed to recover the lower molecular weight protein studied. Proteins interact with hydrophobic surfaces by adsorption and use of hydrophobic interaction chromatography would require careful consideration of ionic strength, pH, and temperature for eluting proteins with non-denaturing conditions. Attempting to selectively elute target proteins is impractical off-line because gradient conditions are necessary to lower ionic strength for elution (Simpson, 2004), which would require multiple sample manipulations. Reversed-phase chromatography has been investigated for separation and fractionation of proteins under gradient conditions with a macroporous column (300 Å) (Martosella et al., 2005). This approach is sufficient for characterization of low abundance proteins; however, quantitative analysis would be inefficient because of the several fraction collection steps.

Molecular recognition involves a selective interaction of the target protein and its antibody through multiple noncovalent interactions. Table 2 (Protein ImmunoPurification (Antibody-Based) Applications) offers representative methods for purification of proteins using antibody-antigen recognition. The two types of antibody-based purification strategies are immunodepletion of major abundant proteins or immunopurification of the target protein through antigen-antibody complex formation. Immunodepletion removes major abundant plasma proteins using antibodies against each protein. Twenty two abundant proteins represent approximately ninety-nine percent of total protein mass in human plasma (Anderson et al., 2002). Most of this total protein mass is albumin, which accounts for approximately 50% of protein mass in plasma

(Issaq et al., 2007). Understandably, removal of the most abundant proteins would be a reasonable approach for protein purification. Several papers have applied immunodepletion techniques as shown in Table 1.2 and recent reviews have compared depletion strategies (Bjorhall et al., 2005; Polaskova et al., 2010). In these studies, most of the focus has been centered on the efficiency of removal of abundant proteins using commercially available kits. However, more investigation is needed to evaluate the effectiveness of immmunodepletion for enrichment of target proteins, and ultimately improving sensitivity of signature peptide quantification. Depletion kits can be purchased as spin columns or LC columns. Normally polyclonal anti-human antibodies (IgG) are employed for these kits; however, chicken derived immunoglobulin yolk (IgY) antibodies are also available, which have been known to provide less cross reactivity with mammalian proteins (Simpson, 2004). Agilent offers a multiple abundant removal column (MARS), which uses up to 14 immobilized polyclonal immunoglobulin's (antibodies) to remove abundant background proteins. A two buffer system is applied to the column which captures the abundant antigens that bind to their specific antibodies. Proteoprep, Seppro, Qproteome, Vivapure, and Aurum are other depletion kits available that vary according to the number of abundant proteins removed, price, sample volume, and throughput capability. Kits can cost as much as $14,000 and only allow low sample volumes (~10 μL), and minimal usage (<200 samples). Other caveats that should be considered for immunodepletion are: loss of target protein bound to endogenous depleted proteins and the multiple dilutions involved may hinder sensitivity. The potential of immunodepletion is evident in Anderson et al.'s 2006 publication, which demonstrated the ability to quantify 47 different proteins using signature peptides obtained from immunodepleted plasma (Anderson et al., 2006).

Immunopurification (immunoprecipitation) of target proteins allows for selective isolation with antibody capture of the target antigen. There are four main processes involved in immunoprecipitation, which typically include immobilization of antibodies, loading of antigen in a complex matrix for capture at physiological pH, washing non-specific components, and finally elution of target antigen. Immobilization of antibodies can be either direct or indirect, depending on the binding process (Guzman et al., 2005; Masseyeff, 1993). Noncovalent binding of antibodies to solid supports (i.e., silica, plastics) occurs due to hydrophobic interactions between the surface and amino acid residues (Masseyeff, 1993), where antibodies will bind "directly" to the surface of the solid support or free amine groups and carbohydrate residues (Peoples et al., 2008). However, direct adsorption of antibodies can result in random orientation and hinder binding activity (Jung et al., 2008; Lu et al., 1996; Peoples et al., 2008). The solid support or stationary phase system should be stable chemically, possess little non-specific binding, be mechanically stable, and provide sufficient surface area for molecular recognition to take place (Peoples et al., 2008). Superparamagnetic and agarose beads are normally employed as solid phase support material for immunoprecipitation. Agarose beads are highly porous with high binding capacity potential (Jung et al., 2008); however, the drawback of these materials is that the antibody may not fully saturate the sponge-like character of the agarose bead, which will lead binding sites free to capture non-specific proteins (Ogert et al., 1992). An alternative bead system is monodisperse superparamagnetic beads, which consist of small, solid, uniform, and spherical shaped beads. Binding occurs on the outer solid surface of these beads. This may allow for faster binding kinetics due to surface only binding; however, the beads cannot compete with the polydisperse agarose beads (Ogert et al., 1992) in terms of binding capacity. During immobilization on any support system, optimal conditions exist when the Fab regions are

exposed and therefore fully active. Indirect immobilization of antibodies requires binding the antibody to a solid phase substrate through a ligand anchor or secondary molecule (Masseyeff, 1993) such as binding to Fc receptors on bacterial cell wall proteins A or G, or recombinant protein A/G (Jung et al., 2008). The molecule biotin's high affinity (Ka – 10^{15}/M) for streptavidin provides another ligand anchor used to bind antibodies to solid phase supports. Whether antibodies are immobilized or not, loading of the antigen containing complex is optimal under physiological conditions. It should be noted that fast and efficient removal of unbound antibodies is essential prior to loading the antigen containing matrix to prevent residual complex formation that will be washed away and reduce target protein yield (Masseyeff, 1993). Once loading and the association of the antigen-antibody complex has taken place, multiple washes are necessary to remove non-specific components of the biological matrix. Washing buffers (normally phosphate buffered saline) may contain detergents or inert proteins to reduce non-specific binding (Masseyeff, 1993). Dissociating the antigen-antibody complex may require acidic elution, use of chaotropes, ionic strength adjustment, or other denaturants (Ogert et al., 1992; Peoples et al., 2008). Optimization of the elution pH (normally 1-3) is necessary to minimize degradation and denaturation of the target protein (Nisnevitch et al., 2001; Ogert et al., 1992; Peoples et al., 2008). Another consideration when performing immunoprecipitation is to avoid elution of the antibody, which will minimize unwanted background. Commercially available kits employ cross-linkers such as disuccinmidyl suberate (DSS) that covalently immobilize the antibody to the support by crosslinking with NHS esters that react with primary amines on the antibody to form stable amide bonds. Crosslinking will take place after capturing the antibody on a Protein A/G agarose resin (http://www.piercenet.com/products/), and this should allow for multiple uses of the immobilized antibody. Immunoprecipitation can offer

selective capture and release of target protein, which can be digested in a relatively "clean" matrix free of background proteins. The drawbacks associated with this technique include: the need for expensive antibodies and the fact that immobilization, capture, and elution optimizations can be laborious. There is also still the potential for capturing non-specific proteins. Table 1.2 provides examples of immunodepletion and immunoprecipitation methods that reach low ng/mL limits of quantification.

1.D PEPTIDE PURIFICATION

Proteolytic digestion of target proteins will further complicate the sample in terms of the number of peptides present by as much as 50 to 100 fold (Picotti et al., 2009). Consequently, there may be a need to continue purification processes at the peptide level of the sample. It is clearly evident in the methods presented in Table 1.2 that there has been minimal investigation into protein purification, and efforts have been focused on the purification of the signature peptides following digestion. Direct assay approaches have yielded LLOQ's in the μg/mL range for some quantitative analyses of signature peptides (Anderson et al., 2006; Williams et al., 2009), suggesting that that additional purification steps are needed to achieve limits of quantification below ng/mL. The intrinsic properties of signature peptides will differ and purifications will need to be optimized accordingly. Conventional SPE methods have shown the most promise in recent works, which have reached ng/mL limits in serum or plasma (Yang et al., 2007; Yang, Z. et al., 2009). Somatropin, and its analogue internal standard bovine fetuin, were digested and subjected to a 2D solid phase extraction for purification of the signature peptides prior to LC-MS/MS analysis (Yang et al., 2007). The digested samples were loaded onto a reversed phase SPE cartridge (SPEC C18), washed, and the eluate was loaded into the second

dimension, a 96-well mixed mode strong cation exchange cartridge (Waters Oasis® MCX). The lower limit of quantification was 500 ng/mL, and analysis required no immunodepletion or immunoaffinity purification. Yang et al. also demonstrated SPE for protein purification followed by signature peptide purification to quantify pegylated-inteferon-α_{2a} in human serum. In this study, serum samples fortified with pegylated-inteferon-α_{2a} and its internal standard (somatropin) were denatured, reduced, and alkylated. Following alkylation, samples were pH adjusted prior to loading onto an equilibrated 96-well SPEC C_{18} plate. The eluate was dried and subsequently concentrated in a buffer, which was ultrafiltered and digested. Finally the digested samples were again extracted using a 96-well mixed mode strong cation exchange cartridge (Waters Oasis® MCX) to achieve an LLOQ of 3.6 ng/mL. Keshishian et al. employed strong cation exchange SPE to purify signature peptides in order to achieve low (1-10 ng/mL) LLOQs for six proteins in plasma; however, plasma samples were immunodepleted prior to digestion (Keshishian et al., 2007).

An alternative to conventional peptide purification is the use of immunoaffinity purification with peptide antibodies. Anderson et al. have pioneered the use of this approach for candidate cardiovascular biomarkers by employing a technique his group has coined Stable Isotope Standards and Capture by Antipeptide Antibodies (SISCAPA) (Anderson et al., 2004). This technique makes use of antibodies against tryptic peptides along with stable isotope internal standards, which act to correct for ion suppression. In SISCAPA, antipeptide antibodies are synthesized by conjugation of peptides to protein carriers for immunization (Anderson et al., 2004). Whiteaker et al. demonstrated this technique by using α1-antichymotrypsin (AAC) and tumor necrosis factor alpha (TNFα) as model compounds. Their results demonstrated quantification of these biomarkers within the physiological range with acceptable precision

(<10%) and accuracy (<20%) (Whiteaker et al., 2007). Their ability to capture tryptic peptides of AAC and TNFα with antibodies followed by elution allowed for more than a 500-fold increase in sensitivity. Table 1.3 presents SISCAPA applications, which reveal quantification of proteins using signature peptides at low ng/mL concentrations.

Offline purification steps prior to LC-MS/MS analysis will improve the sensitivity and robustness of the analytical method. Online separation offers another means to reduce matrix effects, improve sensitivity, and provide selective methods for quantification. Most of the applications presented in Tables 1.1 to 1.3 employ one dimension reversed phase chromatography for separation of signature peptides prior to MS ionization. There are several applications that use 2DLC, column trapping, or switching (Desouza et al., 2008; Hagman et al., 2008; Kay et al., 2008; Williams et al., 2009) which have been shown to minimize matrix effects associated with mass spectrometry and improve column life. Hydrophilic interaction chromatography (HILIC) for separation of signature peptides was first investigated by Alpert et al. (Alpert, 1990). HILIC is similar to normal phase chromatography (NPLC), where a polar stationary phase is used but the non-aqueous mobile phase is replaced with a water miscible organic solvent and low water content is used. The interaction of the polar stationary phase occurs through analytes partitioning into a water-rich layer generated through chemisorption of the stationary phase (Alpert, 1990; Boutin et al., 1992; Yang, Y. et al., 2009). Yang et al. recently employed HILIC for the separation of digested peptides of cytochrome C and β-lactoglobulin; however, the study was performed in buffered solutions and more investigation is needed with complex matrices. Several publications use nano-liquid chromatography coupled to a nano-electrospray, which can result in much greater sensitivity (1-2 orders of magnitude).

Separation mechanisms are similar to conventional 2DLC; however, the run times can be significantly longer (> 45 minutes).

Table 1.3 Peptide Immunoaffinity Applications

Target Protein(s)/quantitative description	**~Molecular Weight (kDa)**	**Limit of Quantification (LLOQ) (ng/mL or as reported)**	**SISCAPA Sample preparation/comments**	**Reference**
Tumor necrosis factor alpha (TNFα) Antichymotrypsin (AAC)	25.6 47.6	1 fmol (~1 pg) of peptide on column	20 µL of serum was immunodepleted using the MARS column removal of six abundant proteins. SISCAPA was continued using magnetic beads coated with peptide antibodies to capture tryptic peptides of natural and isotope labeled peptides. Samples were loaded onto nano-LC for trapping and final elution to MS.	(Whiteaker et al., 2007)
Thyroglobulin	304.7	2.6 ng/mL	Following DRAD of 100 µL, signature peptides for thyroglobulin and internal standard peptide were captured using paramagnetic beads, washed, and injected into LC-MS/MS	(Hoofnagle et al., 2008)

Table 1.3 Continued

Target Protein(s)/quantitative description	~Molecular Weight (kDa)	Limit of Quantification (LLOQ) (ng/mL or as reported)	SISCAPA Sample preparation/comments	Reference
TIMP1	23.1	0.8 ng/mL	Serum was immunodepleted prior to SISCAPA technique for enrichment of peptides.Column trapping was used with nano-LC for separation.	(Ahn et al., 2009)
Troponin I Interleukin-33	21.7 30.7	2.8 ng/mL 1 ng/mL	Following DRAD of plasma, samples were desalted with C18 Empore material and then signature peptides for both biomarkers and their respective internal standard peptides were captured using paramagnetic beads, washed, and injected into LC-MS/MS	(Kuhn et al., 2009)

Table 1.3 Continued

Target Protein(s)/quantitative description	~Molecular Weight (kDa)	Limit of Quantification (LLOQ) (ng/mL or as reported)	SISCAPA Sample preparation/comments	Reference
Calumenin Disulfide isomerase Fibulin-2 Hypoxia up-regulated Legumain L-plastin Osteopontin Plectin Tumor Protein D52	37.2 56.8 126.6 111.3 49.4 70.3 35.4 ~500 24.3	1-166 ng/mL	Automated use of Stable Isotope Standards with Capture by Antipeptide Antibodies (SISCAPA) to quantify multiple proteins in plasma.	(Whiteaker et al., 2010)

1.E MULTIPLY CHARGED INTACT POLYPEPTIDE QUANTIFICATION

Low molecular weight proteins or polypeptides may be quantified without enzymatic digestion by reducing the mass to charge ratio sufficient for limited mass range tandem mass spectrometry. During electrospray ionization, proteins and polypeptides exist as intact ions with multiple charges separated by their mass to charge ratio (m/z) (Fenn et al., 1990). Therefore, it may be possible to reduce m/z within a limited range instrument (2800 m/z) for quantification of intact polypeptides and proteins in biological fluids. Manipulation of charge states through solvent additives such as glycerol or 3-nitrobenzyl alcohol during electrospray ionization has been shown to increase charge state and intensity, which will be beneficial for m/z reduction and improving detectability (Lavarone et al., 2001; Samalikova et al., 2005). However, reproducing multiple charge state precursor ions and the formation of reliable product ions can be affected by ion suppressors, instrument parameters, electrospray solvents, and the target protein's (or polypeptide's) primary structure and conformation (Krusemark et al., 2009; Lavarone et al., 2001). Therefore, mass spectrometer parameters should be optimized by ramping analyte specific parameters such as declustering potential to minimize in-source fragmentation and collision energy voltage to obtain selective product ions. In addition, prior to electrospray ionization and tandem mass spectrometry, extracted samples should undergo extensive sample purification processes to minimize matrix effects.

Purification and quantification of intact polypeptides (peptides greater than 1000 Daltons) has been discussed (Van Den Broek et al., 2008). Similar purification techniques are applied to intact polypeptides as compared to proteolytic peptides described in the previous section. Some examples of quantification of polypeptides are listed in Table 1.4 below.

Table 1.4 Recent Intact Polypeptide Applications

Target Peptide(s)/ quantitative description	**~Molecular Weight (kDa)**	**Limit of Quantification (LLOQ) (ng/mL or as reported)**	**Sample preparation/comments**	**Reference**
bivalirudin	2.2	1.25 ng/mL	A 0.2 mL aliquot of human plasma underwent PPE with methanol, evaporated with vacuum centrifugal concentrator, reconstituted in mobile phase, and injected onto LC-/MS/MS. The sample was diverted to waste the first 4 min.	(Pan et al., 2010)
bradykinin	1.1	10 ng/mL	1 mL of rat plasma was protein precipitated with 1% TFA, and the supernatant was loaded onto an SPE cartridge (STRATA X, Phenomenex), evaporated, reconstituted with water.	(Baralla et al., 2011)
PEGylated Human Calcitonin gene peptide antagonist	20	5 ng/mL	A 0.1 mL monkey serum sample was extracted with an HLB SPE μelution plate	(Li et al., 2011)

Table 1.4 Continued

Target Peptide(s)/ quantitative description	~Molecular Weight (kDa)	Limit of Quantification (LLOQ) (ng/mL or as reported)	Sample preparation/comments	Reference
Angiotensin-II Neurotensin 5-leu-enkephalin Somastatin Bradykinin	1.0 1.7 0.55 1.6 1.1	13 ng/mL 22.4 ng/mL 3.85 ng/mL 40.5 ng/mL 7.75 ng/mL	0.2 mL human plasma was precipitated with acetonitrile and formic acid, then filtered through a 10 kDa MWCO filter, evaporated, reconstituted in water, and injected onto LC-MS (SIM was used).	(Fang et al., 2012)
Peptides I, II, III, IV (proprietary)	1.5 to 1.9	10 pmol/mL	0.1 mL mouse plasma was precipitated in a filter plate containing acetonitrile, extracts were evaporated, reconstituted, and injected onto LC-MS/MS.	(Zhang et al., 2011)
Octreotide	1.0	0.025 ng/mL	0.2 mL human plasma was subjected to a weak cation exchange SPE μelution (WCX, Waters), followed by column trapping, and UPLC-MS/MS	(Ismaiel et al., 2011)

1.F INTERNAL STANDARD SELECTION AND QUANTITATIVE LC-MS/MS

Current applications involving absolute quantification employ a synthetic stable isotopic peptide internal standard to quantify the "natural" or unlabeled signature peptide. The peak area ratios of signature peptide and internal standard are plotted against the theoretical concentration ratios of peptide and internal standard used for calibration. Isotopic peptide internal standards are produced by incorporating an isotope (normally ^{13}C and ^{15}N) on selected amino acid residues of the signature peptides chosen. These isotopes will provide a mass shift to distinguish the internal standard and its natural signature peptide by mass spectrometry, yet physiochemical properties (i.e., chromatography, ionization, fragmentation patterns) remain the same. Internal standards are introduced into the sample at a known concentration to compensate for matrix effects and track recovery if added before digestion. A peptide internal standard is limited however because it does not track recovery during the digestion of the protein. Addona et al. made a significant observation in a multisite assessment that revealed absolute quantification likely needs a labeled protein internal standard which is added at the start of sample processing (Addona et al., 2009). Use of a protein internal standard will allow for sufficient recovery tracking during the entire sample processing. However, synthesizing and purifying a labeled protein could be expensive and time consuming. Brun et al. investigated isotope labeled proteins for quantification of urine-based biomarkers at the picomolar level (Brun et al., 2007). An alternative to isotopically labeled protein internal standard may be to employ a protein analogue internal standard. Yang et al. produced quantitative analysis of two different proteins in separate methods using protein analogue internal standards (Yang et al., 2007; Yang, Z. et al., 2009). Bovine fetuin and somatropin were protein analogue internal standards used for the quantification of somatropin and pegylated interferon-α_{2a}, respectively. Finding appropriate analogue internal standards that

can track recovery and produce signature peptides with similar retention times for all therapeutics and biomarkers may be challenging however. A recent approach that may replace conventional labeled peptides is an extended isotope labeled peptide, also known as digestible internal standard (Plumb et al., 2012). These internal standards offer the same benefits as an isotope labeled signature peptide internal standard; however the extended amino acid residues, which are part of the protein sequence, will offer the ability to track digestion. Ultimately a labeled protein internal standard would be preferable.

1.G CONCLUSIONS

Quantitative analysis of macromolecules using signature peptides and hyphenated techniques with liquid chromatography tandem mass spectrometry presents an attractive approach that can ultimately lead to a new standard of practice for quantifying large molecules. The ability to quantify multiple proteins offers a unique advantage and multiplexing biomarker candidates will likely expedite the process.

While employment of this approach is very promising, it is clear that challenges exist. The lack of quantitative analytical validation data for many reported methods brings into question their actual usefulness for practical analysis. LC-MS/MS continues to be the primary choice for small molecule quantification in biological fluids. Well documented matrix effects involving ion suppression or enhancement with small molecule LC-MS/MS will also be an issue with proteins. Technology for reduction of matrix effects for small molecule has improved but most of these technologies are inadequate for proteins, especially when purifying at the protein level. Considering the concentration range of background protein concentrations and the difficulty involved with reproducibly digesting signature peptides, it will be challenging to

obtain precise and accurate data. High throughput analysis is also a challenge, and reducing the rate limiting step of enzymatic digestion may be the answer. A significant caveat to this methodology is employment of a signature peptide that may result in false identification or lack selectivity for quantitative analysis. During small molecule method development and validation, various experiments are performed to evaluate selectivity, matrix effects, and recovery. The presence of endogenous levels of target protein versus background proteins in plasma necessitates similar experiments for determining selective signature peptides. Multiple lots of plasma should be evaluated for the presence of background that may interfere with the ability to quantify proteins in individual sources of plasma (Ismaiel et al., 2008). Matrix effects evaluations should be performed, and obtaining signature peptides will be an iterative process requiring significant observations in silico, database searching, and spectral data derived from samples in matrix to avoid these effects.

The currently obtainable lower limits of quantification for signature peptides liberated from proteins has hindered the replacement of traditional immunoassays although further research and improvements are expected. Technological advances for the purification of proteins and their liberated signature peptides will enable improvements in detectability. Use of online separations of background matrix components to further purify signature peptides has significantly improved quantification limits. Purification methods using antibodies at the protein and/or peptide level provides optimal isolation and purification of peptides and proteins ultimately reducing matrix effects and improving sensitivity. Technological advances in mass spectrometry such as the ability to perform MRM cubed (MRM^3) with a hybrid triple quadrupole-linear ion trap instrument have offered another solution to improving quantification limits (Fortin, Salvador, Charrier, Lenz, Bettsworth et al., 2009) as shown in Table 1.1. This

technology allows for secondary fragmentation in the third quadrupole by trapping the product ions , which leads to a second generation product ion formed which is subsequently sent to the detector, further enhancing selectivity. Utilization of protein internal standards is beginning to become established and will ultimately allow for significant improvement in the tracking the recovery of target proteins which will lead to better precision and accuracy in protein quantification. It is likely that application of multiple processes will be necessary to obtain reliable methods at lower levels of quantification that are currently achieved using immunochemical methods.

CHAPTER 2

DISSERTATION OBJECTIVES

The first part of this dissertation research will involve investigating the quantification of a therapeutic protein using a signature peptide as a quantitative surrogate for the protein of interest in human plasma. Included with this objective will be creation of an analytical method without the need for antibody purification. An internal standard will be necessary for quantitative analysis to compensate for matrix effects and losses associated with digestion and/or sample purification. Therefore, a protein analogue internal standard will be employed for these purposes. The protein of interest and its analogue internal standard will have signature peptides determined by in silico processes. Proteolytic digestions of the therapeutic protein and its analogue internal standard will be used for optimization of mass spectrometer conditions for analysis in human plasma. Furthermore this analytical method will be validated according to current FDA Bioanalytical Guidance. This method will aim to be suitable for monitoring therapeutic levels of the target protein.

The second part of this dissertation will investigate quantification of an intact polypeptide. Polypeptides will be investigated to determine the formation of sensitive and selective product ions which can form selected reaction monitoring transitions. Solvents and different mass spectrometer parameters will be investigated to determine charge state distribution

of polypeptides. Multiple charge states of an intact polypeptide (oxyntomodulin) will be optimized and used for quantitative analysis in rat plasma. The method will be validated and used for rat samples in a pharmacokinetic study.

The third part of this dissertation will aim to show utility of a liquid chromatography tandem mass spectrometry analytical method by applying that method to a pharmacokinetic study. Non-compartmental pharmacokinetic parameters will be generated from plasma concentration-time profiles. Parameters will be compared following intravenous and intratracheal instillation routes of administration of oxyntomodulin into rats.

CHAPTER 3

QUANTIFICATION OF ALEFACEPT, AN IMMUNOSUPPRESSIVE FUSION PROTEIN IN HUMAN PLASMA USING A PROTEIN ANALOGUE INTERNAL STANDARD, TRYPSIN CLEAVED SIGNATURE PEPTIDES AND LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY.

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3.A INTRODUCTION

Autoimmune diseases rank third behind heart disease and cancer in the United States population and are the most common diseases in the world (Cooper et al., 2003). Psoriasis is one of these immune mediated diseases, which affects approximately 7.5 million Americans (Langley et al., 2005). It affects the skin and joints when the immune system conveys incorrect signals that accelerate the growth cycle of skin cells. Treatments include topical, phototherapy, traditional systemic and biological medications (biologics). Currently the biological medication of choice for treatment of psoriasis is injectable Alefacept, sold under the brand name Amevive.

Alefacept is a dimeric immunosuppressive fusion protein that blocks the activation of T white blood cells, and results in a reduction in skin inflammation. Alefacept consists of fused extracellular CD2-binding portion of the human leukocyte function antigen-3 (LFA-3) linked to the Fc (hinge, CH2 and CH3 domains) portion of human IgG1(Vaishnaw et al., 2002). It is approximately 91 kilodaltons (kDa), with therapeutic levels between 500 to 6000 ng/mL (Ellis et al., 2001).

Pharmacokinetic studies of biologics such as Alefacept are currently performed with immunoassays that can be costly and may require time-consuming method development. Immunoassays also are often associated with selectivity problems related to cross reactivity in biological fluids that may result in imprecise data and/or falsely elevated results (Ackermann et al., 2007). It has been well established that liquid chromatography tandem mass spectrometry is the method of choice for small molecule clinical studies and has been gaining ground for large molecule quantification (Anderson et al., 2004; Parker et al., 2010). Use of signature peptides that act as surrogates for targeted protein quantification when coupled to liquid chromatography tandem mass spectrometry offers a potentially superior methodology for clinical studies and biomarker validation (Abbatiello et al., 2010). This approach requires proteolytic digestion to yield signature peptides that will ultimately be quantified using a triple quadrupole mass spectrometer with multiple reaction monitoring. Gerber et al. pioneered an absolute quantification (AQUA) strategy in 2003, which quantifies proteins using signature peptides and a synthetic, isotopically labeled peptide internal standard (Gerber et al., 2003a). Quantification with synthetic peptide internal standards has been carried out successfully, however the lack of tracking the enzymatic digestion is a potential source of error. Addona et al. performed a multi-laboratory assessment of quantification using synthetic peptide internal standards for three experimental designs (Addona et al., 2009). All laboratories yielded good precision and accuracy when synthetic internal standard peptides were incorporated into digested plasma. The imprecision proved significant however when synthetic peptide internal standards were added post-digestion for target proteins that were spiked into non-digested plasma. This indicates that a synthetic peptide internal standard may not sufficiently track digestion and therefore overall recovery.

Isotopically labeled internal standards can be added before digestion however, most published works have done this after digestion. Protein internal standards may be more suitable because they can be digested with the target protein and therefore track digestion recovery

Several approaches have been investigated for purification of target proteins. Immunodepletion of the highly abundant proteins (HAP) in human plasma prior to enzymatic digestion is an effective method for reducing superfluous background in the matrix (Anderson et al., 2006; Keshishian et al., 2007; Kuhn et al., 2004; Lin et al., 2006; Whiteaker et al., 2007). The drawbacks associated with this technique include expensive kits, possible imprecision, minimal volume applied, and minimal column life (<200 samples) (Seam et al., 2007). Immunopurification of the target protein can also be accomplished and has also been shown to yield lower limits of detection (Berna et al., 2008; Dubois et al., 2008; Ho et al., 2008; Kumar et al., 2010; Winther et al., 2009). However, the requirement for antibodies for immunopurification makes this technique less desirable. Stable Isotope Standards and Capture by Antipeptide Antibodies (SISCAPA) is an approach which provides an alternative through employing anti-peptide antibodies to capture the signature peptide and reduce matrix effects (Anderson, Anderson et al., 2004). The SISCAPA approach has been shown to be effective in reaching low ng/mL levels in human plasma or serum (Ahn et al., 2009; Hoofnagle et al., 2008; Kuhn et al., 2009; Whiteaker et al., 2010; Whiteaker et al., 2007), but production of antibodies for peptides is also time-consuming and costly. Other methods have employed two-dimensional solid phase extractions (Yang et al., 2007; Yang, Z. et al., 2009) for protein and/or signature peptide purification, which may require several optimizations. Yang et al. were able to reach low ng/mL concentrations (3.6 ng/mL LLOQ) for pegylated-

interferon alpha (α_{2a}) using a monolithic C_{18} solid phase extraction for target protein enrichment and mixed mode cation exchange (Waters Oasis MCX) for digested sample clean-up prior to LC-MS/MS (Yang, Z. et al., 2009).

Control of digestion and tracking of signature peptides is critical to ensure precise and accurate results. Protein internal standards offer tracking of the digestion step due to introduction at the beginning of sample preparation. Under ideal conditions where all preparation steps are stoichiometric, an isotopically labeled protein internal standard may be appropriate as demonstrated in previous studies (Brun et al., 2007; Heudi et al., 2008); however, synthesis time and expense may still be limitations. Protein analogue internal standards are good candidates for this process since they may have similar intrinsic properties (pI, hydrophobicity) as the target protein. Protein analogue internal standards undergo the same preparation steps as the target protein, and signature peptides are generated in a similar fashion for quantification. The key to making this work is to match similar retention times for the signature peptides representing the target protein and analogue internal standard, respectively. Retention time similarities will facilitate correction of matrix effects. Yang et al. have successfully employed a protein analogue internal standard to yield precise and accurate results at ng/mL concentrations in biological fluids (Yang et al., 2007; Yang, Z. et al., 2009).

In the present work, our current method combines selective protein precipitation with use of a protein analogue internal standard (horse heart myoglobin) to quantify Alefacept in human plasma.

3.B EXPERIMENTAL

3.B.1. CHEMICALS AND REAGENTS

Alefacept (Amevive) was purchased from Virginia Commonwealth University Medical Center Pharmacy. Horse heart myoglobin, sodium hydroxide, glacial acetic acid, hydrochloric acid, iodoacetamide, dithiothreitol, proteomics grade trypsin, and ammonium bicarbonate were obtained from Sigma Aldrich (St.Louis, MO, USA). Deionized water was obtained in-house using a Nanopure Diamond water system from Barnstead International (Dubuque, IA, USA). Methanol, isopropanol, acetonitrile were purchased from Burdick and Jackson (Muskegon, MI, USA). Acetic acid was procured from Curtin Matheson Scientific Inc. (Houston, TX, USA). Formic Acid was obtained from EMD Chemicals Inc. (Gibbstown, NJ, USA). K_2EDTA Human plasma was obtained from BioChemed Services (Winchester, VA, USA).

3.B.2. MATERIALS AND EQUIPMENT

Plasma samples were aliquoted into 1.5 mL microcentrifuge tubes purchased from VWR International (Westchester, PA, USA). Samples were filtered using nanosep MF 0.2 μm filters from Pall Life Sciences (Ann Arbor, MI, USA). All centrifugation was carried out using an Eppendorf 5804R centrifuge (Hamburg, Germany). All mixing was performed using a multi-tube vortex mixer from VWR International (Westchester, PA, USA). A syringe pump from Harvard Apparatus (Holliston, MA, USA) was used to infuse solutions for tuning optimization and post-column infusion.

3.B.3. INSTRUMENTS AND HPLC CONDITIONS

High Performance Liquid Chromatography (HPLC) separations were performed using the following equipment: Shimadzu system controller SCL-10A VP, pumps LC-10AD VP, solvent degasser DGU14A (Shimadzu, Kyoto, Japan). An HTS PAL autosampler from CTC Analytics (Zwingen, Switzerland) and a CH-30 column heater from Eppendorf (Westbury, NY, USA) were used. Column trapping was performed using a Phenomenex Security Guard column (Gemini C18, 4 X 2.0 mm) as the loading column and a Gemini C-18 column (100 mm x 2.0 mm I.D., 5.0 μm) as the elution column, both from Phenomenex (Torrance, CA, USA). Three Shimadzu pumps were operated with one controller to apply the gradient conditions. Mobile phases from pumps A and C consisted of 0.1% Formic Acid and Mobile Phase B (0.1% formic acid in acetonitrile) was delivered from Pump B. The initial loading conditions used 100% aqueous mobile phase (0.1% formic acid) from pump C. Following a short loading time (0.5 minutes), the diverter valve switched to position B and the gradient initiated. Mobile phase A and B were coupled together with a mixer and the elution conditions started with 5% mobile phase B (0.1% formic acid in acetonitrile). During loading of the sample, the mobile phases were delivered at 0.5 mL/min to provide sufficient flow through the trapping column along with continuous flow into the mass spectrometer. The flow was reduced to 0.25 mL/min during elution onto the analytical column and the column temperature was maintained at 45 ˚C. Gradient conditions are plotted in Figure 3.1 for mobile phases A, B and C.

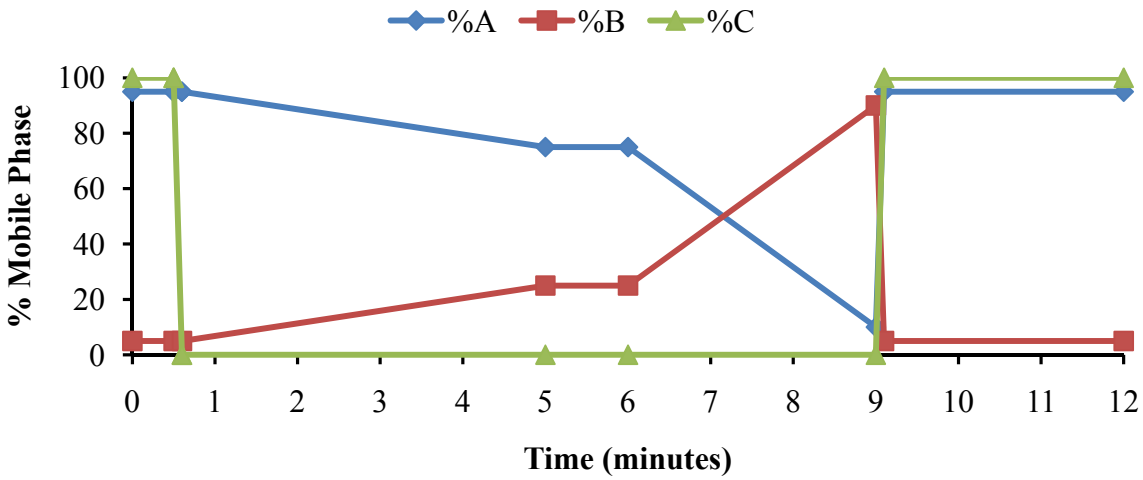

Figure 3.1 Gradient conditions are plotted for mobile phases A, B, and C.

A 10-port Cheminert switching valve and a microelectric actuator obtained from Valco Instruments Co. Inc. (Houston, TX, USA) was used to perform on-line column trapping. There were two autosampler rinses. The first rinse consisted of a cocktail of acetonitrile:methanol:isopropanol: water in the ratio of 40:20:20:20 and the second rinse was 1:1 mobile phase A and B. The mass spectrometer was an API 4000Qtrap hybrid triple quadrupole/ linear ion trap from AB Sciex (Foster City, CA, USA) with Analyst 1.5 data acquisition software. The data analysis was performed using Quantitation Wizard processing software that accompanies Analyst. MRM pilot 2.0 (AB Sciex-Foster City, CA, USA) and was used to facilitate modeling of signature peptides. All nitrogen gas was generated from a Parker Hannifin (Haverhill, MA, USA) Tri-Gas Generator LC/MS 5000. Figure 3.2 represents an overall schematic diagram of the instrument including the plumbing for column trapping.

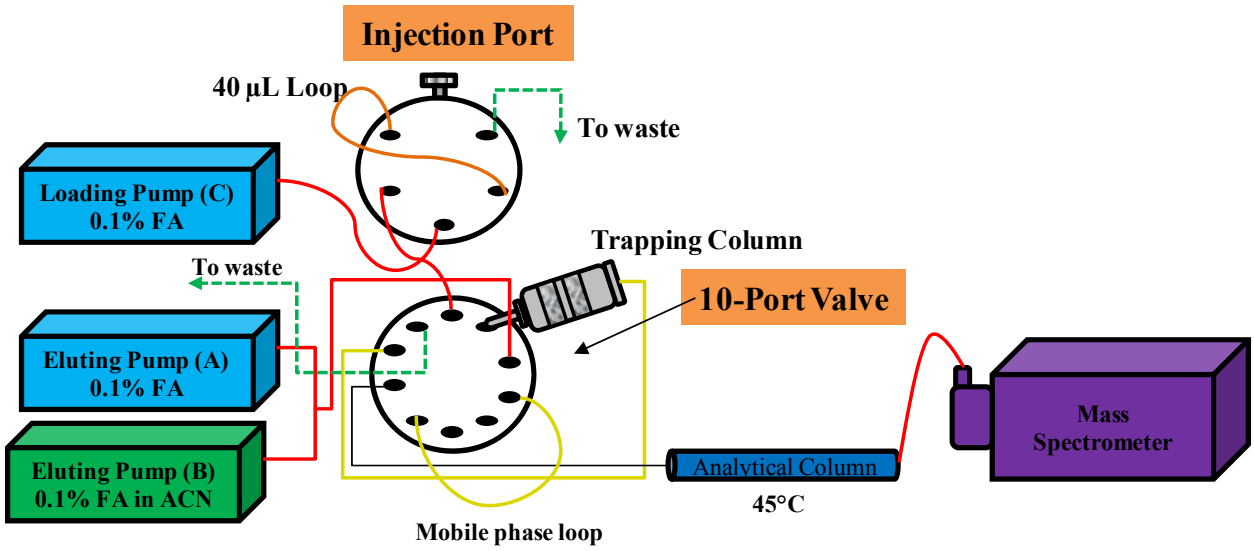

Figure 3.2 Schematic of the overall instrument set-up including column trapping plumbing.

3.B.4. MASS SPECTROMETER PARAMETERS

The mass spectrometer was operated in the positive electrospray ionization (ESI) mode with selected reaction monitoring (SRM) of signature peptides for Alefacept and horse heart myoglobin. Potential signature peptides were obtained through *in silico* digestions using MRMPilot 2.0 and sequence homology evaluations were performed using the basic local alignment search tool (BLAST-http://blast.ncbi.nlm.nih.gov/Blast.cgi). Peptide Cutter (http://www.expasy.org/peptide_cutter/) was also used for predicting yield of signature peptides from an enzymatic digestion with trypsin. Signature peptides for Alefacept and myoglobin were optimized using multiple iterative processes of tuning, *in silico* predictions of cleaved peptides, SRM transitions, and collision energy voltages, and finally LC-MS/MS data collection. Tuning was performed two ways, direct infusion and LC-MS/MS injection by evaluation of the following scan types: Q1 full scan, precursor ion scan, product ion scan, and finally SRM to determine the final SRM(s) transitions that would represent Alefacept and myoglobin for signature peptide quantification. A 5 µg/mL solution of Alefacept and myoglobin prepared in 50

mM ammonium bicarbonate was digested with trypsin. This digested sample was used as a tuning solution for direct infusion at 10 μL/min tee'd with mobile phase at a flow rate of 0.20 mL/min. Mobile phase A and B composition was varied to evaluate intensity changes during tuning optimization. The same solution was also injected with similar gradient conditions described in section 3.B.3 to determine the optimal conditions. The mass spectrometer parameters were tuned and optimized to achieve maximum sensitivity of selected signature peptides which resulted in the following parameters: source temperature 325 ˚C, ion spray voltage 5500 V, gas 1 (GS1) 38, gas 2 (GS2) 28, collision activated dissociation (CAD) was set on high, channel electron multiplier (CEM) 2200 V, declustering potential (DP) 109, entrance potential (EP) 15, and collision exit potential (CXP) set on 13. Alefacept and myoglobin signature peptides eluted at approximately 4.22 and 3.65 minutes, respectively. Table 3.1 presents the SRM transitions, collision energies (CE), signature peptide information, and dwell times used during this study.

Table 3.1 Selected Reaction Monitoring (SRM) Transitions and Selected Parameters

Signature Peptide	Q1	Q3	Signature Peptide Information	Dwell (msec)	Collision Energy (v)
AQSP[a]	597.4	894.5	VAELENSEFR→LENSEFR (+2/y7)	200	31
AMSP 1[b]	597.4	781.8	VAELENSEFR→ENSEFR (+2/y6)	200	31
AMSP 2[c]	597.4	652.4	VAELENSEFR→NSEFR (+2/y5)	300	31
Myoglobin[d]	636.3	716.3	LFTGHPETLEK → ETLEK (+2/y6)	200	50

[a] Alefacept quantified signature peptide
[b] Alefacept monitored signature peptide 1
[c] Alefacept monitored signature peptide 2
[d] Myoglobin signature peptide (analogue internal standard)

3.B.5. STOCK SOLUTION AND WORKING SOLUTION PREPARATION

The Amevive (Alefacept) vehicle was diluted with sterile water to yield a concentration of 7.5 mg/mL. This stock solution was subsequently diluted with water to prepare an

intermediate working solution of 1 mg/mL. This working solution was then spiked in plasma to prepare the high standard (10,000 ng/mL) and high quality control (8000 ng/mL), which was then used to prepare the remaining calibration points and quality controls. Horse heart myoglobin was diluted in deionized water for the internal standard solution at a concentration of 1 mg/mL. Internal standard spiking solution was prepared fresh daily. Alefacept working solutions were prepared immediately prior to spiking into the plasma.

3.B.6. PREPARATION OF CALIBRATION STANDARDS AND QUALITY CONTROL SAMPLES IN HUMAN PLASMA

Pooled dipotassium EDTA human plasma from at least two donors was used to prepare the calibration standards and quality controls. A volume of 0.250 mL of the intermediate solution (1.0 mg/mL) of Alefacept was spiked into 25 mL plasma to obtain the high standard (10,000 ng/mL). The high standard was spiked into 10 mL volumetric flasks to prepare the remaining seven calibration standards (250, 500, 750, 1000, 2500, 5000, and 9000 ng/mL). A volume of 0.200 mL of the intermediate solution (1.0 mg/mL) of Alefacept was spiked into 25 mL plasma to obtain the highest quality control (HQC = 8,000 ng/mL). The HQC standard was spiked into 25 mL volumetric flasks to prepare the remaining quality control samples representing the lower limit of quantitation (LLOQ) QC, low QC (LQC), medium QC (MQC) and high QC (HQC) quality controls, which were prepared at 250, 600, 2000, and 8000 ng/mL, respectively. A dilution control was prepared at 2.5 times the highest calibration standard (25,000 ng/mL), to evaluate dilution of samples within the calibration range. The dilution control was diluted fivefold with blank plasma to obtain a concentration within the calibration range (5000 ng/mL). Calibration standards (STDs) and quality control samples were freshly prepared for the first

analytical run to establish a day zero nominal value for storage stability studies. The calibration standards and QC samples were prepared in a similar fashion to contain less than or equal to 5% (v/v) of the intermediate solution in order to simulate real matrix samples as much as possible. The STDs and QC samples were divided into aliquots of 0.750 mL each, and stored at -20 ˚C until analysis.

3.B.7. SAMPLE PREPARATION

The sample preparation procedure involved a selective precipitation followed by reduction, alkylation, trypsin digestion, dilution, and filtration prior to LC-MS/MS analysis. Each STD, QC, or plasma blank were aliquotted (0.35 mL) into 1.5 mL microcentrifuge tubes and diluted to 0.500 mL with 50 mM ammonium bicarbonate. Twenty microliters of myoglobin internal standard spiking solution (solution = 1 mg/mL or 58.9 µM) was added into the tube followed by brief vortex mixing. The pH was then adjusted to 5.1 with 1.0 M acetic acid and samples were incubated at 45 °C for approximately 10 minutes. The supernatant from the samples was then chemically reduced with 0.040 mL of dithiotreitol (100 mM of DTT) and incubated for 10 minutes at 45 °C. Following reduction, samples were alkylated with 0.080 mL 100 mM iodoacetamide and incubated for an additional twenty minutes in the dark. Samples were allowed to equilibrate to room temperature, and the pH was adjusted to 8.5 for optimal pH conditions for trypsin digestion with an approximate enzyme to substrate ratio of 1:20 (w/w). Samples were digested overnight for approximately 16 hours at 37 °C. Digestion was stopped with 1 M acetic acid, followed by a dilution with 0.150 mL of 1:1 mobile phase A:mobile phase B. Samples were mixed thoroughly and filtered through 0.2 µm filters, pipet transferred to a 96-well 2 mL plate, and injected into the LC-MS/MS using a 50 µL injection.

3.B.8. SELECTIVE PRECIPITATION (PARTIAL-PROTEIN PRECIPITATION EXTRACTION)

One protein purification technique that exploits a protein's solubility and stability is selective precipitation, also known as partial protein precipitation extraction. Employing pH adjustment coupled with heat incubation offers an effective method where the target protein will undergo minimal denaturation, and background proteins can be more completely denatured and precipitate (Simpson, 2004). This denaturation process implies destruction of the tertiary structure of a protein, with the formation of random polypeptide chains (Matulis, 1997). These chains tangle and aggregate, and to some extent, will form disulfide bonds (Matulis, 1997). Using temperature and pH adjustments concurrently, conditions may be created well away from the target protein's isoelectric point (pI) to keep the target protein in solution (Lovrien et al., 1997; Van Holde, 2006). The closer the pH is to the pI of the target protein, the more likely it will aggregate (Simpson, 2004). As temperature is increased, the hydrogen bonds of the protein are weakened (Lovrien et al., 1997) and the internal electrostatic forces are eliminated.

The goal of this purification process is to maximize the recovery of target proteins and minimize the recovery of background proteins. Purification of target proteins will allow for a more efficient digestion and a reduction of matrix effects. Five different pH adjustments (pH 3.0, 4.7, 5.1, 7.6, 9.0) were applied to plasma samples incubated at five varying temperatures (22, 37, 45, 65, 95 °C) over the course of ten minutes. The two variables (pH and temperature) were evaluated together with all possible combinations. The isoelectric points of two of the most abundant background proteins were investigated along with the average isoelectric points (pI) of Alefacept and myoglobin. Albumin (pI = 4.7), IgG (pI = 7.2), and the average pI of Alefacept (pI

= 7.8) and myoglobin (pI = 7.2) were evaluated (average pI = 7.6). Adjustment of the pH to 5.1 was also evaluated based on previous experimental results with myoglobin. A recent study by Saguer et al. indicated the secondary structure of serum albumin begins to unfold at pH 5, which leads to protein aggregation (Saguer, 2009). Employment of pH adjustments of 3.0 and 9.0 were evaluated to determine if pH adjustment well away from the pI is necessary to maintain the solubility of target proteins. Signature peptides were monitored for target and background proteins (albumin, IgG, transferrin) to evaluate the change in peak area response with pH adjustment and various temperature incubations. A plasma sample was fortified with myoglobin and Alefacept at a concentration of 10 µg/mL and analyzed in triplicate for each condition. Background signature peptides were identified from *in silico* predictions, evaluations and literature sources (Anderson et al., 2006), and were optimized accordingly. The background signature peptides monitored were: albumin (575→937), IgG (839→262), and transferrin (815→693).

3.B.9 DIGESTION TIME STUDY

The trypsin incubation time was evaluated to determine if the digestion time could be reduced to improve throughput efficiency. The majority of the literature indicates overnight incubation times are needed; however, accelerated trypsin digestion by employment of microwave irradiation and convection has been successfully demonstrated by Lesur et al (Lesur et al., 2010); however, this was not available in our laboratory. A study was performed with 5 incubation time points (0.08, 1, 2, 4, and 20 hours) to evaluate precision of quality control samples and the internal standard. Quality controls 1-3 were extracted in plasma as described above in triplicate with the different incubation periods for enzymatic digestion. Signature

peptides were monitored for Alefacept and myoglobin. The +1 and +2 charge states of the myoglobin signature peptide were monitored, but the intensity for other myoglobin peptides was not sufficient for quantification or for any of the other experiments such as the digestion time study.

3.B.10 MATRIX EFFECTS EVALUATIONS AND RECOVERY

Matrix effects were evaluated for potential ion suppression or enhancement of signature peptides along with monitoring of phospholipids as a surrogate for matrix effects (Little et al., 2006). A post-column infusion study was performed similarly to previous methods (Bonfiglio et al., 1999; Shah et al., 2009). This experiment employed a sample that was extracted from 50 mM ammonium bicarbonate according to the sample preparation described previously as a clean matrix while injecting a blank plasma extracted sample as the matrix sample. The resulting profile was evaluated for any change in the ESI response of signature peptides for Alefacept and myoglobin. Phospholipids were monitored using the quasi-SRM transition 184→184 during this post-column infusion study as a likely candidate for suppression or enhancement of Alefacept and/or myoglobin signature peptides as suggested by previous phospholipid monitoring studies (Ismaiel et al., 2007; Little et al., 2006). A post-extraction addition study to evaluate absolute matrix effects was also assessed using a similar method as suggested by Matuszewski et al (Matuszewski et al., 2003). The experiment was performed by comparing the peak areas of processed blank plasma samples spiked with low, medium, and high concentrations of Alefacept (600, 2000 and 8000 ng/mL) in triplicate. Spiking was performed by diluting extracted blank plasma samples with a ratio of 1:1 with clean matrix extracted samples. A dilution factor of two

was applied to the post-spike samples in order to compare the peak areas to the same non-diluted clean matrix extracted samples.

In addition to post-extraction addition investigations, a multiple donor source matrix evaluation was carried out as recommended previously (Ismaiel et al., 2008). Six different sources were fortified with Alefacept at the LQC level (600 ng/mL) and analyzed in triplicate. Concentrations were calculated from the calibration curves analyzed in the same run.

A relative recovery experiment was performed for Alefacept in human plasma. Signature peptide peak areas of plasma extracted and solvent (50 mM ammonium bicarbonate) extracted quality control samples were compared. All samples were analyzed in triplicate at three different concentrations (LQC, MQC, and HQC). Absolute recovery was not performed due to the lack of standard peptides to represent the target and internal standard signature peptides.

3.B.11 LINEARITY

Eight calibration standards were prepared by serial dilution at concentrations of 250, 500, 750, 1000, 2500, 5000, 9000, and 10,000 ng/mL in dipotassium EDTA human plasma. All standards were analyzed in duplicate, and were calculated by using the Alefacept signature peptide and myoglobin signature peptide internal standard peak area ratios for each concentration level. Standard curves were constructed using linear regression and a $1/x^2$ weighing factor was employed for the determination of Alefacept concentrations.

3.B.12 PRECISION AND ACCURACY

Precision and accuracies were determined by analysis of quality control (QC) samples at three different concentrations and were analyzed in three separate runs. QC concentrations were

calculated from the calibration curves analyzed in the same run. Intra- and inter-assay precision and accuracy were determined by extracting LLOQ, low (LQC), medium (MQC), and high quality controls (HQC) in six replicates for intra-assay performance. A dilution control was also evaluated for intra-assay performance with six replicates. Quality controls were extracted in triplicate in two additional runs for a total of three analytical runs for inter-assay performance.

3.B.13 SELECTIVITY

Human plasma samples from six different sources were analyzed in duplicate to evaluate selectivity with regard to interferences. Each individual lot was extracted as described above (section 3.B.7) with and without the addition of the myoglobin internal standard. Selectivity requirements were that the peak areas co-eluting with Alefacept must be less than 20% of the peak area of the average of LLOQ samples of Alefacept for all six lots of blank plasma sources. Crosstalk interference was evaluated in pooled plasma requiring no more than 5% contribution from Alefacept to the myoglobin internal standard peak area. A high standard (10,000 ng/mL) was extracted absent of myoglobin in duplicate to evaluate the Alefacept contribution to the internal standard. The myoglobin internal standard contribution to Alefacept was evaluated in each run and in six different sources.

3.B.14 STABILITY AND CARRYOVER

The stability evaluations of stock solutions were minimized due to daily preparation. Following the first validation run with freshly prepared calibration standards and quality controls, STDs and QCs were frozen at -20°C and sub-aliquots were used each day for analysis. Long term storage stability was evaluated by using the day zero nominal value established by

extraction of freshly prepared low and high controls. Post-preparative stability (PPS) was performed to evaluate extracted samples stored in the autosampler beyond 24 hours at 5°C in the event of an instrument malfunction requiring re-injection of samples. PPS was assessed from re-injection reproducibility after storage of quality control samples in the auto sampler for 48 hours.

Carryover was assessed by injecting blank and/or extracted buffer samples immediately after each of the highest calibration standards (10,000 ng/mL) in an analytical run. The criterion for carryover in this experiment was the LQC must be accurate to within 15%.

3.C RESULTS AND DISCUSSION

Signature peptides for target protein quantification have been evaluated various ways. Here, we present an alternative technology by coupling selective precipitation with a protein internal standard for the quantitative analysis of our target protein, Alefacept. Use of controlled pH and temperature allowed for a reduction in the major background proteins and maintained the target protein. The myoglobin internal standard was critical in order to obtain precise and accurate results. This approach is a simple process and offers an alternative to isotope labeled proteins for internal standards and more complex purification processes such as immunoprecipitation.

3.C.1 LC-MS/MS

Signature peptides were identified using *in silico* predictions, direct infusion, and injection of solvent based samples to obtain the most intense signature peptides using positive electrospray ionization. Each precursor ion underwent extensive evaluation of gas pressures and voltages to obtain the most intense product ion peaks. Optimized collision energy voltages for product ions were comparable with the model generated recommendations made using

MRMpilot. Optimization of the collision energies was critical in achieving maximum sensitivity. It was observed that changing collision energy +/- 10 units decreased intensity significantly. Additionally, injection of clean digested samples to evaluate potential product ions yielded greater intensity than direct infusion experiments. Injecting a clean digested sample through an analytical column while performing a gradient elution may reduce the background interferences associated with the components in an enzymatic digestion even though the samples were prepared in a clean matrix. This digested sample is unlike a synthetic signature peptide due to the presence of components like DTT, iodoacetamide, and trypsin which are necessary in order to obtain a signature peptide for LC-MS/MS optimization. The final signature peptides are presented in Table 3.1 as mentioned in section 3.B.4.

Chromatographic analysis of Alefacept and myoglobin signature peptides was investigated with various columns to resolve matrix peaks and ultimately produce similar retention times. An Ace C8, Aquasil C18, and a Phenomenex Gemini C18 were evaluated with a gradient of 0.1% formic acid and 0.1% formic acid in acetonitrile to achieve maximum sensitivity. The Phenomenex Gemini C18 provided the highest sensitivity, good peak shape, and closely related retention times for Alefacept and myoglobin signature peptides (3.6 versus 4.2 minutes). The first attempt at a validation run the method resulted in complete loss of signal after 39 injections; therefore, to improve ruggedness of the method, on-line column-trapping was employed. Similar analytical columns that were initially evaluated for separation of Alefacept and myoglobin were evaluated again for trapping (loading) and analytical (elution) columns; however, high back pressure and longer retention times lead to the investigation of using a Phenomenex security guard column as a trapping column. The Gemini C18 guard column allowed for a short elution time onto the analytical column, which resulted in a 12 minute run

time. Loading time (elution time off the trapping column) was evaluated for intensity and peak shape, and resulted in an optimized loading time of 0.5 minutes before the valve switch onto the analytical column. Introducing the on-line column trapping into the method improved the overall ruggedness. Following approximately 60 injections, the guard column was replaced; however, the same analytical column was used for the entire validation and has maintained performance for more than 400 injections. Examples of a blank chromatogram (A) and an LLOQ sample showing the Alefacept and myoglobin signature peptides (B, C), respectively are presented in Figure 3.3.

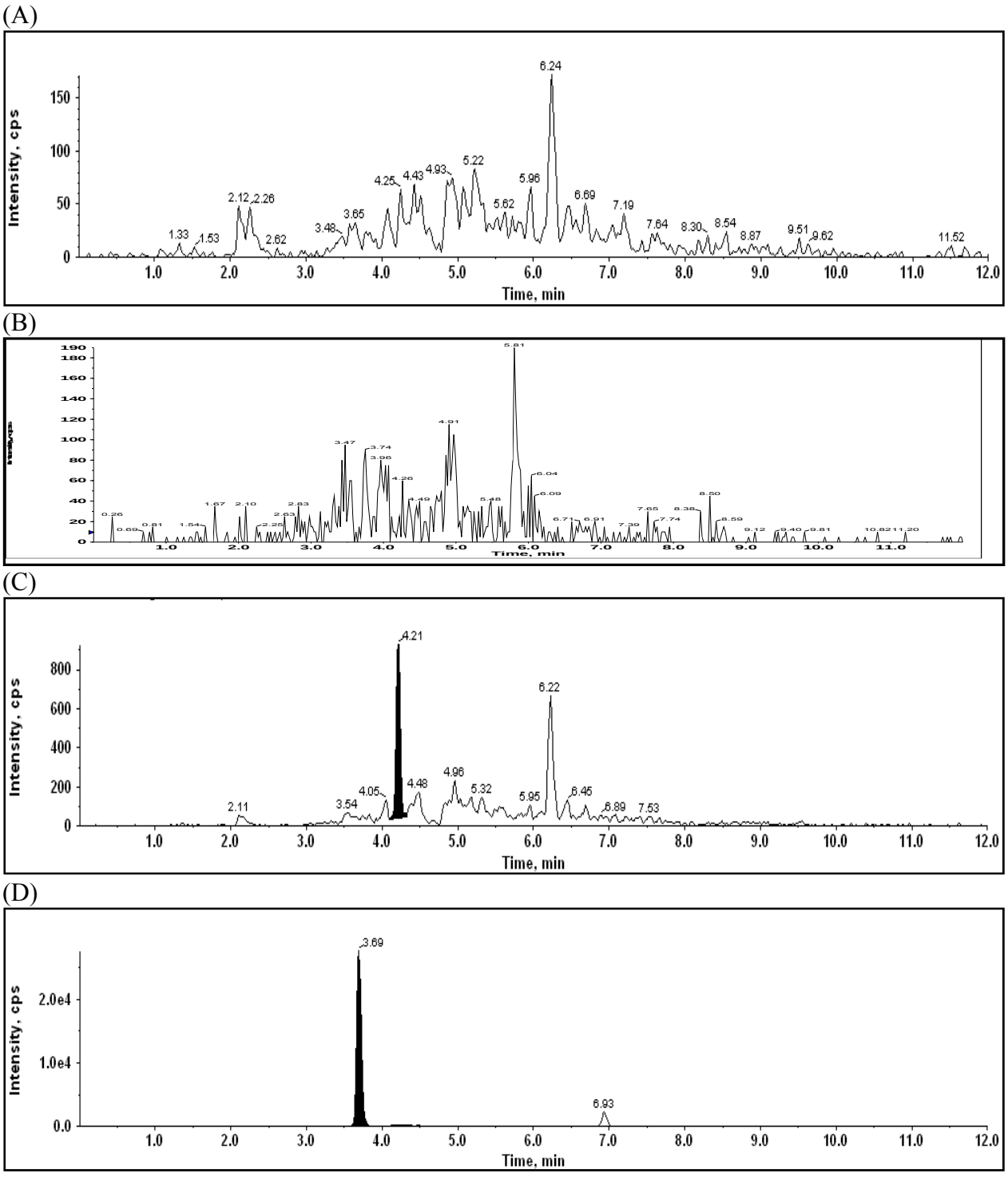

Figure 3.3 Representative chromatograms of (A) blank human plasma monitoring alefacept SRM: 597→894, (B) blank human plasma monitoring myoglobin SRM: 636→716, (C) extracted LLOQ (250 ng/mL) monitoring alefacept SRM: 597→894, (D) myoglobin signature peptide internal standard monitoring SRM: 636→716.

3.C.2 SELECTIVE PRECIPITATION RESULTS (PARTIAL PROTEIN PRECIPITATION EXTRACTION)

Conditions were created where the target protein would undergo minimal denaturation, and representative background proteins would be more completely denatured and precipitated (Simpson, 2004). Using elevated temperature and pH adjustment sequentially, conditions were obtained away from the target protein's (alefacept and myoglobin) isoelectric points (pIs), in order to keep the target proteins soluble (Simpson, 2004). Charge repulsion between the charged random polypeptides in a protein keeps these peptides from aggregating however, the closer the pH is to the pI of the target protein, the more likely aggregation will occur (Simpson, 2004). The main goal was to use pH and temperature (which are generally not independent) to precipitate background proteins (mainly human serum albumin) and avoid precipitation of target proteins. As temperature is increased, the hydrogen bonds of the protein are weakened, and adjustment of the pH would be expected to reduce the internal electrostatic forces of the protein and open it up to complete denaturation (Lovrien et al., 1997; Simpson, 2004). An initial study with myoglobin spike into human plasma revealed potential reduction in major background proteins when the pH was adjusted to 5.1 at a temperature of 45 °C. Therefore, as a control alongside more plasma experiments, myoglobin and alefacept were evaluated in deionized water adjusted to pH 5.1 and each temperature (22, 37, 45, 65, and 95˚C) was evaluated to determine if similar trends would occur in a clean matrix for the target proteins. This data are plotted in figure 3.4. Data represent mean±SD, with water samples n =3.

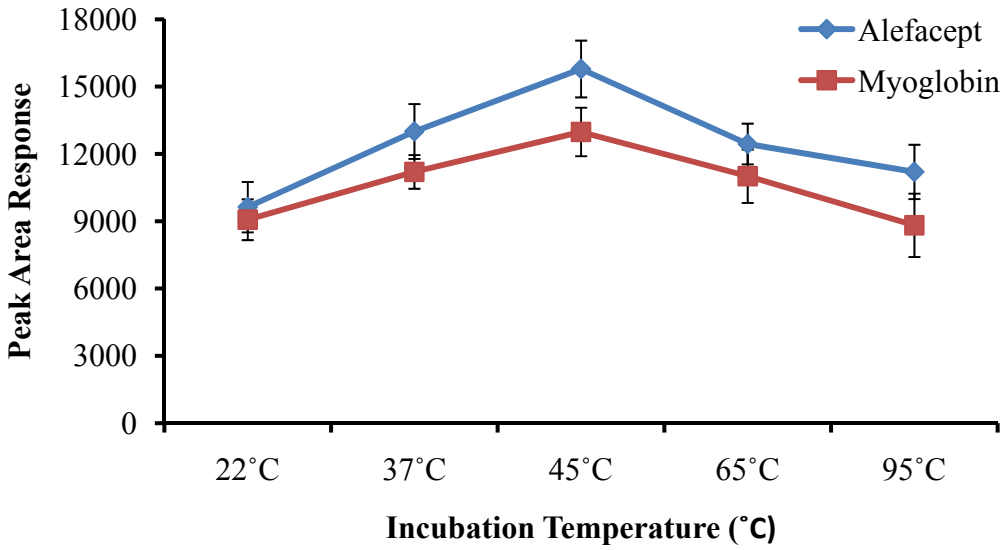

Figure 3.4 Alefacept (monitored SRM: 597→894) and myoglobin (monitored SRM: 636→716) peak areas following a partial precipitation using pH 5.1 and 45˚C in deionized water. Data represent mean±SD, with samples n =3.

The similar trends for myoglobin and Alefacept agreed with the original study carried out with myoglobin in plasma. This guided further investigation of these conditions with our target protein since Alefacept has a relatively similar pI to myoglobin (7.2 versus 7.8). As mentioned in section 3.B.8, six pH adjustments were evaluated with five different temperatures to attempt to denature background proteins and maintain target protein's structure in the purification step. Optimal conditions were determined by assessing the most intense peak areas obtained for the target proteins along with a reduction in major background proteins. Adjusting the pH to 3 under the five different temperatures yielded negligible differences in peak area response for both alefacept and myoglobin signature peptides, and background protein signature peptides monitored showed negligible loss of response. Adjusting the pH to 7.6 or pH 9.0 resulted in loss of all signature peptide responses (including background signature peptides) below detection limits at temperatures of 22, 37, and 45 °C. Background protein and target signature peptide

responses appeared again at 65 and 95 °C temperature conditions at both pHs. The increase in signature peptide peak area response under these temperatures may be due to a concentration effect associated with increased aggregation; these samples yielded supernatant volumes approximately three fold less. The data was normalized to reflect the varying supernatant volumes. Alefacept data and human serum albumin are shown in three dimensional graphs (Figures 3.5 and 3.6) to illustrate the trends of peak area response under different pH and temperature conditions. Data shown represent mean±SD for plasma samples n =3. At pH 4.7 (albumin pI) and 5.1, similar results in peak area responses for all temperature conditions were observed. Background protein signature peptides however were reduced more using the pH adjusted to 5.1. Under these conditions, IgG background was reduced 25% as compared to 37 °C, and as much as a 10-fold reduction compared to 65 °C. However, denaturing at 65 °C with the pH adjusted to 5.1 did not result in acceptable target protein yields. The albumin peak area response indicated that the presence of albumin was reduced to more than three-fold less than that of the target protein responses at pH 5.1 and 45 °C. Data showing the peak area response differences of each protein at pH 5.1 and 45 °C are shown in Figure 3.7. Using these results, it was decided to adjust pH to 5.1 and denature at 45 °C as an initial purification step. Transferrin background was a lesser concern under any conditions since the peak area response was consistently lower than the target protein signature peptide response.

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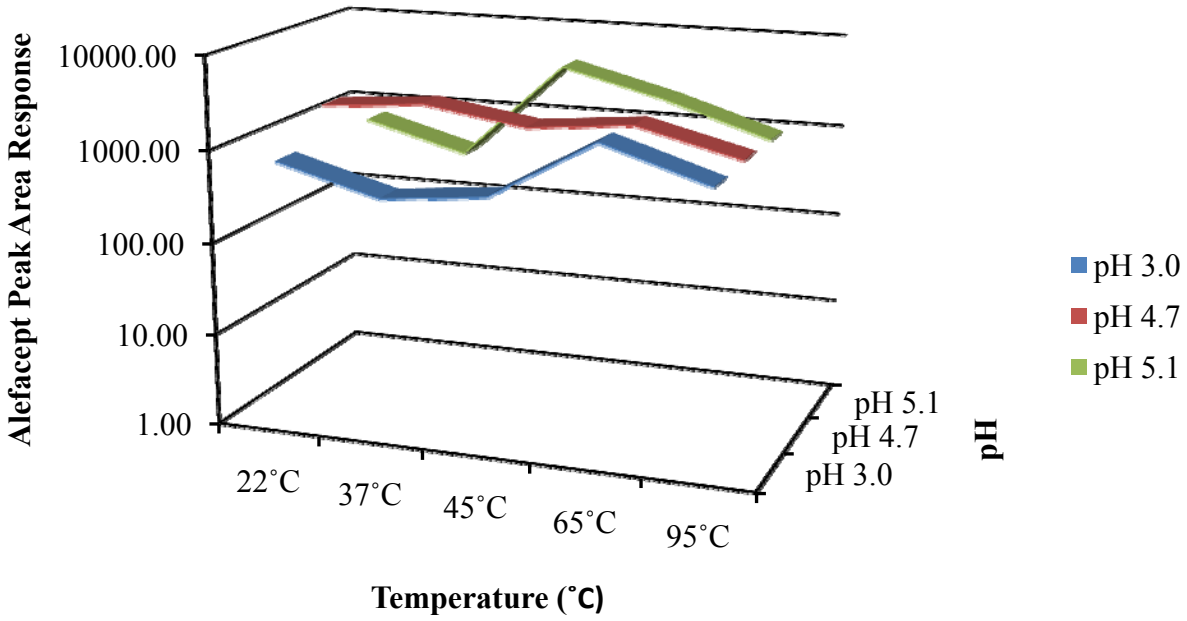


Figure 3.5 Three-dimensional graph of Alefacept (monitored SRM: 597→894) peak area response versus pH (3.0. 4.7, and 5.1) and temperature (22, 37, 45, 65, and 95˚C). Data shown represent mean±SD with samples n =3.

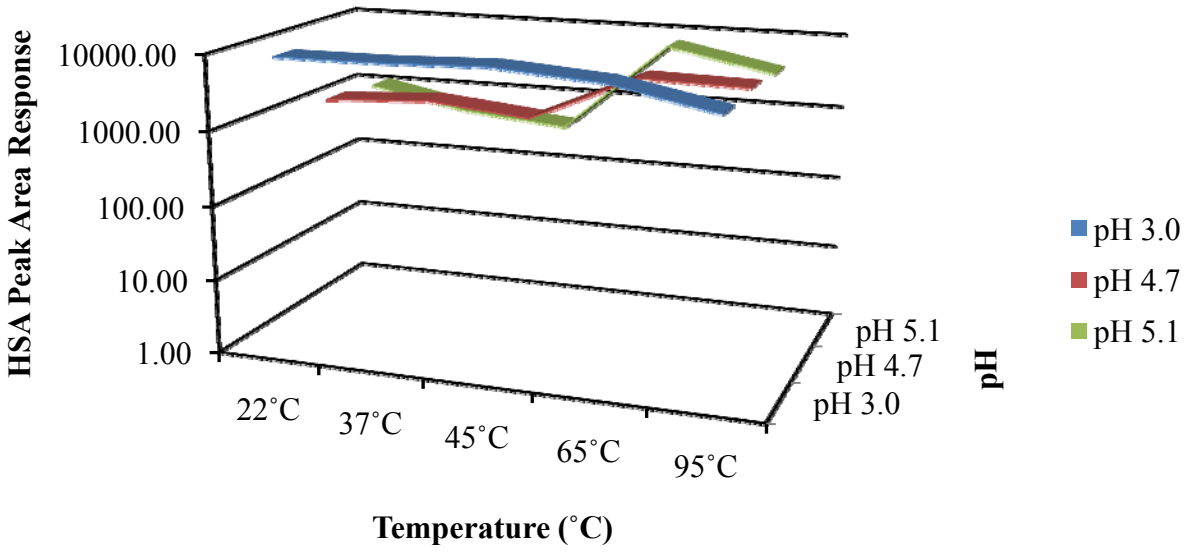


Figure 3.6 Three-dimensional graph of human serum albumin (HSA), (monitored SRM: 575→937) peak area response versus pH (3.0. 4.7, and 5.1) and temperature (22, 37, 45, 65, and 95˚C). Data shown represent mean±SD with samples n =3.

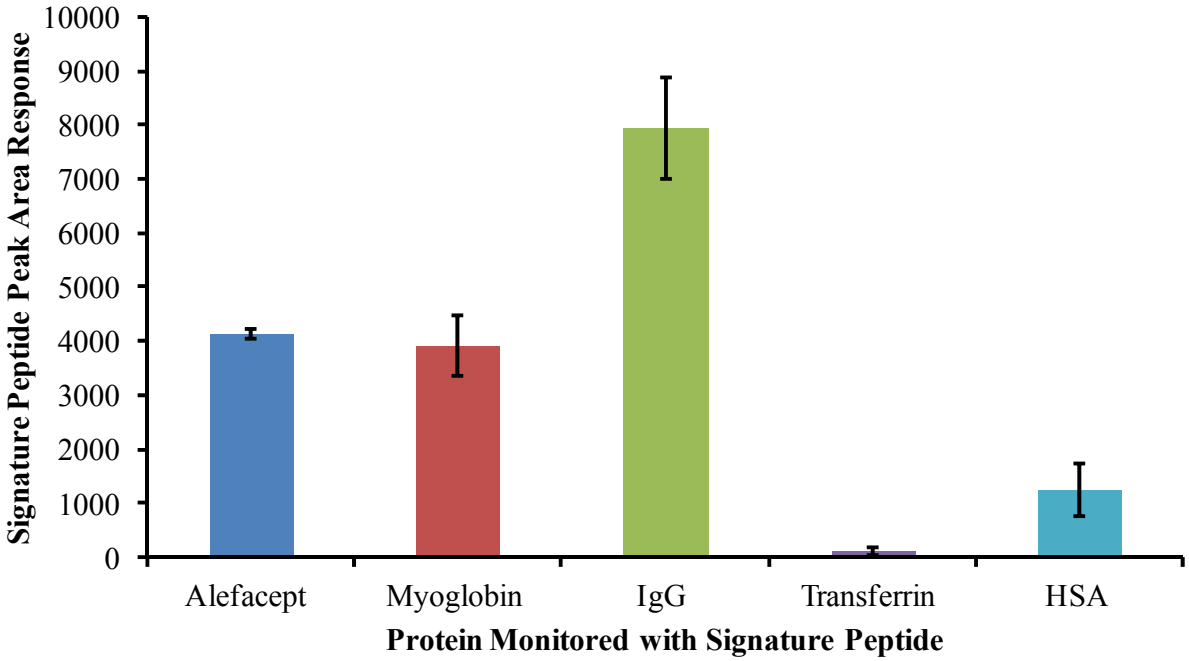


Figure 3.7 Maximum response of target proteins (Alefacept and myoglobin) monitoring signature peptides and minimum response of background proteins (immunoglobulin-IgG, transferrin, and human serum albumin-HSA) with optimal precipitation conditions of pH 5.1 and 45˚C. Data shown represent mean±SD error bars generated from samples n =3.

3.C.3 DIGESTION TIME

Figure 3.8 represents the results of the evaluation of trypsin digestion time at five different time points for triplicate analysis of controls (LQC, MQC, and HQC). The internal standard was also evaluated by averaging the peak area response obtained from the quality control samples for each incubation period. Analysis of variance was performed to determine if each time point was significantly different for quality controls and internal standard. This revealed the quality controls under these conditions are not equal and less incubation time yielded high variability. In order to further explain the results, the relative standard deviations (%RSD) were plotted in Figure 3.9. The comparison of %RSD revealed that the precision was less than 15% RSD employing 20 hour incubation for all quality controls and less than 10% RSD

for the internal standard response. Consequently, the 20 hour incubation time for trypsin digestion was chosen to be more appropriate.

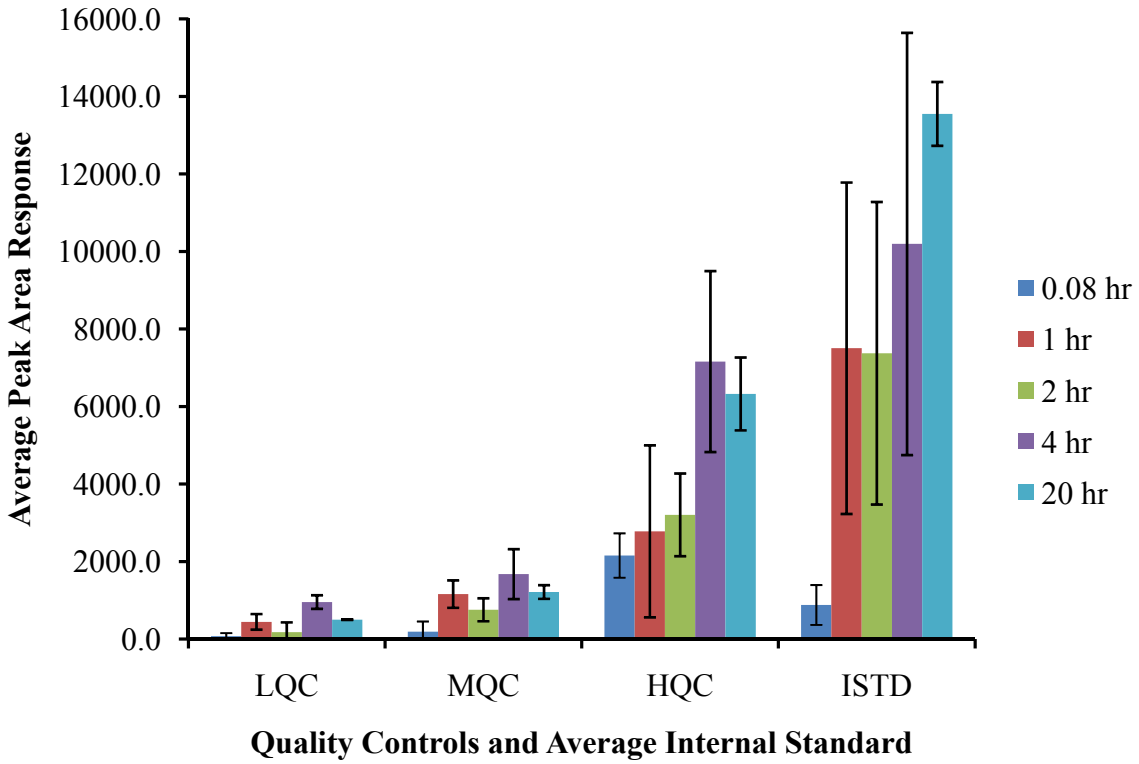


Figure 3.8 Incubation time for trypsin digestion results for LQC (600 ng/mL), MQC (2000 ng/mL), HQC (8000 ng/mL), and internal standard (ISTD) signature peptide peak area response with standard deviation error bars. Data represent QC's extracted n =3 and internal standard response with each respective digestion time point.

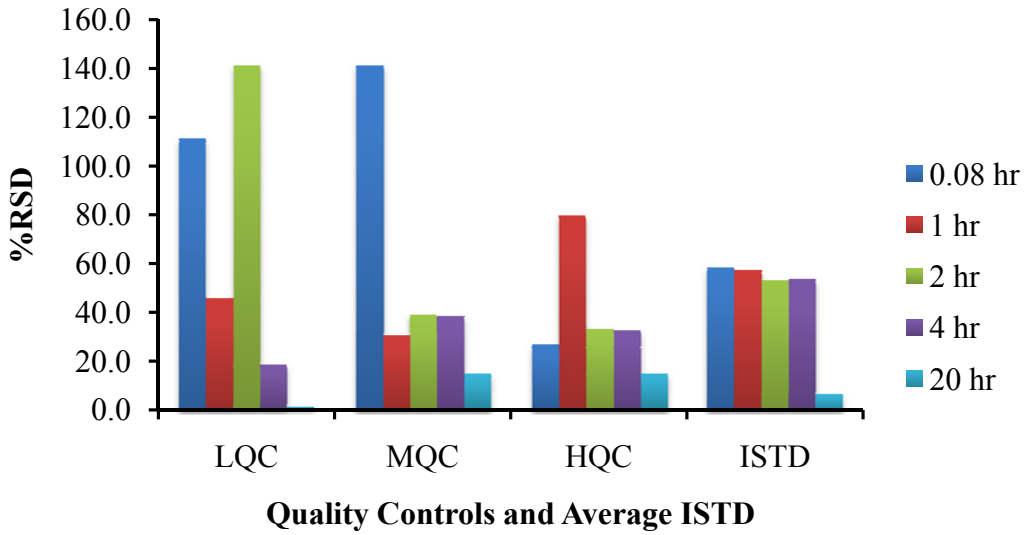


Figure 3.9 Comparison of relative standard deviation of 4 hr and 20 hr incubation times for trypsin digestion for each quality control and internal standard response.

3.C.4 MATRIX EFFECTS RESULTS

Ion profiles from the post-column infusion study revealed no clear suppression or enhancement at the retention times of myoglobin or Alefacept signature peptides. Phospholipids were monitored during the post-column infusion, which indicated their presence following extraction; however, all peaks were chromatographically resolved from Alefacept and myoglobin signature peptides. Even though phospholipids were present, ion profiles showed no suppression or enhancement as a result of phospholipids. In order to further evaluate matrix effects, a post-extraction addition study was performed as described in section 3.B.11. The percent matrix effect was calculated as follows: Matrix effects = 100 × (post spike peak area -solvent extracted peak area)/solvent extracted peak area. The studies revealed the presence matrix effects with the percent matrix effects resulting in -50.8% for LQC, -73.3% for MQC, and -70.8% for HQC. A multiple source matrix effect study was also performed as discussed in section 3.B.11. The results were acceptable (± 15% for precision and accuracy) for all individual human plasma lots and are shown in Table 3.2.

Table 3.2 Multiple Source Matrix Effect Evaluation, with each human plasma lot analyzed n =3.

	LOT 1	LOT 2	LOT 3	LOT 4	LOT 5	LOT 6
Average measured concentration (ng/mL) ± SD	636.77 ± 55.28	652.77 ± 40.81	658.76 ± 49.86	687.00 ± 93.36	639.33 ± 26.84	650.77 ± 40.55
%RSD	8.77	6.35	7.67	13.67	4.20	6.22
%DFN	6.11	8.88	9.88	14.50	6.66	8.44

3.D METHOD VALIDATION

3.D.1 LINEARITY AND LIMIT OF DETECTION

The peak area ratio of Alefacept to myoglobin internal standard signature peptides in human plasma was linear as a function of concentration over the range 250 to 10,000 ng/mL. The calibration curves yielded acceptable reverse calculated residuals ranging from -3.7% to 2.9%. Without use of the peak area ratios of analyte and internal standard signature peptides, calibration curves produced from absolute responses of Alefacept signature peptides versus actual concentration yielded reverse calculated residuals between -66% to 77%. Precision measured in terms of percent relative standard deviation ranged from 7.2% to 14.8%. The LLOQ for Alefacept was established at 250 ng/mL using the signature peptide approach. Data are presented Table 3.3.

Table 3.3 Reverse predicted concentrations for alefacept calibration standards (ng/mL)

	Alefacept Concentration (ng/mL)								
RUN Number	**250.00**	**500.00**	**750.00**	**1000.00**	**2500.00**	**5000.00**	**9000.00**	**10000.00**	**r^2**
Run 1	222.24	577.14	670.99	766.97	2210.20	4850.90	9322.03	9450.76	0.990
	294.54	430.22	752.00	988.89	2960.23	5970.43	9370.22	11200.03	
Run 2	264.53	479.54	707.01	1140.01	2330.22	4820.33	10300.01	8500.32	0.998
	248.37	470.23	795.20	839.22	2820.22	5480.33	7270.99	11500.01	
Run 3	236.87	570.26	659.99	1090.00	2560.00	4650.43	9930.01	8770.22	0.998
	252.32	508.02	747.44	990.44	2522.22	5100.23	8997.03	10102.01	
Mean	253.15	505.90	722.11	969.26	2567.18	5145.44	9198.38	9920.56	0.995
StdDev	24.85	58.17	52.11	143.11	284.32	496.45	1053.48	1243.04	0.005
%RSD	9.81	11.50	7.22	14.76	11.07	9.65	11.45	12.53	0.47
%DFN	1.26	1.18	-3.72	-3.07	2.69	2.91	2.20	-0.79	

Limit of detection (LOD) was determined by extracting three blank plasma samples and determining the peak to peak noise height at the elution time of alefacept signature peptide. Standard deviation of these samples was calculated and LOD calculation was performed by: 3 times the standard deviation of the blank divided by the slope of the calibration curve. The LOD was 44 ng/mL and was assessed to determine if detectability could potentially be lower than the current immunoassay approach (80 ng/mL).

3.D.2. SELECTIVITY

The selectivity of the method was evaluated in six different lots of blank human plasma. No endogenous peaks at the retention time of alefacept or myoglobin signature peptide were observed for any of the lots. Figure 3.3(A) shows a blank plasma representative chromatogram. Signature peptide selectivity was monitored throughout the entire study with three different product ions for the signature precursor ion. The MRM transitions monitored were 597→652, 597→894, and 597→781, which are depicted with the internal standard in Figure 3.6. No apparent interferences were present with any of the transitions and 597→894 was chosen as the quantitative signature peptide because of its greater intensity. The 636→716 transition was chosen for the myoglobin signature peptide. The +1 charge state of the myoglobin precursor ion (1272) was monitored but was not used due to its lower intensity than the +2 charged precursor ion (636). Figure 3.10 (A, B, C) shows raw chromatograms which are labeled according to Table 3.1. Analyte interference studies were also acceptable with no peaks detectable at the retention time of alefacept or myoglobin during these experiments.

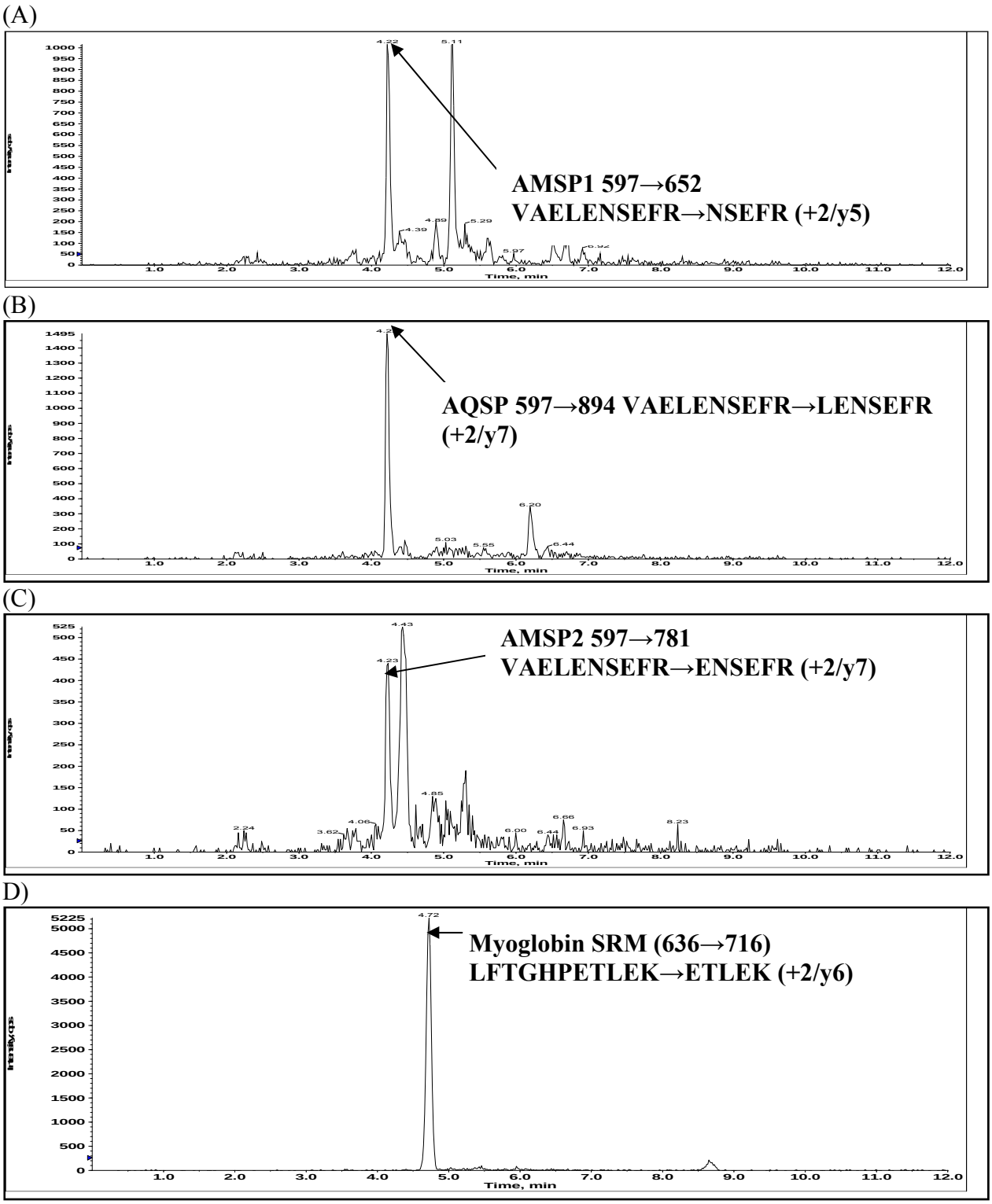


Figure 3.10 (A) alefacept monitored signature peptide 1 (AMSP1: 597→652), (B) alefacept quantitative signature peptide (AQSP: 597→894), (C) alefacept monitored signature peptide 2 (AMSP2: 597→781), (D) myoglobin internal standard signature peptide (ISTD: 636→716).

3.D.3 RECOVERY AND CARRYOVER

Relative recovery was performed to evaluate signature peptide recovery. Solvent extracted versus matrix extracted quality controls samples (LQC, MQC, and HQC) were analyzed in triplicate. Percent recovery was obtained by dividing the matrix extracted samples by the solvent extracted samples and multiplying by 100. The results revealed 33.5, 24.9, 25.0% recovery for the LQC, MQC, and HQCs, respectively. Carryover was evaluated and no response (below detection limits) was present at the retention times for alefacept or myoglobin signature peptides, and no biased versus LQC accuracy was observed.

3.D.4. PRECISION AND ACCURACY

The precision and accuracy data are summarized in Table 3.3. Inter and intra-day precision and accuracy were determined for the LLOQ QC, LQC, MQC and HQC samples. The intra-run accuracy (% bias) was within ± 10.6% (maximum RSD of 11.2%) for all the concentrations including the LLOQ. Additionally, inter-run accuracy was within ±10.9% (maximum RSD of 16.2%) for all concentrations. The dilution quality control precision and accuracy were less than 10%, 6.3 and 5.7, respectively. The results indicate that the use of a signature peptide for alefacept with an analogue internal standard was both accurate and precise according to established acceptance criteria.

Table 3.3 Inter and Intra-assay Precision and Accuracy*

QC (ng/mL)	Average measured concentration (ng/mL) ± SD	Inter-assay % bias	Intra-assay % bias	Intra-assay precision (%RSD)	Inter-assay precision (%RSD)
LLOQ (250.00)	242.42 ± 39.35	-3.03	-10.00	10.33	16.29
LQC (600.00)	665.35 ± 80.51	10.98	6.94	9.70	12.10
MQC (2000.00)	2086.77 ± 242.15	4.33	10.68	11.20	11.60
HQC (8000.00)	8169.27 ± 1033.35	2.11	1.95	9.03	12.65

*QCs analyzed n =6 in 3 separate analytical runs

3.D.5 STABILITY

PPS was assessed from re-injection reproducibility after storage of quality control samples in the auto sampler for 48 hours at 5°C. Processed LQC, MQC, and HQC samples were stable and %bias was 10.1, 6.7, and 11.3%, respectively. Storage stability was evaluated in the final validation run by using the freshly prepared day zero nominal concentrations to quantify the frozen quality controls processed. Processed LQC and HQC samples were stable for 21 days at -20°C and the %bias from day zero was 11.6 and 12.1%, respectively.

3.E CONCLUSIONS

Signature peptides employed as surrogates for target protein quantification is an attractive alternative to traditional immunoassays in biological fluids. Current methods for protein quantification by LC/MS have employed antibody-based purification through molecular recognition of target proteins and/or peptides in addition to immunodepletion of major background proteins (Berna et al., 2008; Ho et al., 2008; Kumar et al., 2010). Two dimensional solid phase extractions has been used to reach low ng/mL levels and achieve precise and accurate results without the use of antibodies(Yang, Z. et al., 2009). In this study, selective denaturation purification combined with the use of a carefully chosen protein analogue internal standard and

on-line extraction yielded precise and accurate results that meet FDA guidance acceptance criteria without the use of molecular recognition, immunodepletion, or off-line solid phase extraction. Internal standardization at the protein level was found to be necessary to compensate for matrix effects which hindered the ability to achieve precise and accurate data. Using Alefacept as a model therapeutic protein, the current method was able to achieve quantification limits necessary for evaluation of therapeutic levels (500-6000 ng/mL) without the need for multiple dilutions required in an ELISA assay that has a more limited range (80-900 ng/mL (Vaishnaw et al., 2002)). Employment of a more sensitive mass spectrometer may further lower quantification limits to be comparable to the enzyme-linked immunosorbent assay (ELISA) method (LLOQ = 80 ng/mL) (Vaishnaw et al., 2002).

CHAPTER 4

DETERMINATION OF OXYNTOMODULIN, AN ANORECTIC POLYPEPTIDE, IN RAT PLASMA USING 2D-LC-MS/MS COUPLED WITH ION PAIR CHROMATOGRAPHY
Drawn from article submitted to *J. Chromatograph B.*(April 2012)

4.A INTRODUCTION

The prevalence of obesity is evident, as over one-third of adults in the United States are considered obese. At present, there are no effective pharmacotherapy's to treat obesity and/or reduce its prevalence (CDC, 2011). In this context, however, recent studies have advocated that manipulation of the gut-brain axis for appetite control can be a physiologically natural means to reduce body weight. Gut-secreting anorectic polypeptides produced post-prandially in response to meal intake have received considerable attention as potential therapeutic drug entities for obesity (Wynne & Bloom, 2006). Oxyntomodulin (OXM) is one of such anorectic polypeptides, composed of 37-amino acids with a molecular weight of 4449 Daltons (Wynne & Bloom, 2006; Wynne, Park et al., 2006). In humans, its systemic blood levels under fasting condition are ~50 pM (0.2 ng/mL), which are elevated to ~150 pM (0.7 ng/mL) within 60 min in response to meal intake [7, 8]. Central and peripheral injections of OXM have demonstrated significant food intake suppression and reduced body weight in rodents and humans (Dakin et al., 2001; Dakin et al., 2004; Dakin et al., 2002; Wynne & Bloom, 2006; Wynne, Park et al., 2006). Nevertheless, OXM has also shown to be metabolized quite rapidly by dipeptidyl peptidase (DPP-IV) and neutral endopeptidase (NEP), apparently suffering from a short half-life in the systemic circulation and thus, short-lived pharmacological effects (Druce et al., 2009).

If OXM is to be developed as a therapeutic drug, one of the critical hurdles would be determination of OXM concentrations in biological matrices, to assess its pharmacokinetics following administration. Conventionally, highly sensitive immunoassays such as radioimmunoassay and enzyme-linked immunosorbent assays (ELISA) have been used for this purpose, even though their cross-reactivity to related molecules and time-consuming procedures are disadvantageous (Cohen et al., 2003). These methods are used primarily because liquid chromatography tandem mass spectrometry (LC-MS/MS) commonly used for small molecule drugs has not been considered suitable for quantification of large molecule drugs such as proteins and peptides until recently (Chang et al., 2005; Ismaiel et al., 2011; Pan et al., 2010; Xu et al., 2010; Yang et al., 2007).

LC-MS/MS quantitative analysis of intact polypeptides traditionally involves four processes, which include: 1) sample pre-treatment, 2) chromatography, 3) ionization, and 4) detection for quantification (Van Den Broek et al., 2008). Sample pre-treatment from plasma has included protein precipitation (PPE), liquid-liquid extraction (LLE), solid phase extraction (one and two-dimensional SPE), immunoaffinity purification (IAP), and online extraction using two-dimensional high-performance liquid chromatography (2D-LC), (Chang et al., 2005; Delinsky et al., 2004; Farthing et al., 2004; Ismaiel et al., 2011; Pan et al., 2010; Raffel et al., 1994; Van Den Broek et al., 2007, 2008; Yin et al., 2003). Each sample preparation has its advantages and disadvantages, with difficulty, method development time, and overall costs considered in method development as illustrated in Figure 4.1.

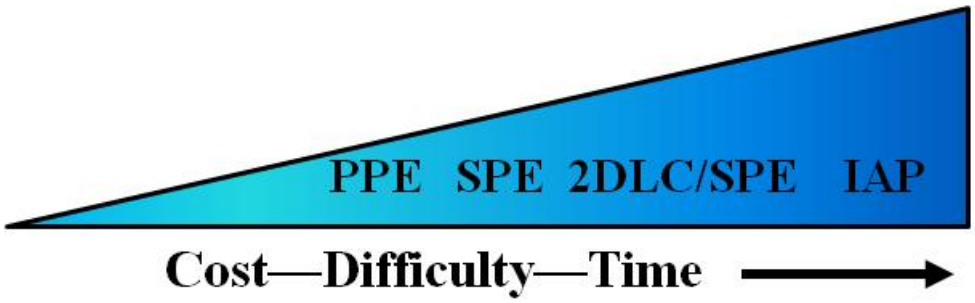


Figure 4.1 Sample preparations PPE, SPE, 2DLC/SPE, and IAP considerations scaled in order from least to most for cost, difficulty, and method development time.

Protein precipitation has been employed for peptide analysis previously; however, losses of target peptide in precipitate and/or insufficient reduction in matrix effects are significant disadvantages. Liquid liquid extraction has limitations for sample pretreatment since it is more suitable for nonpolar compounds, rather than peptides that have ionic functional groups (Van Den Broek et al., 2008). Solid phase extraction provides sufficient reduction in matrix effects and improved recovery compared to PPE; however, multiple steps can require lengthy optimization. Immunoaffinity purification (IAP) requires antibodies that can be expensive, require significant optimization, and may not be necessary.

Two-dimensional liquid chromatography (2D-LC) has recently emerged because of its ability to provide separation of protein or peptide mixtures (Ismaiel et al., 2011; Liu et al., 2009; Van Den Broek et al., 2008). One-dimensional reversed phase chromatography may be sufficient for chromatographic separation; however, 2D-LC offers the additional dimension that may be used as an online sample purification to reduce matrix effects and improve sensitivity. Ionization of peptides may be accomplished using electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), atmospheric pressure photoionization (APPI), or matrix-assisted laser desorption ionization (MALDI). However, positive electrospray ionization (ESI) has been well established as the method of choice for ionization of polar, large molecules such as peptides

(Hao et al., 2011). ESI enables multiply charged positive ions $[M + nH]^{n+}$ to form, which is suitable for the ionization of polypeptides. Formation of a multiple charge state ion will reduce the mass to charge ratio (m/z), which may allow for a polypeptide to be analyzed with a mass spectrometer with limited mass range (~ 2800 m/z).

Mass spectrometric detection of proteins and peptides has traditionally employed ion trap, time-of-flight (TOF), or hybrid quadrupole time-of-flight (Q-TOF) mass analyzers. These mass analyzers offer accurate mass measurements for the identification and relative quantification of proteins and peptides (Plumb et al., 2012). However, these mass analyzers lack the sensitivity necessary for absolute quantification of peptides due to their significantly lower duty cycle as compared to triple quadrupole mass spectrometers operating in selection reaction monitoring mode (Plumb et al., 2012). The duty cycle difference of 100% for tandem mass spectrometers versus 20% active for TOF type instruments can result in a 5 to 10-fold difference in sensitivity (Plumb et al., 2012). Van den Broek et al. revealed in a recent review that larger peptides (> 3200 Da) normally do not employ SRM (or multiple reaction monitoring: MRM) for quantification of peptides in biological fluids. It is understandable that the formation of MRM transitions for large peptides is challenging due to the potential to produce too many fragments from high collision energy, which yields lower sensitivity (Rogatsky et al., 2007).

In this chapter, OXM determination in rat plasma by LC-MS/MS has been achieved, and the method has been validated using the FDA bioanalytical guidelines (FDA, 2001). This method was performed without enzymatic digestion to obtain signature peptides as described in chapter 3. The method was unique in the employment of an optimized μelution anion exchange SPE, two-dimensional LC-MS/MS, and reversed phase ion pair chromatography. The method required 100 μL of rat plasma to obtain a lower limit of quantification of 1 ng/mL and a chromatographic

run time of less than 10 minutes. A stable isotope-labeled internal standard was spiked into each sample. Following validation, the method was used for determination of the pharmacokinetic profile of OXM in rats following intravenous injection.

4.B EXPERIMENTAL

4.B.1. CHEMICALS AND REAGENTS

Oxyntomodulin (OXM: molecular weight of 4449.9 g/mol) was purchased from Bachem Americas (Torrance, CA, USA). Isotope-labeled OXM (OXM_{IL}) was synthesized by Open Biosystems, Inc. (Huntsville, AL, USA), replacing all arginines (R) at 17^{th}, 18^{th}, 31^{st} and 33^{rd} positions with heavy arginine isotopes. This labeling resulted in an average mass of 4491.0 g/mol, which was approximately a +40 Da shift from native OXM. DPP IV inhibitor was purchased from Millipore (St. Charles, MO, USA). Formic acid, trifluoroacetic acid (TFA), 3-nitrobenzyl alcohol (3-NBA), sodium hydroxide, hydrochloric acid, bovine serum albumin (BSA), and ammonium hydroxide were obtained from Sigma-Aldrich (St.Louis, MO, USA). Polypeptides angiotension-1, obestatin, rat ghrelin, horse heart myoglobin, polypeptide YY (PYY_{1-36}), insulin growth factor-1 (IGF-1), parathyroid hormone (PTH), and leptin were also purchased from Sigma-Aldrich (St.Louis, MO, USA). Polypeptide YY_{3-36} (PYY_{3-36}) was purchased from Bachem Americas (Torrance, CA, USA). Deionized water was obtained in-house using a Nanopure Diamond water system from Barnstead International (Dubuque, IA, USA). Methanol, isopropanol, and acetonitrile were purchased from Burdick and Jackson (Muskegon, MI, USA). Sodium heparinized rat plasma was obtained from BioChemed Services (Winchester, VA, USA). All reagents were HPLC grade unless otherwise noted.

4.B.2 MATERIALS AND EQUIPMENT

Heparinized rat plasma samples were aliquoted into 2 mL conical bottom 96-well plates (VWR International, Westchester, PA, USA). Oasis® brand mixed-mode anion exchange (MAX) µelution plates (30 µm, 2mg/well; Waters Corporation, Milford, MA, USA) were used for solid phase extraction (SPE) of the samples using automated pipetting on a Quadra 96 Model 320 Tomtec (Hamden, CT, USA). Centrifugation and mixing, respectively, were carried out using an Eppendorf 5804R centrifuge (Hamburg, Germany) and a multi-tube vortex mixer (VWR International or a Talboys Advanced Model 1000MP Microplate Shaker (Troemner: Thorofare, NJ, USA). A syringe pump from Harvard Apparatus (Holliston, MA, USA) was used to infuse OXM solutions for tuning optimization and post-column infusion studies.

4.B.3 CHROMATOGRAPHY AND MASS SPECTROMETRY EQUIPMENT

The first dimension (loading step) of 2D-LC was performed on a Waters Acquity UPLC®. The second dimension (eluting pump) used high performance liquid chromatography (HPLC) with the following equipment: Shimadzu system controller SCL-10A VP, two LC-10AD VP pumps, solvent degasser DGU14A (Shimadzu, Kyoto, Japan). A 10-port Cheminert switching valve and a microelectric actuator obtained from Valco Instruments Co. Inc. (Houston, TX, USA) was used to perform on-line column trapping. The mass spectrometer was an API 4000Qtrap hybrid triple quadrupole/ linear ion trap from AB Sciex (Foster City, CA, USA) with Analyst 1.5 data acquisition software. The data analysis was performed using Quantitation Wizard processing software that accompanies Analyst. Bioanalyst software AB Sciex (Foster City, CA, USA) was used for peptide reconstruction to evaluate charge state distribution during

tuning optimization. All nitrogen gas was generated from a Parker Hannifin (Haverhill, MA, USA) Tri-Gas Generator LC/MS 5000.

4.B.4 CHARGE STATE DISTRIBUTION AND PRODUCT ION FORMATION STUDY OF OXYNTOMODULIN AND COMPARISON TO OTHER POLYPEPTIDES

Electrospray ionization is a soft ionization technique that has the capability of producing intact multiply charged ions from biomolecules (Fenn et al., 1990). The charged state distribution of an intact biomolecule will normally yield mass to charge ratios (m/z) between 500 and 3000 Daltons depending on the zero charge mass (Iavarone et al., 2001). Having this range of m/z offers an advantage to quantification of large molecules using lower mass range mass analyzers (< 3000 Daltons) such as a triple quadrupole mass spectrometer. Part of this investigation was to evaluate the factors that may affect charge state distribution and product ion formation of OXM. Along with OXM, ten other polypeptides were evaluated to observe possible trends with charge state distribution and the formation of product ions. Polypeptides were chosen spanning a range of 1296 to 16,951 Daltons, which are listed in section 4.B.1. These polypeptides were chosen to represent a diverse group of peptides with a range of molecular weights that may be feasible to produce a m/z ratio fit for the mass spectrometer and yield product ions to form SRM transitions. Given that the sequence of OXM and the selected peptides contain basic amino acid residues, positive ions tend to be localized on these residues and they will most likely ionize better with positive polarity (Krusemark et al., 2009). It has been established that multiple factors can affect charge state distribution such as solvents, instrument parameters, primary structure and conformation of a protein (Krusemark et al., 2009). Solvents such as glycerol and 3-nitrobenzyl alcohol have been shown to increase charge state and improve fragmentation (Krusemark et al.,

2009; Sze et al., 2002). Therefore, we investigated the charge state distribution of ten polypeptides, including oxyntomodulin to determine the feasibility for quantification of the intact biomolecule using a triple quadrupole mass spectrometer with these additives in mind. Our intention was to maximize the detectability of a particular charge state (m/z) and form product ions for selected reaction monitoring (SRM).

The first part of this study was to determine the charge state distribution of polypeptides in various solvent mixtures and optimize precursor ions (intact ionized polypeptide without fragmentation). Different combinations of acetonitrile (ACN) or methanol (MeOH), water, 3-nitrobenzyl alcohol (3-NBA), glycerol, and formic acid (FA) were prepared. Each solvent combination was used as a diluent for each polypeptide to prepare a final concentration of 5µg/mL. The solvent (S1 to S12) ratios and their description are shown in Table 4.1.

Table 4.1 Twelve Solvent Mixtures used in charged state distribution evaluation of polypeptides

Solvents	Description
S1	25:75 ACN:H2O
S2	25:75 MeOH:H2O
S3	25:74:1 ACN:H2O:3-NBA
S4	25:74:1 MeOH:H2O:3-NBA
S5	25:74:1 ACN:H2O:glycerol
S6	25:74:1 MeOH:H2O:glycerol
S7	25:74:1 ACN:H2O:formic acid
S8	25:74:1 MeOH:H2O:formic acid
S9	24:74:1:1 ACN:H2O:3-NBA:formic acid
S10	24:74:1:1 MeOH:H2O:3-NBA:formic acid
S11	24:74:1:1 ACN:H2O:glycerol:formic acid
S12	24:74:1:1 MeOH:H2O:glycerol:formic acid

Note: ACN = acetonitrile, MeOH = methanol, 3-NBA = 3-nitrobenzyl alcohol, H_2O = deionized water

The solvent mixture containing a polypeptide at 5 µg/mL was infused into the mass spectrometer at a flow rate of 10µL/minute. This experimental schematic is shown in Figure 4.2.

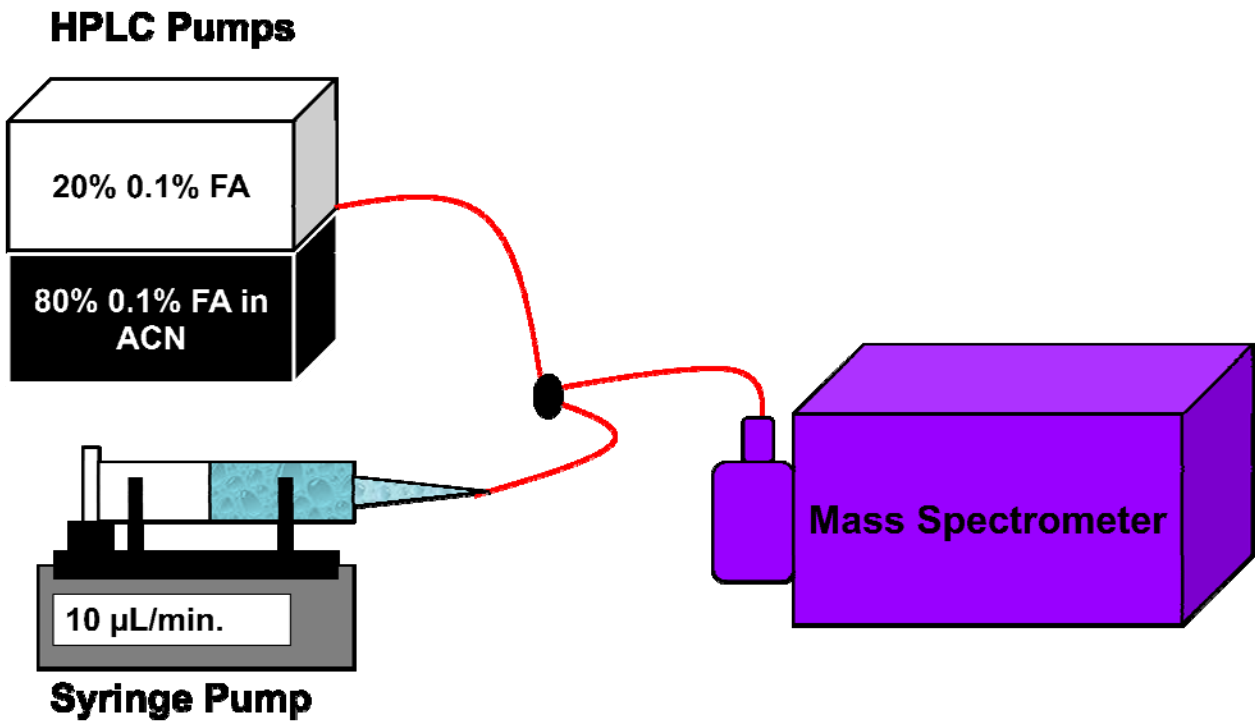


Figure 4.2 Experimental schematic of charge state distribution experiment for polypeptides. Each peptide was diluted into mixtures at 5 µg/mL, placed into a 1 mL syringe, and infused at 10 µL/minute coupled with mobile phase at a flow of 200 µL/min. with 80% 0.1% formic acid in acetonitrile (ACN) and 0.1% formic acid (FA).

Mobile phase was also tee'd into the infusion experiment at a flow rate of 0.200 mL/min with 80% acetonitrile and 20% 0.1% formic acid. Infusion experiments were performed with and without mobile phase flow by evaluation of Q1 (1^{st} quadrupole) MS scans (i.e. full scan). Each mixture underwent optimization of each instrument parameter, which included: ramping declustering potential and entrance potential, optimization of gases, source temperature, and turbo ion voltage. Following the observation of maximum Q1 full scan intensity, a peptide reconstruction was performed using Bioanalyst to determine the charge state distribution of the mass spectrum obtained. The list of ions reconstructed from the mass spectrum was evaluated for the presence of the polypeptide, and the charge state distribution was observed. Following optimization of precursor ions for each solvent, formation of product ions was determined for the minimum, maximum, and most intense charge state of each polypeptide. In product ion mode, instrument parameters were again ramped for optimal performance. Collision energy was ramped slowly from (0 to 150) due to the potential to generate multiple fragments with weak intensity that are from multiply charged biomolecules (Chen, 2010). As a final tuning optimization, selected reaction monitoring (SRM) transitions were evaluated for maximum intensity with optimal parameters applied for each solvent and respective charge state that formed product ions for each polypeptide (if applicable).

4.B.5 OPTIMIZATION OF REVERSED PHASE ION PAIR CHROMATOGRAPHY

Reversed phase chromatography was investigated with and without the presence of an ion pairing reagent by monitoring the SRM transition of oxyntomodulin for chromatographic performance (i.e., peak shape and intensity). It has been observed that incorporation of modifiers into reverse phase chromatography may improve sensitivity and resolution for intact proteins

(Valeja et al., 2010). Traditionally, 3-NBA ($C_7H_7NO_3$), with a pKa of 13.82, has been used as a matrix for fast atom bombardment and matrix assisted laser desorption ionization (MALDI) (Cerveau, 1992; Chan, 1992). Following optimization of SRM transitions, it was observed that signal intensity improved more than an order of magnitude in the presence of 3-nitrobenzyl alcohol (3-NBA) in the solvent mixture. Therefore, 3-NBA was evaluated as a potential ion pairing agent to improve chromatography and enhance sensitivity. Initially, 3-NBA was added into the aqueous (mobile phase A) and organic mobile phase (mobile phase B) at seven molar concentrations (0.0, 1.3, 3.3, 6.5, 13.1, 32.7, 65.3 mM). A slow gradient (5% A over 1 minute, 80% B from 1 to 5 minutes, hold for 1 minute at 80% B, and equilibrate column for 2 minutes again at 5% A) onto the eluting column described in the next section (4.B.6) was used to evaluate response and chromatographic performance. Following the evaluation of each molar concentration of 3-NBA, six pH adjustments (pH = 2.3, 4.7, 5.7, 6.4, 7, 10) to the mobile phase were evaluated.

4.B.6 GRADIENT METHOD FOR 2D-LC ANALYSIS OF OXYNTOMODULIN

Samples were injected (30 µL injection) with a Waters Acquity UPLC® integrated autosampler. In the first dimension of 2D-LC, the loading column was an ACE C8, 5 um, 2.1 x 50 mm, 300 Å. (MacMod, Chadds Ford, PA, USA). The second dimension of 2D-LC used a Waters XBridge BEH300 C18 Column, 5 µm, 2.1 x 100 mm, 300 Å as the analytical (eluting column). Mobile phase A consisted of 0.1% (6.5 mM) 3-NBA (pH 4.7) v/v and mobile phase B consisted of 0.1% (6.5 mM) 3-NBA in acetonitrile v/v. The Acquity employed two washes: a weak wash consisted of 1:1 acetonitrile:water and the strong wash consisted of 80:20 acetonitrile:water with 0.1% 3-NBA and 0.1% formic acid. In the first dimension, the samples

were loaded onto the ACE C8 column maintained at 50 ºC using a flow of 0.250 mL/min. and mobile phase composition of 95% A for 1 minute. After a 1 minute loading time, the diverter valve switched to position B and the gradient initiated. Mobile phase A and B were combined together with a mixer and the elution conditions started with 5% mobile phase B (0.1% 3-NBA in acetonitrile) and increased to 90% B over 3.5 minutes. Isocratic conditions were held for two minutes at 90% B, followed by a quick switch (5.6 minutes) back to the original conditions of 95% A. The columns were equilibrated for approximately 2.5 minutes for a total run time of 8 minutes. During the elution step while the diverter valve was in position B from 5.6 to 7 minutes, the loading column was washed at a mobile phase composition of 50% A at a flow of 0.4 mL/minute. Continuous flow was also permitted into the mass spectrometer with the eluting pumps.

4.B.7 MASS SPECTROMETER PARAMETERS

The mass spectrometer was operated in the positive electrospray ionization (ESI) mode with selected reaction monitoring (SRM) of OXM and its isotope labeled internal standard (OXM_{IL}). Charge state distribution and evaluation of the most intense SRM transitions was finalized following the investigation discussed in section 4.B.4. Tuning was performed on several potential SRM transitions and all parameters were re-evaluated such as gases, source temperature, declustering potential, collision energy, ion spray voltage, and collision exit potential.. A 2 µg/mL solution of OXM or its labeled internal standard was prepared in solvent 2 (25:74:1 ACN:H_2O:3-NBA) and was used as a tuning solution for direct infusion at 10 µL/min. tee'd with mobile phase at a flow rate of 0.20 mL/min using a composition of 80% Mobile phase B. The mass spectrometer parameters were tuned and optimized to achieve maximum sensitivity

for each SRM transition which resulted in the following parameters: source temperature 325 ˚C, ion spray voltage 5500 V, gas 1 (GS1) 66, gas 2 (GS2) 30, collision activated dissociation (CAD) was set on high, entrance potential (EP) was set to 9 V, collision exit potential (CXP) 16 V, and channel electron multiplier (CEM) was set at 2500 V. Oxyntomodulin and OXM_{IL} eluted at approximately 3.95 and 3.91 minutes, respectively. SRM transitions, collision energies (CE), declustering potential (DP), and dwell times used during this study are shown in Table 4.2. Each peptide was given a name with its precursor ion charge state $(M+nH)^{n+}$ in parentheses. Four OXM transitions (SRM's) were monitored; however, only the 636.9→666.9 was used for quantification due to its sensitivity and selectivity.

Table 4.2 Selected reaction monitoring (SRM) transitions and selected mass spectrometer parameters of OXM and OXM_{IL}.

Peptide	Q1	Q3	DP (v)	Collision Energy (v)	Dwell time (msec)
OXM (+7)	636.9	666.9	70	27	350
OXM_{IL}	642.3	676.6	76	29	200
OXM $(+8)^{m1}$	557.4	666.9	70	27	200
OXM $(+8)^{m2}$	557.4	225.4	45	54	200
OXM $(+7)^{m3}$	636.9	110.1	45	78	200

m1, m2, m3 = SRM's used as monitoring transitions and not quantified

4.B.8 STOCK SOLUTION AND WORKING SOLUTION PREPARATIONS

OXM was diluted with 1% BSA in deionized water to prepare a stock solution at 0.5 mg/mL. This stock solution was subsequently diluted with 1% BSA to prepare working solutions. All solutions were subaliquotted and stored at -70 ºC. The working solutions were used to prepare fresh calibration standards and frozen quality controls. As OXM_{IL} was provided at 5 pmol/µL in 5% acetonitrile (22.45 µg/mL) by Open Biosystems, 10 aliquots of 0.2 mL were prepared and stored at -70 ºC. OXM_{IL} spiking solution was prepared fresh daily at 2.25 µg/mL

by diluting the 0.2 mL aliquots with 2 mL of 5% acetonitrile. All working solutions were prepared just before spiking into rat plasma.

4.B.9 PREPARATION OF CALIBRATION STANDARDS AND QUALITY CONTROL SAMPLES IN RAT PLASMA

Pooled sodium heparinized rat plasma from at least two donors was used to prepare the calibration standards and quality controls. A volume of 0.020 mL of each OXM intermediate solution (20, 40, 100, 200, 500, 1,000, 2,000, 5,000, 10,000, 20,000 ng/mL) was spiked into 0.4 mL of rat plasma for each calibration standard. A total of ten calibration standards were freshly prepared for each analytical run at concentrations of 1, 2, 5, 10, 25, 50, 100, 250, 500, and 1000 ng/mL. Quality controls were prepared in two different pools of rat plasma at the same concentrations. One pool was the same rat plasma used to prepare the calibration standards. The other plasma pool contained DPP-IV inhibitor at a concentration of 20 µL per milliliter of rat plasma. A volume of 0.4 mL of a 20,000 ng/mL OXM intermediate solution was spiked into 10 mL of rat plasma in a volumetric flask to obtain the highest quality control (HQC = 800 ng/mL). A 10,000 ng/mL intermediate solution was spiked (0.060 mL) into a 10 mL volumetric flask and diluted to 10 mL with rat plasma to make a medium QC (MQC) at 60.0 ng/mL. A 1,000 ng/mL intermediate solution was used to prepare the lower limit of quantitation (LLOQ) QC and the low QC (LQC), by spiking 0.025 mL into a 10 mL volumetric flask for the LQC (2.5 ng/mL) and 0.010 mL into a 10 mL volumetric flask to prepare the LLOQ (1.0 ng/mL). A dilution control was prepared at 5 times the highest calibration standard (5,000 ng/mL), to evaluate dilution of samples into the calibration range. The dilution control was prepared by performing a ten-fold dilution with blank rat plasma to obtain a concentration within the calibration range (50.0

ng/mL). Quality control samples were freshly prepared for validation to determine the day zero nominal value for storage stability studies. QC samples were sub-aliquotted for daily use (0.250 mL) into lobind polypropylene tubes (Eppendorf, Hauppauge, New York, USA) and stored at -70ºC until analysis.

4.B.10 SAMPLE PREPARATION

All samples were thawed in an ice water bath and vortex mixed. A 100 µL sample of rat plasma was aliquotted into a 2 mL conical bottom 96-well plate, along with 100 µL of 4% phosphoric acid, and 20 µL of internal standard spiking solution (2.25 µg/mL). While samples mixed for 10 minutes, the MAX µelution was conditioned with 200 µL of methanol followed by 200 µL of 4% phosphoric acid. Samples were centrifuged prior to loading, and then slowly loaded onto the SPE (2 x 110 µL) by increasing the vacuum pressure from 0 to 5 psi after each loading step. The SPE was then washed with 200 µL of 5% ammonium hydroxide and 200 µL 5% acetonitrile. The plate was blotted dry and samples were eluted with 5% TFA in 75:25 acetonitrile/water v/v (2 x 25 µL). Eluent was collected into a 1 mL 96-well plate (Waters Corp.). Finally, samples were diluted with 25 µL of 25:74:1 acetonitrile/water/3-NBA and mixed briefly. A 25 µL sample was injected onto the LC-MS/MS.

4.C VALIDATION AND SAMPLE ANALYSIS

4.C.1 MATRIX EFFECTS EVALUATIONS AND RECOVERY

Post-column infusion studies, post-extraction addition, and evaluation for phospholipids as potential matrix effects were all evaluated. A post-column infusion study was performed by injecting an extracted blank rat plasma sample while infusing a 2 µg/mL oxyntomodulin solution

at 10 µL/min. The resulting profile was evaluated for chromatographic co-elution with OXM and changes in the ESI response of OXM that may indicate suppression or enhancement. During the post-column infusion study, phospholipids were monitored using the in source-SRM transition 184→184 as suggested by Little et al (Little et al., 2006). As suggested by Matuszewski et al, a more quantitative approach to assessing matrix effects was carried out using a post-extraction addition study, where a percent matrix effects (%ME) is determined (Matuszewski et al., 2003). Unextracted "clean" samples were prepared as final extracted concentrations (3.33, 80.0, 1067 ng/mL) from the LQC (2.5 ng/mL), MQC (60 ng/mL), and HQC (800 ng/mL) in the final sample solvent (75% of: 5% TFA in 75:25 acetonitrile/water /25% of: 25:74:1 acetonitrile/water/3-NBA) and compared to post-spiked blank extracts with the same quality control concentrations. A percent matrix effect was determined using the following equation: %ME = ((peak area response of post-extracted sample/peak area response of clean sample) -1)*100.

A relative recovery experiment was also performed for OXM in rat plasma, by comparison of pre- and post-spiked extracted quality control samples. QC's were extracted n =6 and percent recovery was calculated as follows: %recovery = (extracted mean peak are response/unextracted mean peak area response) x100.

4.C.2 LINEARITY

A total of ten calibration points were freshly prepared as described in section 4.B.9 in sodium heparin treated rat plasma. Each analytical run employed duplicate analyses of calibrators. Peak area ratios of OXM:OXM_{IL} and each concentration level was used to construct the calibration curve. Calibration curves used linear regression with a $1/x^2$ weighing factor for the determination of OXM concentrations.

4.C.3 PRECISION AND ACCURACY

Three quality controls were used to assess inter-day precision and accuracy, which were analyzed in at least three separate runs. QC concentrations were calculated from the calibration curves analyzed in the same run. Intra- and inter-assay precision and accuracy were determined by extracting LLOQ, low (LQC), medium (MQC), and high quality controls (HQC) in six replicates for intra-assay performance. Furthermore, a dilution control (DQC, 5000 ng/mL) was evaluated for intra-assay performance with six replicates. Quality controls were extracted in triplicate in five additional runs.

4.C.4 SELECTIVITY

Six individual rat plasma samples were used to assess selectivity. Each individual rat plasma sample was extracted in duplicate with and without the presence of internal standard as described in section 4.B.10. Acceptable selectivity was established by the fact that chromatographic peaks that co-elute with OXM were less than 20% of the peak area of the average LOQ samples. No more than 5% contribution from OXM to its isotope labeled internal standard signal was acceptable. This experiment was performed by extracting a double blank blank with no internal standard added as well as a high standard (1,000 ng/mL) without internal standard.

4.C.5 STABILITY EVALUATIONS AND CARRYOVER

Stock solution stability was evaluated in a stress test. The stress test experiment was performed by comparison of a 0.5 mg/mL stock solution kept at -70ºC to an aliquot of the same stock solution that was left at room temperature for four hours. Both aliquots of the stock solution were diluted in the final extraction solvents described in section 4.B.10 and injected in triplicate. Average peak area responses of the diluted stock solutions were compared.
The first validation run was used to determine day 0 nominal concentrations of quality controls for long term storage stability. Each QC was stored at -70ºC in sub-aliquots for daily use. Storage stability was determined by using the day zero nominal values established for the low and high QC's. Following an appropriate storage time, low and high QC's were analyzed and concentrations were determined with freshly prepared calibrators. Post-preparative stability (PPS) was performed to determine an extended amount of time that extracted samples could be left in the refrigerated autosampler in the event that re-injection was necessary due to instrument malfunction. Carryover was assessed by injecting final extraction solvent samples immediately after the highest calibration standards (1,000 ng/mL) in an analytical run.

4.C.6 CROSS TECHNOLOGY VALIDATION OF OXM IMMUNOASSAY

As part of this study, we also wanted to compare sample results using a peptide immunoassay versus our validated LC-MS/MS assay. A commercially available competitive immunoassay was used according to the manufacturer's instructions for rat serum and plasma (Bachem Americas (Torrance, CA, USA). Briefly, the peptide enzyme immunoassay employed an antibody coated plate that was spiked with antiserum and incubated at room temperature for an hour. Standards and samples (in diluent) were then aliquotted into the wells and incubated for

another two hours. A biotinylated tracer was added and the plate was incubated overnight in the refrigerator. The plate was equilibrated to room temperature and washed five times with buffer using an automated plate washer. Streptavidin-horseradish peroxidase (HRP) was then added, and the plate was mixed for one hour. After another five plate washes, the substrate TMB (3,3′,5,5′-tetramethylbenzidine) was added and the plate was mixed for forty-five minutes. The reaction was terminated with 2 N hydrochloric acid (HCl) and the absorbance was read at 450 nm within ten minutes.

4.D RESULTS AND DISCUSSION

In this study, we employed a modified solid phase extraction coupled with 2D-LC/MS/MS to monitor a multiply charged peptide for quantitative analysis of OXM in rat plasma samples. The combination of these parameters allowed for a robust method to quantify OXM in rat plasma with a lower limit of quantification (LLOQ) at 1 ng/mL (Figure 4.7A). Optimization of solid phase extraction, multiply charged SRM, and two-dimensional reversed phase ion pair chromatography was necessary to meet the demands of quantifying this polypeptide.

4.D.1 MASS SPECTROMETRY OF OXYNTOMODULIN AND OTHER POLYPEPTIDES

Charge state distribution and product ion formation were investigated using different solvents and eleven polypeptides as described in section 4.B.4. The objective was to achieve conditions that would reduce the predominate m/z to achieve a more suitable mass range and ultimately improve the intensity for this m/z for formation of SRM's. Solvent effects proved to

play a role in charge state distribution and product ion formation for most polypeptides investigated. Lavarone et al. first observed this with cytochrome c using 3-NBA and glycerol to increase charge state and abundance (Lavarone et al., 2001). This increase in charge state may have been the result of using a low vapor pressure solvent, which could have determined the electrospray droplet evaporation characteristics (Samalikova et al., 2005). Solvent additives such as 3-NBA have a high surface tension, which also may play a role in increased charge states and abundances (Krusemark et al., 2009).The solvent mixture (S2) which contained 25:74:1 (ACN:H_2O:3-NBA), improved intensity of the most abundant charge state of OXM by as much as one order of magnitude. The charge state (z) distribution for this solvent mixture consisted of a lowest charge state equal to +4, a highest charge state equal to +9, and the most abundant charge state equal to +8. This mass spectrum is labeled for each OXM charge state in Figure 4.3. Note that the spectrum shown in Figure 4.3 is from a form of OXM lacking part of the N-terminus peptide bond (C-NH_2), which is -28 daltons less, making the molecular weight 4421 Daltons. This standard is no longer commercially available and the entire OXM structure (4449 Da) was used for the remaining parts of this study, including the validation.

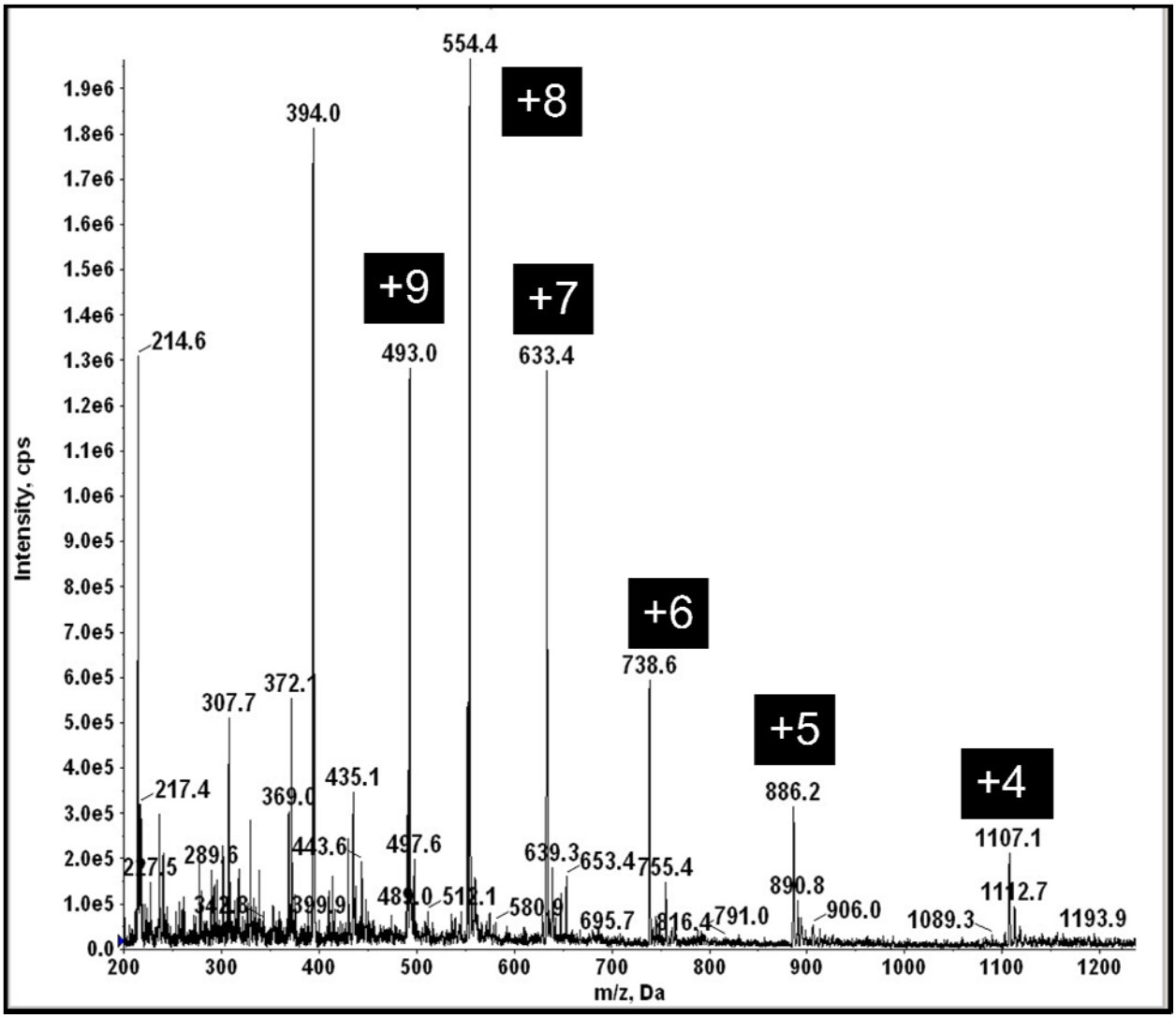


Figure 4.3 Mass Spectrum illustrating charge state distribution of OXM in solvent 3 (25:74:1 ACN:H_2O:3-NBA).

Depending on the polypeptide, the most abundant charge state was +3 to +16 for any solvent mixture. Therefore, it may be feasible to reduce m/z sufficient enough for polypeptides in the range evaluation, which was 1296 to 16,951 Daltons. The +6 or +8 charge state of OXM was the most abundant depending on the solvent; however, the lowest and highest charge also varied between each solvent and had an effect on the formation of reliable product ions. This solvent dependency on charge state distribution was similar with all polypeptides. These results are shown in Table 4.3, where the charge state distributions of polypeptide are highlighted. In addition, the intensity and charge state distribution as a result of solvent mixtures are shown for all eleven polypeptides in Appendix 1.

Overall conclusions made from the charge state distribution, product ion formation, and solvent comparisons of the ten selected peptides were six-fold. 1) The average abundant charge state (z) of the polypeptides (mean±SD) was +8z±3.5 for any solvent mixture. 2) The median charge states of each polypeptide formed more product ions than any other charge states other than parathyroid hormone, leptin, and myoglobin. The median was evaluated for each polypeptide because there was a correlation with middle charge states (i.e. +7 and +8 for OXM) and the formation of product ions. It has been observed that charge state distribution for polypeptides is gaussian shaped (Strupat, 2005). Therefore, average and median charge states may be used as charge states that will most likely form product ions. 3) The relative intensity of the most abundant charge state of each polypeptide was compared in the presence of acetonitrile or methanol. Acetonitrile as a solvent additive showed slightly more intensity (~10%) than methanol for the polypeptides evaluated as shown in Appendix 1. This may be due to the slightly lower vapor pressure (9.73 versus 9.76 kPa), which can affect charge state intensity (Krusemark et al., 2009). 4) The presence of 3-NBA improved signal for most of the peptides, including two

orders of magnitude for PYY_{1-36}. It was found to improve the OXM signal one order of magnitude, which lead to a 1 ng/mL LLOQ. 5) More charge states were present with 3-NBA and/or FA present in each solvent mixture; however, larger peptides (> 9400) did not form reliable fragment ions. The larger the polypeptide the fewer product ions were formed. 6) Similar abundant charge states and product ion formation were observed with the peptides ghrelin, PYY_{1-36}, PYY_{3-36}, and OXM. Coincidentally, these peptides have similar molecular weights (ranging from 3314 to 4449 Da) and similar isoelectric points (9.2 to 10.6). Using these similar peptide characteristics (i.e. molecular weight and isoelectric point) may help predict charge states of multiple peptides that can be used for the formation of SRM transitions.

Table 4.3 Results of charge state distribution as a result of solvent mixtures following mass spectrometer optimization. Note that PTH, Leptin, and Myoglobin did not form product ions (NPIF) for any solvent at the concentration infused (5 µg/mL).

Polypeptide	Molecular weight (Daltons)	Lowest and Highest Charge State	Most Abundant Charge State (MACSz)	Solvent Resulting in MACSz	Most intense SRM
Angiotension-1	1296	+1, +4	+3	S9	433→110
Obestatin	2547	+2, +5	+3	S11	851→262
Ghrelin	3314	+3, +9	+8	S3	416→207
Glucagon	3483	+4, +6	+5	S11	698→1002
Polypeptide YY (PYY_{1-36})	4410	+4, +7	+7	S9	617→136
Polypeptide YY (PYY_{3-36})	4149	+3, +9	+7	S9	580→70
Oxyntomodulin (OXM)	4449	+4, +11	+8	S9	554→120
Insulin growth factor-1 (IGF-1)	7655	+5, +10	+7	S7	1094→1199
Parathyroid hormone (PTH)	9425	+8, +10	+9	S1	NPIF
Leptin	16,026	+10, +20	+15	S7	NPIF
Myoglobin	16,951	+11, +26	+11	S7	NPIF

The selected charge states evaluated for product ion formation from each polypeptide in each solvent were chosen because they represented the most abundant charge state precursor ions which yielded more product ions and ultimately lead to more useful SRM transitions. Solvents 2 and 5 produced the most intense product ions to form SRMs using the +7 (636 m/z) and +8 (557 m/z) charge states of oxyntomodulin. The immonium ion at 120 m/z was the most intense product ion for OXM. An immonium ion, represented by RCH=NH2+, with R indicating the amino acid side chain, has been used for quantification previously (Murao et al., 2007). However, it was not selective as a fragment for SRM transitions of OXM due to interferences present in plasma. The product ion mass spectrum for OXM from the +8 charge state (557 m/z) shows the prevalence small ions, such as the immonium ion 120 m/z in figure 4.4. Each abundant product ion was evaluated as SRM transitions for OXM that would be the most sensitive and selective SRM. This investigation resulted in a transition of 636.9→666.9 m/z which was used for quantification purposes. Other SRM's in Table 4.2 were monitored as qualification transitions.

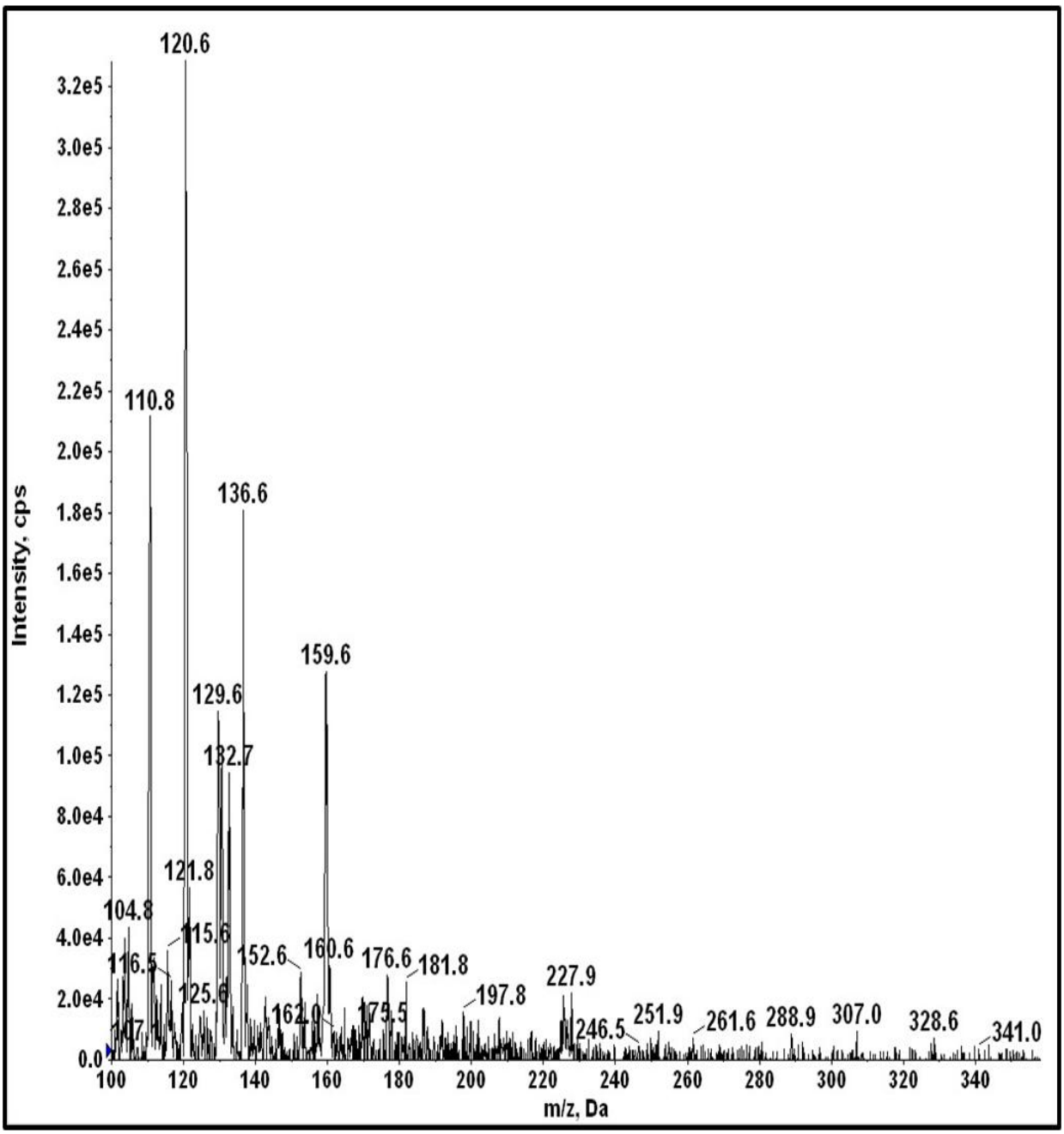


Figure 4.4 Product Ion spectrum for the OXM +8 charge state precursor ion (557 m/z).

4.D.2 2D-LC-MS/MS USING REVERSED PHASE ION PAIR CHROMATOGRAPHY

Two-dimensional reversed phase chromatography is the standard of practice for quantification of peptides (Ismaiel et al., 2011; Van Den Broek et al., 2008). Use of column trapping allows for an additional sample purification online, which should reduce matrix effects and improve robustness. The results from the charge state distribution discussed in section 4.D.1 lead us to use 3-NBA as a mobile phase additive (Valeja et al., 2010). The addition of 3-NBA allows provides for ion pairing with the zwitterionic peptide species in an equilibrium process. Peak shape, response, and reproducibility were investigated during the optimization of molar concentration of 3-NBA in the mobile phase. Consistent peak shape and the most intense response was produced with 0.1% (6.5 mM) 3-NBA in the mobile phase. The pH of the mobile phase can also play a role in ion pair chromatography; therefore, three molar concentrations (3.3, 6.5, 65 mM) of 3-NBA were then evaluated for pH adjustment (pH 2.3, 4.7, 5.7, 6.4, 7, 10) as shown in Figure 4.5.

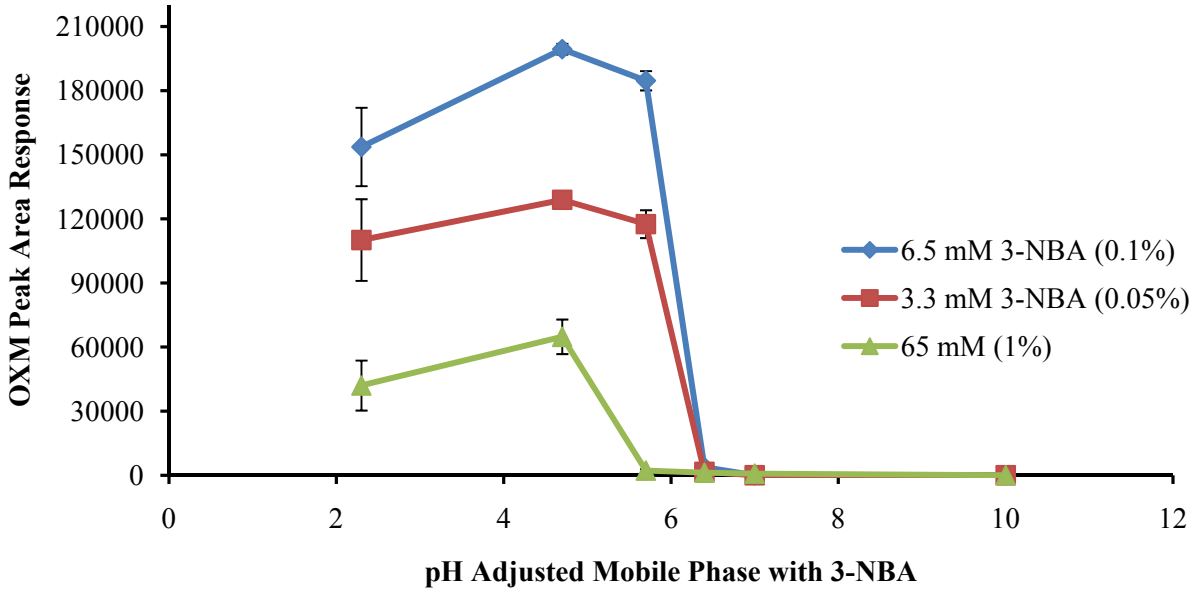


Figure 4.5 Assessment of pH adjustment to mobile phase containing 3-NBA versus mean peak area response of oxyntomodulin (monitored SRM: 636.9→666.9 m/z), with samples injected n =3 Data shown are mean±SD (with standard deviation error bars).

The most intense and reproducible peak shape was generated using 6.5 mM 3-NBA with the pH adjusted to 4.7. During this study, various gradients were attempted with single and two-dimensional reverse phase chromatography. The increase to 90% organic (0.1% 3-NBA in acetonitrile) provided ideal retention (tr = 3.93 minutes), good peak shape, and the highest peak area response. Part of this investigation also revealed that the presence of 3-NBA in the final sample extract must be optimized. Three different proportions of 3-NBA (0.05%: 3.3 mM, 0.1%: 6.5 mM, 1.0%: 65 mM) were evaluated in the final sample diluent, which contained 25% acetonitrile. The thought was having more reagent (3-NBA) would help force the equilibrium to the ion pairing of the peptide and 3-NBA. The results show that the peak area response was more than 2.5 fold higher with more 3-NBA (1.0%: 65 mM) in the final sample extract as shown in Figure 4.6. It should be noted that 1.5% 3-NBA was prepared, but it was not fully soluble in the final sample mixture; therefore, no further 3-NBA concentrations were evaluated.

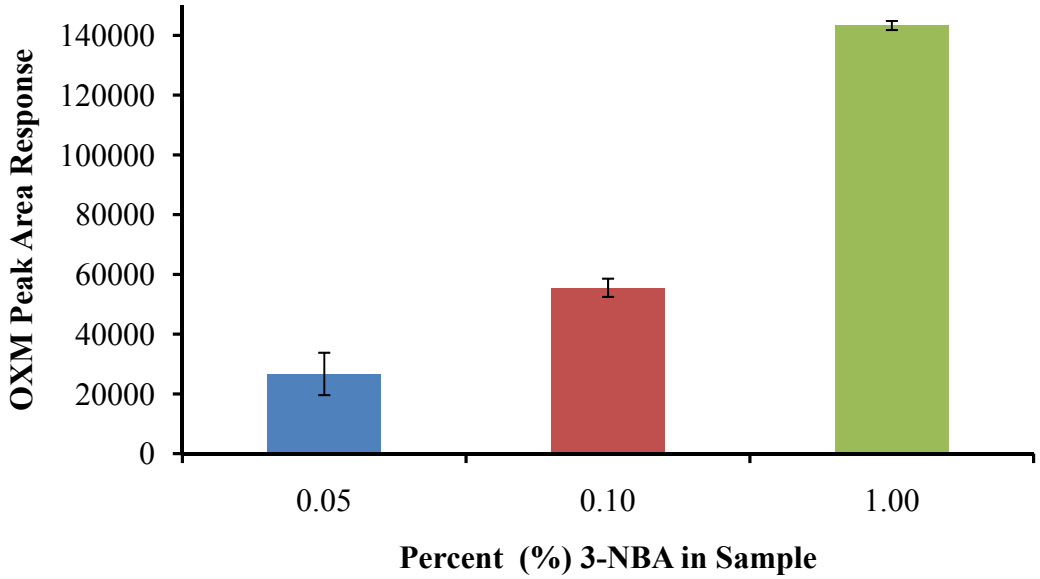
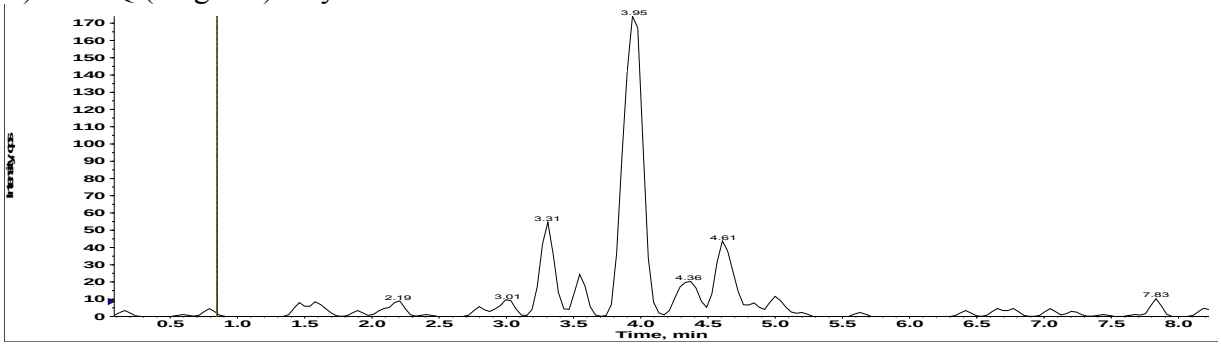


Figure 4.6 OXM (monitored SRM: 636.9→666.9 m/z) mean peak area response versus percent 3-NBA (or mM) in the final sample solvent extract. Data shown are mean±SD (with standard deviation error bars).

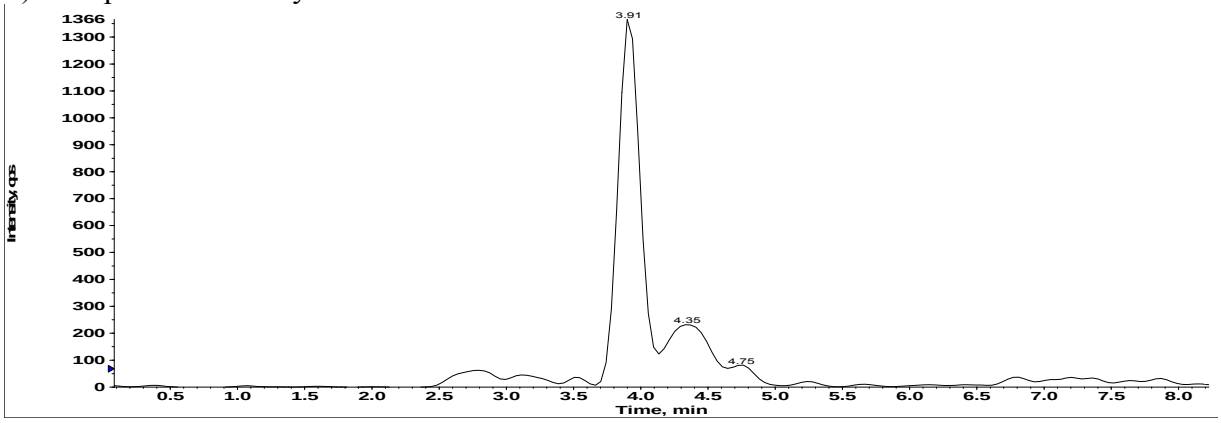
During chromatographic investigations, a single reversed phase C18 UPLC® column was originally used for oxyntomodulin with a gradient similar to that described in section 4.B.5. However, during sample extraction development, this column quickly (<200 injections) showed poor peak shape, high back pressure, and proved to be inefficient for quantification purposes. Our previous successes with column trapping lead us to investigate 2D-LC for chromatographic separation of OXM. Initially, C18 guard columns were attempted as the loading column to provide a short loading time as demonstrated previously (Halquist et al., 2011). The mobile phase optimization study of 3-NBA described in section 4.B.5 provided compelling evidence to continue use of the Waters XBridge BEH300 C18 for the analytical column. A Phenomenex Gemini C18 (4.0 x 2.0 mm) security guard, an Agilent ZORBAX 300SB-C3 Guard 5um, 2.1 x 12.5mm, and a Waters Acquity HSS C18 VanGuard Pre-column, 1.8 µm, 2.1 x 5mm were evaluated as the loading column. All loading columns showed minimal retention of

oxyntomodulin, with the maximum retention time being 42 seconds. Therefore, it was decided to investigate a more traditional length (50 mm) column that may retain OXM long enough to remove unwanted matrix components prior to switching to the analytical column. A C8 and C18 column (both 2.1 x 50 mm, 5 um, 300Å) were evaluated. It was concluded that the C8 provided better overall performance with consistent retention, and ruggedness after multiple injections. The combination of an ACE C8 column in the first dimension with a loading time of 1.5 minutes, followed by a switching valve to change the direction of the mobile phase and initiate the gradient onto the analytical column (Waters XBridge BEH300 C18) was sufficient for methods validation. The loading and analytical column washes post-elution increased the longevity of the column, which allowed for more than 900 injections. Chromatographic examples are shown in Figure 4.7 (A, B, and C). Representative blank chromatograms are shown in Figure 4.8 (A and B).

A) LLOQ (1 ng/mL) Oxyntomodulin



B) Isotope Labeled Oxyntomodulin Internal Standard



C) Rat sample dosed with Oxyntomodulin

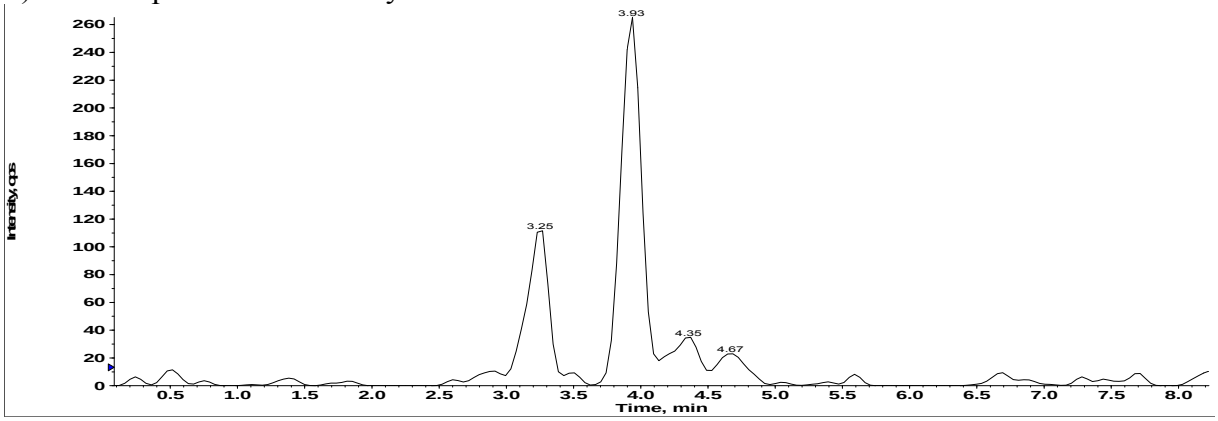
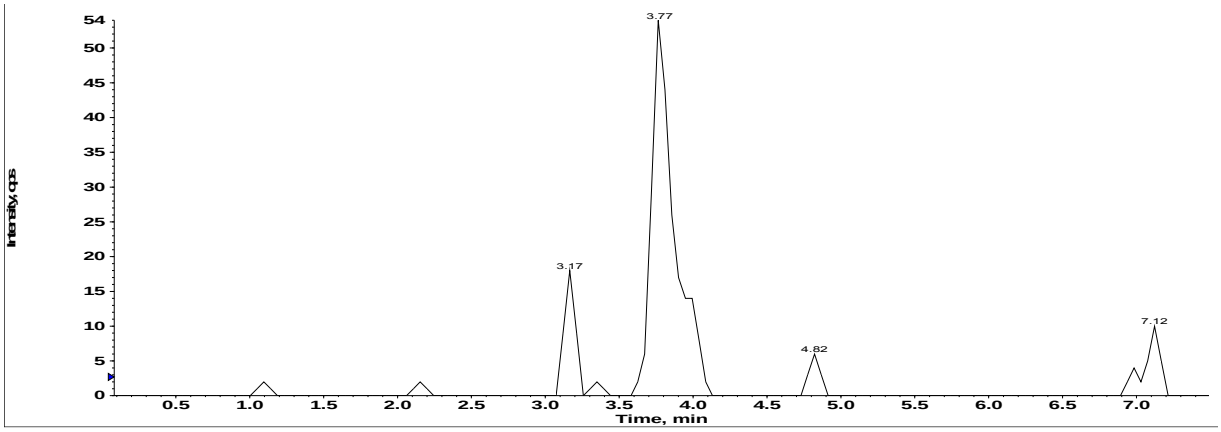


Figure 4.7 Representative chromatograms of the (A) lower limit of quantification of OXM (1 ng/mL), monitoring SRM: 636.9→666.9 m/z , (B) OXM_{IL} isotope labeled internal standard, monitoring 642.3→676.6 m/z (B), (C) rat sample dosed with OXM monitoring SRM: 636.9→666.9 m/z.

A) Blank Rat Plasma



B) Blank Rat Plasma OXM_{IL}

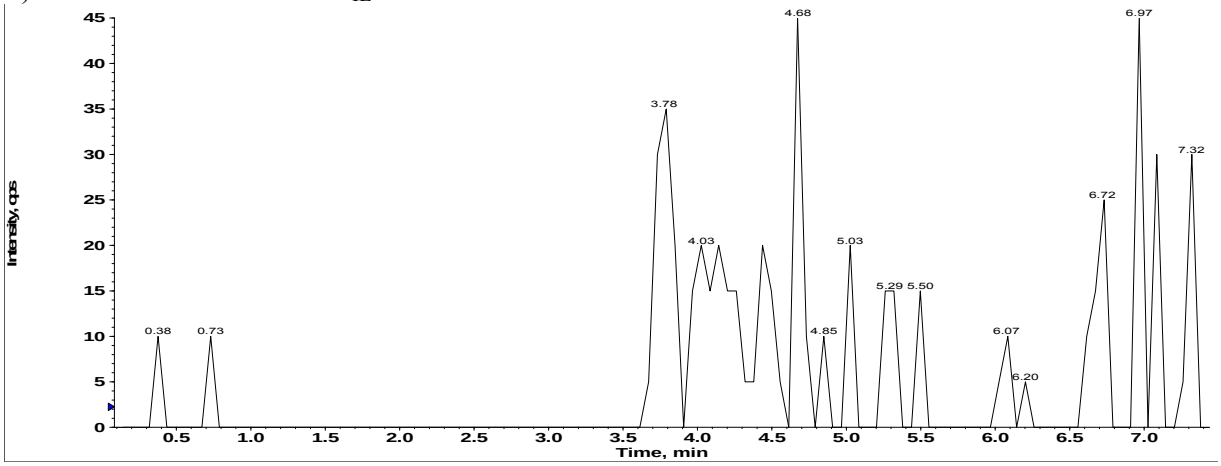


Figure 4.8 Representative blank rat plasma chromatograms of (A) OXM, monitoring SRM: 636.9→666.9 m/z and (B) OXM_{IL} isotope labeled internal standard, monitoring 642.3→676.6 m/z.

4.D.3 SAMPLE PREPARATION SELECTION AND OPTIMIZATION

During this investigation protein precipitation (PPE) and solid phase extraction (SPE) were evaluated. Precipitating reagents acetonitrile, perchloric acid (7%) and methanol were tested as solvents for sample purification. Glucagon is a similar polypeptide to OXM due to its sequence containing the first 29 amino acids of OXM. Glucagon has been quantified using a protein precipitation with acetonitrile in rat plasma (Delinsky et al., 2004); however, the recovery in all precipitating reagents tested for OXM was low (<20%). Similar recovery results were

observed when using reversed phase SPE and strong cation-exchange SPE. Mixed mode weak cation (WCX) and anion (MAX) microelution solid phase extractions were further optimized to improve recovery. Recovery using the manufacturer's protocol's for MAX and WCX was approximately 10 to 15%, respectively. An investigation was performed to determine where the loss was occurring. The first investigation included collection of all loading, washing, and elution solvents to evaluate the presence of OXM in any of these solvents. It was observed in the manufacturer's protocol for wash 2 (20% acetonitrile) that the presence of OXM was prevalent in the collected wash; therefore, wash 2 was reduced to 5% acetonitrile, which yielded negligible recovery of OXM during analysis. The loading application for each SPE was 4% phosphoric acid, which may not adequately disrupt OXM from other proteins. Consequently, nonspecific protein binding was investigated. It was first observed that recovery of OXM in water as a matrix was significantly lower (5% versus 15%) than rat plasma, thus nonspecific binding may be an issue. Chaotropes such as guanidine hydrochloride and UREA were added to the plasma to facilitate disruption of OXM binding to proteins; however, these reagents had negligible effects as compared to the 4% phosphoric acid that was currently being added to the plasma prior to loading onto the SPE. Lastly, a stronger elution solution was tried. Instead of 1% TFA in the eluent, the TFA percentage was increased to 5% which resulted in improved recovery. MAX was chosen due to slightly better recovery (~55%) than WCX (~45%).

4.D.4 VALIDATION RESULTS

4.D.4a LINEARITY

Linearity was determined using the peak area ratio of OXM and its isotope labeled internal standard as a function of concentration over the range 1 to 1,000 ng/mL. Calibration

curves had acceptable reverse calculated residuals between -8.6% and 6.0%. Inter-run precision measured in terms of percent relative standard deviation ranged from 6.5% to 11.0% over six analytical runs. Data are present in Table 4.4.

Table 4.4 Reverse predicted concentrations for oxyntomodulin calibration standards (ng/mL)

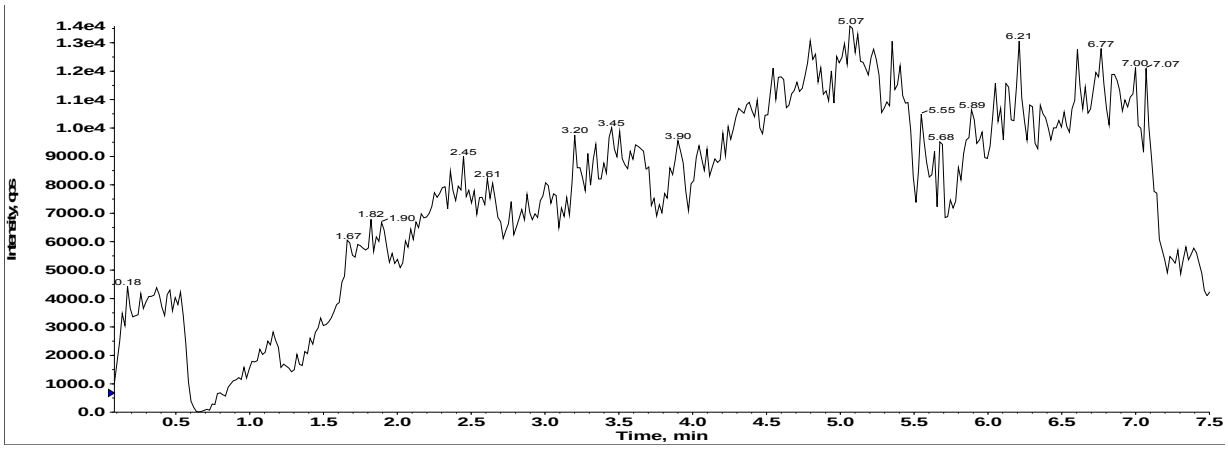
	Oxyntomodulin Concentration (ng/mL)										
RUN Number	**1.00**	**2.00**	**5.00**	**10.00**	**25.00**	**50.00**	**100.00**	**250.00**	**500.00**	**1000.00**	**r^2**
Run 1	0.81	1.98	4.90	7.94	24.30	56.00	86.40	189.00	567.81	992.23	0.992
	0.97	1.90	5.79	11.00	23.30	51.40	114.00	251.00	552.00	1070.00	
Run 2	0.99	2.10	4.37	8.78	22.40	50.40	95.40	284.00	573.33	1144.22	0.993
	0.89	1.93	5.09	9.99	25.44	43.10	93.67	255.55	506.33	1008.40	
Run 3	0.96	1.80	5.43	8.20	26.20	55.70	87.70	241.00	443.00	1132.22	0.995
	1.02	1.91	5.31	9.84	25.68	56.31	102.42	257.77	502.77	1011.42	
Run 4	0.82	2.07	5.04	9.72	24.82	48.22	88.76	250.67	509.99	979.23	0.990
	0.86	2.42	5.10	9.88	26.32	49.21	99.32	246.55	576.44	1005.66	
Run 5	0.94	1.98	4.91	8.89	24.25	55.44	86.03	206.66	491.35	1014.44	0.994
	0.97	1.94	5.81	8.25	25.00	53.09	90.24	281.22	567.34	997.33	
Run 6	0.92	1.88	4.31	8.55	26.31	52.22	95.34	241.99	571.22	999.03	0.991
	0.83	1.95	4.30	9.09	28.74	51.89	99.11	267.99	498.09	1020.22	
Mean	0.91	1.99	5.03	9.18	25.23	51.92	94.87	247.78	529.97	1031.20	0.992
SD	0.071	0.16	0.52	0.91	1.64	3.88	8.11	27.38	43.51	54.65	0.002
%RSD	7.73	7.93	10.30	9.96	6.51	7.47	8.54	11.05	8.21	5.30	0.18
%DFN	-8.57	-0.55	0.60	-8.23	0.92	3.83	-5.13	-0.89	5.99	3.12	

4.D.4b SELECTIVITY

Six different lots of rat plasma were evaluated for selectivity of the method. No significant (<10% of LLOQ) endogenous peaks at the retention time of OXM were observed for any of the lots. Figure 4.7(B) shows a representative blank rat plasma chromatogram. Qualification SRM's were monitored throughout the entire study, which are listed in table 1. The SRM transition for OXM 636.9→666.9 was chosen over the other monitored transitions due to superior selectivity and reproducibility. Transitions 557.4→225.4 and 636.9→110.1 had higher response but were not reliable for quantification due to imprecision most likely from matrix effects. A blank sample with internal standard and the upper calibration standard (1,000 ng/mL) were analyzed for analyte interference and found to have only a negligible effect on OXM or internal standard response.

The post-column infusion study described in section 4.C.1 resulted in no obvious suppression or enhancement at the retention time of OXM. Figure 4.9(A) reveals the ion profile for OXM during the post-column infusion, which shows no change in response at the retention time of OXM (tr = 3.93 min.). Figure 4.9 (B) represents the chromatographic profile of phospholipids monitored during the post-column infusion, which indicated their presence following extraction; however, all peaks were chromatographically resolved from OXM. The late eluting peaks present in the phospholipid profile (Figure 4.9 B) are well resolved from OXM and the run time was increased to 8 minutes to avoid late elution phospholipids on subsequent injections.

A) Oxyntomodulin Monitored (636→666)



B) Post-Column Infusion Monitoring of Phospholipids (184→184)

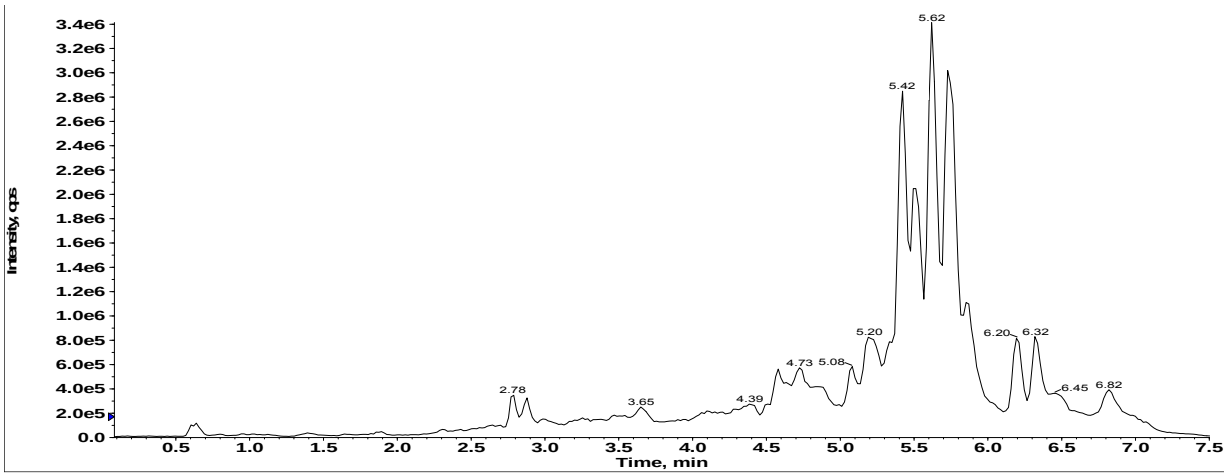


Figure 4.9 Post-column infusion profiles of oxyntomodulin (A) and phospholipids (B).

Matrix effects were further evaluated with a post-extraction addition study performed as described in section 4.C.1. The presence of matrix effects was -33.4% for LQC, -23.2% for MQC, and -40.2% for HQC.

4.D.4c RECOVERY AND CARRYOVER

Recovery was performed following optimization of extraction and chromatographic procedures during the validation. Six Extracted replicates of LQC, MQC, and HQC were compared to post-spiked matrix residue samples as described in section 4.C.1. Calculations were

obtained by dividing the matrix pre-spiked extracted samples by the post-spiked extracted samples and multiplying by 100. The results revealed 54.5, 64.9, 58.7% recovery for the LQC, MQC, and HQCs, respectively. Carryover assessment resulted in no response in solvent blanks following the high calibration standard was observed at the retention time of OXM.

4.D.4.d PRECISION AND ACCURACY

Table 4.5 summarizes the inter- and intra-day precision and accuracy results. Inter-day results were determined based on six separate runs, whereas intra-day results were determined in one run with six replicates of the LLOQ QC, LQC, MQC, HQC and DQC samples. Accuracy (% bias) for inter-day was -11.7 to 12.4 % for all quality controls including the LLOQ. Intra-day accuracy resulted in -13.1 to 8.2% bias. The relative standard deviation (precision) for inter-run results ranged from 4.0 to 14.3% and intra-day precision was 5.6 to 15.3% RSD. The LLOQ was within ±20% for precision and accuracy, and all other controls were within ±15% for inter and intra assay results.

Table 4.5 Inter and Intra-assay Precision and Accuracy

QC (ng/mL)	Average measured concentration (ng/mL) ± SD	Inter-assay % bias*	Intra-assay % bias	Intra-assay precision (%RSD)	Inter-assay precision (%RSD)*
LLOQ (1.00)	0.88 ± 0.13	-11.70	-13.10	15.26	14.30
LQC (2.50)	2.81 ± 0.31	12.40	6.74	11.14	11.03
MQC (60.00)	66.7 ± 2.67	11.16	8.22	5.60	4.01
HQC (800.00)	817.23 ± 59.91	2.15	5.11	8.26	7.33
DQC (5000.00)	5102.31±363.28	N/A	2.04	7.12	N/A

*(QCs analyzed n ≥3 in six separate analytical runs)

4.D.4e STABILITY AND INCURRED SAMPLE RE-ANALYSIS (ISR)

Stability assessments included: post-preparative stability (PPS), storage stability, stock stability, and freeze-thaw stability. PPS was determined from quality controls stored in the

autosampler for approximately 39 hours at 5°C. These stored QC's proved to be stable with accuracy results all within ± 12.4%. Freshly prepared calibration standards were used to calculate the accuracy of stored QC's. Storage stability results ranged from -13.3 to -15.1% biases for LQC and HQC with and without DPP-IV inhibitor samples stored at -70ºC for 64 days. The stress test for stock solution held at room temperature compared to a fresh aliquot of the same stock showed acceptable peak area response differences (5.2% bias). Freeze-thaw stability was assessed for three cycles, with LQC and HQC resulting in -18.1% and -16.3% biases, respectively. One freeze-thaw cycle of the LQC and HQC's resulted in acceptable accuracy (± 15%). Therefore, samples should be sub-aliquotted for daily use and be thawed in an ice water bath as a precaution.

Ten rat samples were analyzed a second time to evaluate incurred sample re-analysis. The latest European Medicines Agency bioanalytical guidance was used to determine the acceptability of ISR results (Ema, 2011). This guidance indicates 67% percent of the ISR samples must be within 20% of the mean of the original and repeat sample. Results in indicated that 7 out of 10 (70%) of the repeated rat samples met this criteria.

4.D.5 CROSS TECHNOLOGY IMMUNOASSAY VALIDATION

A peptide immunoassay kit was validated using reagents provided with the kit procedure. Calibration curves resulted in R^2 values of 0.988 and 0.991, respectively, with a linear range from 0.1 to 7 ng/mL. The curve fitting was a four parameter fit with y = ((A-D)/(1 + (x/C)^B)) + D. Precision (%CV) of these calibration curve replicate points were between 0.4 to 24.5%. The same rat samples that were used for the pharmacokinetic study described in section 2.12 were analyzed with the LC-MS/MS method and this immunoassay. The results of the rat samples

analyzed with the immunoassay were significantly different than the LC-MS/MS results and almost all time points yielded no detectable response for OXM. Furthermore only one rat had a similar pharmacokinetic profile. However, some of the individual time points did not match with this rat either (percent difference as much as 200% at the 5 minute time point). An investigation followed where successive runs with calibration standards and quality controls were evaluated in two different matrices. It was suspected that the rat plasma from the rat species may contain a matrix effect that would hinder this assay. Thus, another investigation was performed using dilution to reduce the suspected matrix effect. The kit contains a peptide-free rat serum for preparation of calibration standards, quality controls, and samples. This rat serum was also used as a diluent to prepare quality control samples (low, 0.2 ng/mL and high, 6 ng/mL), which may dilute out the potential matrix effect from rat plasma obtained from the same rat species used in the pharmacokinetic study. Dilutions of 1, 10, and 100 of blank rat plasma with the kit matrix (peptide free rat serum) supplied with the kit were evaluated at the same concentrations. The results indicate there may be a matrix effect because no result was obtained for 1 and 10-fold dilutions. The 100-fold dilution was acceptable for the high QC (6 ng/mL) with a percent bias of 17.3%; however, no result was obtained for the low QC. Therefore, a significant dilution of this rat plasma may be necessary to achieve reliable results whereas too much dilution would result in poor sensitivity.

4.E. CONCLUSIONS

Polypeptides that are approaching protein sizes are challenging molecules to quantify in the presence of a complex biological matrix such as plasma. Matrix effects and non-specific binding proved to be a challenge during method development and validation. Characteristics of

the peptide such as isoelectric point (pI), hydrophobicity, and individual amino acid residues must be considered for separation, storage, and ionization. All of these characteristics affected the development and optimization for oxyntomodulin. The use of 3-NBA proved to be beneficial for ionization of the peptides and as a mobile phase additive for reversed phase ion pair chromatography. The use of 2D-LC was found to be necessary for peptide quantification due to its reliable removal of matrix effects and superior robustness. Stability experiments indicate that peptide stability can be a concern and storage precautions such as daily use aliquots for samples, quality controls, and stock solutions must be necessary. Another concern was the inability of the immunoassay to yield results that could be correlated with the LC-MS/MS analysis. These results could be an anomaly, but prove the value of using LC-MS/MS as an alternative to immunoassays.

CHAPTER 5

DEMONSTRATING UTILITY OF LC-MS/MS: IN VIVO PHARMACOKINETICS OF OXYNTOMODULIN IN RATS FOLLOWING INTRAVENOUS AND PULMONARY ROUTES OF ADMINISTRATION

5.A INTRODUCTION

In Chapter 4, two-dimensional LC-MS/MS coupled with μelution anion exchange solid phase extraction (SPE) were employed to determine rat plasma concentrations of an anorectic gut-secreting peptide, oxyntomodulin (OXM). The method has been successfully validated, enabling quantification of OXM. A linear range of 1-1000 ng/mL in rat plasma yielded reverse predicted concentrations between -3.7 and 2.9 %DFN (percent difference from nominal). In addition, low, medium, and high quality controls were between 2.2 and 12.4 %DFN, and 4.0 to 11.0 %RSD. Clearly, one of the prospective uses of this validated analytical method is for determination of the plasma OXM concentration versus time profiles following administration, as OXM has been suggested to possess therapeutic potential in obesity treatment by reducing food intake and body weight (Dakin et al., 2001; Dakin et al., 2004; Dakin et al., 2002; Wynne & Bloom, 2006; Wynne, Park et al., 2006). Indeed, Nadkarni has recently demonstrated that needle-free pulmonary administration of OXM at 0.5 mg/kg enabled 30.0±5.4 % food intake suppression for 4-6 hr period in freely-fed rats (Nadkarni, 2009).

Hence, in this chapter, the method validated in Chapter 4 was used to determine the plasma OXM concentration versus time profiles following intravenous and pulmonary routes of administration in rats for conventional noncompartmental pharmacokinetic analysis. These data were then discussed, associated with the absolute bioavailability for pulmonary delivery of OXM, the difference in dose-response pharmacokinetics due to analytical methods and across different species, and bioavailability using pulmonary delivery of similar peptides. Employment of the validated LC-MS/MS method for a pharmacokinetic study of oxyntomodulin further shows the benefits of LC-MS/MS technology.

5.B MATERIALS AND METHODS

5.B.1 MATERIALS

Lyophilized human oxyntomodulin (OXM; molecular weight of 4449 Da) was purchased from Bachem Americas, Inc. (Torrance, CA, USA). It was received as lyophilized powder and certified with an HPLC purity of at least 97%; therefore, it was directly reconstituted for preparation of OXM dosing solutions. Phosphate-buffered saline (PBS; 0.2 M; pH 7.4) was obtained from Invitrogen Corporation (Carlsbad, CA, USA).

5.B.2 ANIMALS

The animal experiments followed the NIH Principles of Laboratory Animal Care (NIH publication # 85-23, revised in 1985). The experiments were approved by VCU's Institutional Animal Care and Use Committee IACUC (AM10038). All animal experiments were carried out by Dr. Masahiro Sakagami and Ms. Hua Li. Sprague-Dawley rats (male; Hilltop Lab Animals

Inc., Scottdale, PA) in a weight range of 291- 454 g were used after at least two days of acclimatization in the animal care facility. Animals had free access to standard rat chow (Harlan Teklad Global Diet, Product # 7012, Boston, MA) and drinking water.

5.B.3 IN VIVO ANIMAL STUDIES FOR OXM PHARMACOKINETICS FOLLOWING INTRAVENOUS INJECTION AND PULMONARY ADMINISTRATION

In vivo experiments were carried out, as described previously with slight modifications (Nadkarni, 2009). Rats were anesthetized with an intraperitoneal injection of 50 mg/kg of sodium pentobarbital (Ovation Pharmaceuticals, Inc. (Deerfield, IL) and then placed on a surgical board for OXM administration and blood sampling. Rats were divided into 3 groups with n=4, first group (1) received an intravenous bolus injection at 0.05 mg/kg (Rat 4, 5, 6, and 7), second group (2) received a pulmonary solution administration at 0.05 mg/kg (Rat 2, 8, 9, and 10) and the third group (3) received a pulmonary solution administration at 0.50 mg/kg (Rat 1, 11, 12, and 13). For intravenous injection, nominal 0.1 ml of OXM solution prepared in saline was injected into the right jugular vein at 0.05 mg/kg. Blood (0.1 mL) samples were withdrawn from the left jugular vein at 2, 5, 10, 20, 30, 45, 60, 90, 120, and 180 min following injection. For pulmonary administration, nominal 0.1 mL of OXM solution prepared in saline was orotracheally instilled at 0.05 mg/kg and 0.5 mg/kg using the MicroSprayer (PennCentury). Blood samples (0.1 mL) were withdrawn from the left jugular vein at 2, 5, 10, 20, 30, 45, 60, 90, 120, and 180 min following administration. During experiments, animals were maintained under sufficiently-deep anesthesia with supplemental pentobarbital injected at 25 mg/kg as needed, and placed under a heating lamp to maintain body temperature. All blood samples were centrifuged

at 12,000 rpm (23,182 xg) for 2 min, and plasma samples were then stored immediately at -70ºC until analysis.

5.B.4 DETERMINATION OF OXM IN RAT PLASMA BY LC-MS/MS

The rat plasma samples taken from the above animal studies were analyzed by the method described and validated in Chapter 4. Briefly, after appropriate dilution with blank rat plasma (Biochemed Services, Inc. Winchester, VA), the rat plasma samples were spiked then spiked with 0.020 mL of 2.25 µg/mL the isotope-labeled OXM internal standard (OXM_{IL}). These samples were then extracted using a Waters Oasis® MAX µelution modified solid phase extraction (SPE) method as described in Chapter 4. Following extraction, samples were injected onto the instrument, which was coupled with two-dimensional reversed phase ion pair chromatography tandem mass spectrometry for analysis of oxyntomodulin in rat plasma. The samples were analyzed with a chromatographic run time of 8 minutes. Multiple charged state forms of OXM and OXM_{IL} were monitored using selected reaction monitoring (SRM), where transitions of 636.9→666.9 (m/z) and 642.3→676.9 (m/z), were selected, respectively, with +7. As described in Chapter 4, the method was validated with acceptable limits for precision and accuracy as shown in Tables 4.3 and 4.4.

Note that the plasma samples were thawed and kept in an ice water bath to prevent potential loss or degradation of OXM.

5.B.5 PHARMACOKINETIC DATA ANALYSIS

5.B.5a NONCOMPARTMENTAL PHARMACOKINETIC DATA ANALYSIS

Plasma concentration versus time profiles of OXM following intravenous injection and pulmonary administration were analyzed in each animal by conventional noncompartmental approach. The maximum plasma peak concentration (Cmax) and the time to reach Cmax (Tmax) were assessed by visual inspection. All other parameters were computed using Microsoft Excel, as follows:

The terminal phase slope (β) was determined, which represents the slope of the terminal phase on the plasma concentration-time profile on a semi-log linear plot, and its half-life ($t_{1/2}$) was calculated from equation 5.1 (Eq. 5.1).

$$t_{1/2} = 0.693/\beta \qquad \text{(Eq. 5.1)}$$

The area under the plasma concentration vs. time curve ($AUC_{0\text{-}inf}$) was determined from:

$$AUC_{0\text{-}inf} = AUC_{0\text{-}tlast} + AUCt_{last\text{-}inf}$$

where $AUC_{0\text{-}tlast}$ was calculated by the trapezoidal method and $AUCt_{last\text{-}inf}$ was the extrapolated residual area calculated from the last plasma concentration (C_{last}) divided by β. For intravenous injection, the plasma concentration at time 0 (C_t=0) was calculated from:

$$C_{t=0} = A + B$$

where A and B are the coefficients of the following 2-compartment model equation, derived from the method of residuals (Gibaldi, 1982):

$$C=A*\exp(-alpha*t)+B*\exp(-beta*t) \qquad \text{(Eq. 5.2)}$$

The apparent clearance (CL/F), volume of distribution of central compartment (Vd_{cc}) and

apparent pseudo steady state (Vd_{pss}/F), are calculated from:

$CL/F = Dose/AUC_{0\text{-}inf}$ (Eq. 5.3)

$Vd_{cc} = Dose/C_{t=0}$ (Eq. 5.4)

$Vd_{pss}/F = (CL/F)/ \beta$ (Eq. 5.5)

Absolute bioavailability for OXM following pulmonary administration (F) was then determined from $AUC_{0\text{-}inf}$ for the individual profiles relative to the mean $AUC_{0\text{-}inf}$ for OXM following intravenous administration with dose normalization from the equation as shown below:

$F = [AUC_{lung}/ AUC_{IV}] \times [Dose_{IV}/ Dose_{lung}]$ (Eq. 5.6)

where subscripts lung and IV represent pulmonary and intravenous administration, respectively. inejection,

All derived parameters were expressed as mean ± standard deviation (SD) with n =4. Statistical significance was determined using an unpaired t-test or analysis of variance (ANOVA) and employed $p<0.05$.

5.C RESULTS

5.C.1 NONCOMPARTMENTAL ANALYSIS OF OXM PLASMA PROFILES FOLLOWING INTRAVENOUS AND PULMONARY ADMINISTRATION

Figure 5.1 illustrates the plasma concentration-time profiles following intravenous administration of OXM at 0.05 mg/kg on a linear scale. The plasma concentrations beyond 90 min were all below lower limit of quantification (LLOQ). The noncompartmental parameters are shown in Table 5.1. The profile was determined to be biexponential (as shown later in Figure 5.3) and it was concluded that there was simultaneous distribution and elimination. The terminal

half life was 20±3.3 min, while CL and Vd_{cc} were determined to be 3.81±1.32 mL/min/kg and 105.8±29.1 mL/kg. It has been reported that rats with similar weights to this study (~300 g) have approximately 16.0 mL of plasma and the glomerular filtration rates are approximately 5 mL/min/kg (Davies et al., 1993; Nadkarni, 2009). Furthermore, when considering body weight and statistical similarities, these physiological parameters are effectively consistent with the results, which indicate multi-compartment distribution and renal elimination.

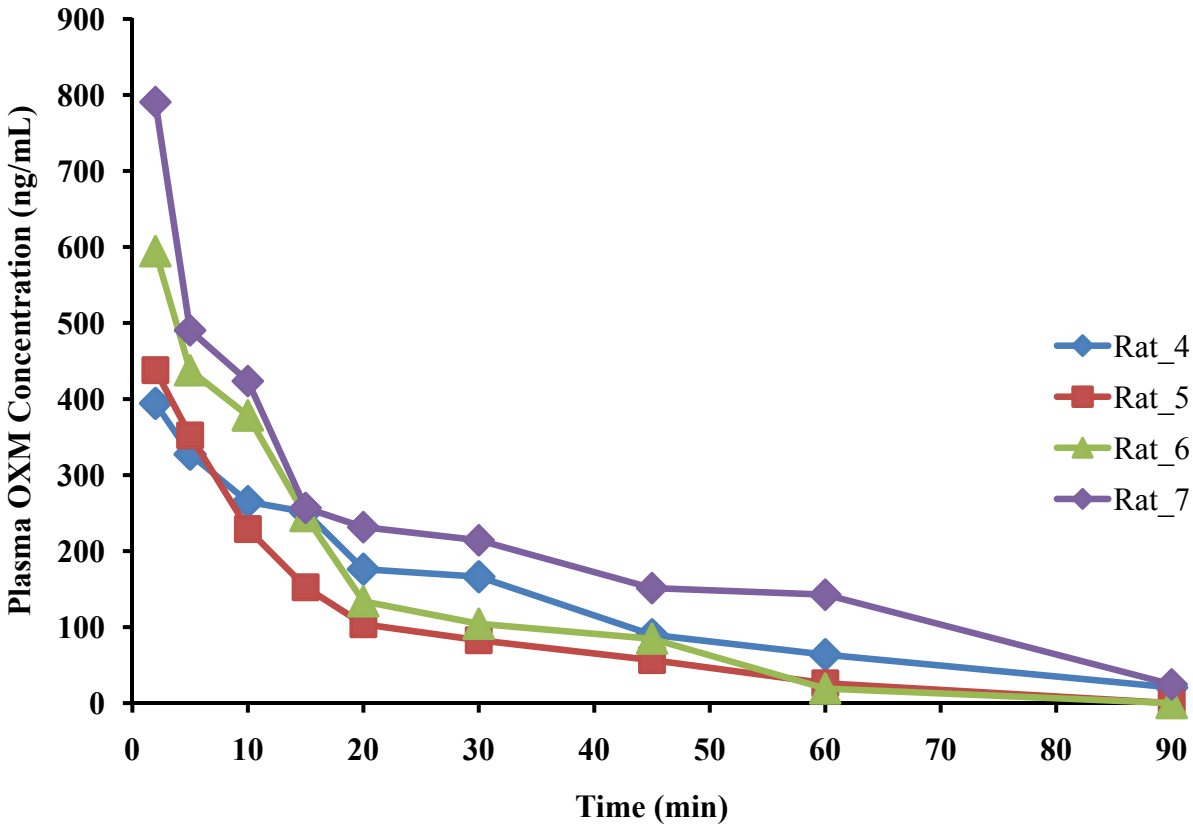


Figure 5.1 Oxyntomodulin (OXM) concentration in plasma vs. time profile following intravenous bolus (IV bolus) injection at 0.05 mg/kg in individual rats with n =4.

Table 5.1 Noncompartmental pharmacokinetic parameters following intravenous bolus injection of oxyntomodulin at 0.05 mg/kg in rats (n=4).

Parameters	**Rat 4**	**Rat 5**	**Rat 6**	**Rat 7**	**Mean±SD**
Dose per body weight (mg/kg)			**0.05**		
C_{max} (ng/mL)	394.4	438.1	594.4	790.8	554.4±179.4
$AUC_{0\text{-}inf}$ (ng x min/mL)	12420	8658	11169	19787	13008±4782
Terminal Slope β (min^{-1})	0.032	0.034	0.046	0.031	0.036±0.0069
$t_{1/2}$ (min)	21.9	20.4	15.1	22.3	20.0±3.3
CL (mL/min/kg)	4.03	4.14	5.11	1.96	3.81±1.32
Vd_{cc} (mL/kg)	126.2	122.2	111.7	63.2	105.8±29.1
Vd_{pss} (mL/kg)	127.5	182.5	130.0	81.4	130.3±41.3

All values are shown with significant digits typically used in pharmacokinetic data.

5.C.2 NONCOMPARTMENTAL ANALYSIS OF OXM PLASMA PROFILES FOLLOWING PULMONARY ADMINISTRATION

The same dose (0.05 mg/kg) that was administered into the lungs yielded much lower plasma concentrations of oxyntomodulin than intravenous administration. Noncompartmental analysis results are presented in Table 5.2 and the respective plasma concentration-time profiles are shown in Figure 5.2. The bioavailability of pulmonary administration of OXM was 0.13±0.04. However, this may be due to enzymatic degradation by dipeptidyl peptidase, which is known to rapidly clear circulating oxyntomodulin by removing the histidine-serine dipeptide from the N-terminus (Santoprete et al., 2011). The pulmonary dose at 0.5 mg/kg showed a ten-fold increase almost proportional to dose (10-fold higher). The bioavailability was similar (F= 0.13±0.03), and the C_{max} and $AUC_{0\text{-}inf}$ were comparable when normalized for dose (Table 5.2, 5.3). Plasma concentration-time profiles are also shown in Figure 5.2. Furthermore, the terminal phase (β) for intravenous and pulmonary doses was statistically consistent ($p>0.05$), which

indicates that lung absorption was not the slowest process (kinetically rate-determining). Consequently, systemic elimination was the slowest process even with pulmonary administration, which is indicative of non-flip flop pharmacokinetics.

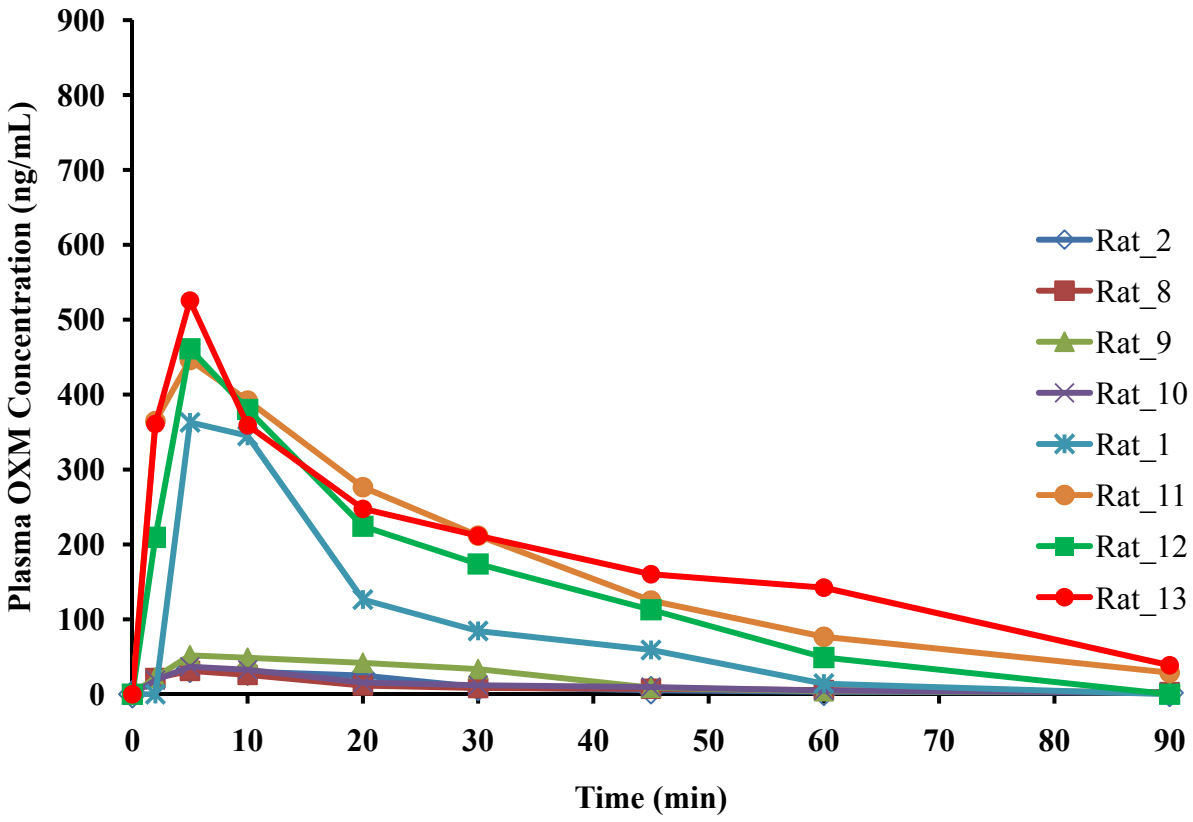


Figure 5.2 Oxyntomodulin (OXM) plasma concentration vs. time profiles following orotracheal instillation (IT) at 0.05 mg/kg (Rats 2, 8, 9, and 10) and 0.50 mg/kg (Rats 1, 11, 12, and 13) in individual rats with n =4.

Table 5.2 Noncompartmental pharmacokinetic parameters following pulmonary administration of oxyntomodulin at 0.05 mg/kg in rats (n=4).

Parameters	Rat 2	Rat 8	Rat 9	Rat 10	Mean±SD
Dose per body weight (mg/kg)			**0.05**		
C_{max} (ng/mL)	33.9	31.2	51.8	36.8	38.4±9.2
T_{max} (min)	5.0	5.0	5.0	5.0	5.0±0.0
$AUC_{0\text{-}inf}$ (ng x min/mL)	1025	1020	1811	1103	1240±383
Terminal Slope β (min^{-1})	0.035	0.019	0.044	0.028	0.032±0.010
t1/2 (min)	29.9	34.2	25.4	21.8	27.8±5.4
CL (mL/min/kg)/F	48.8	49.0	27.6	45.3	42.7±10.8
Vd_{pss} (mL/kg)/F	2105.4	2418.2	1013.4	1423.6	1740.1±638.1
F	0.10	0.10	0.18	0.11	0.13±0.039
C_{max}/Dose (kg/mL)	678.6	623.9	1036.8	736.2	768.9±184.4
$AUC_{0\text{-}inf}$/Dose (kg x min/ml)	20510	20406	36226	22053	24799±7655

All values are shown with significant digits typically used in pharmacokinetic data.

Table 5.3 Noncompartmental pharmacokinetic parameters following pulmonary administration of oxyntomodulin at 0.5 mg/kg in rats (n=4).

Parameters	Rat 1	Rat 11	Rat 12	Rat 13	Mean±SD
Dose per body weight (mg/kg)			**0.5**		
C_{max} (ng/mL)	357.4	444.9	456.8	438.0	424.3±45.3
T_{max} (min)	5.0	5.0	5.0	5.0	5.0±0.0
$AUC_{0\text{-}inf}$ (ng x min/mL)	7714	14349	12737	15136	12484±3333
Terminal Slope β (min^{-1})	0.041	0.033	0.042	0.033	0.037±0.0050
$t_{1/2}$ (min)	27.4	21.3	16.4	20.8	21.5±4.5
CL (mL/min/kg)/F	64.8	34.8	38.9	38.5	44.2±13.8
Vd_{pss} (mL/kg)/F	2560.1	1241.4	1068.6	1140.8	1427.1±761.6
F	0.079	0.15	0.13	0.15	0.13±0.017
C_{max}/Dose (kg/mL)	714.7	889.9	913.6	876.0	848.6±90.6
$AUC_{0\text{-}inf}$/Dose (kg x min/ml)	15427	28698	25475	30272	24968±6667

All values are shown with significant digits typically used in pharmacokinetic data.

The overall mean plasma concentration-time profiles (mean±standard deviation) of both lung doses and the intravenous dose are shown in Figure 5.3. The terminal phase (β) for intravenous and both respective lung doses are illustrated in a semi-log linear plot in Figure 5.4.

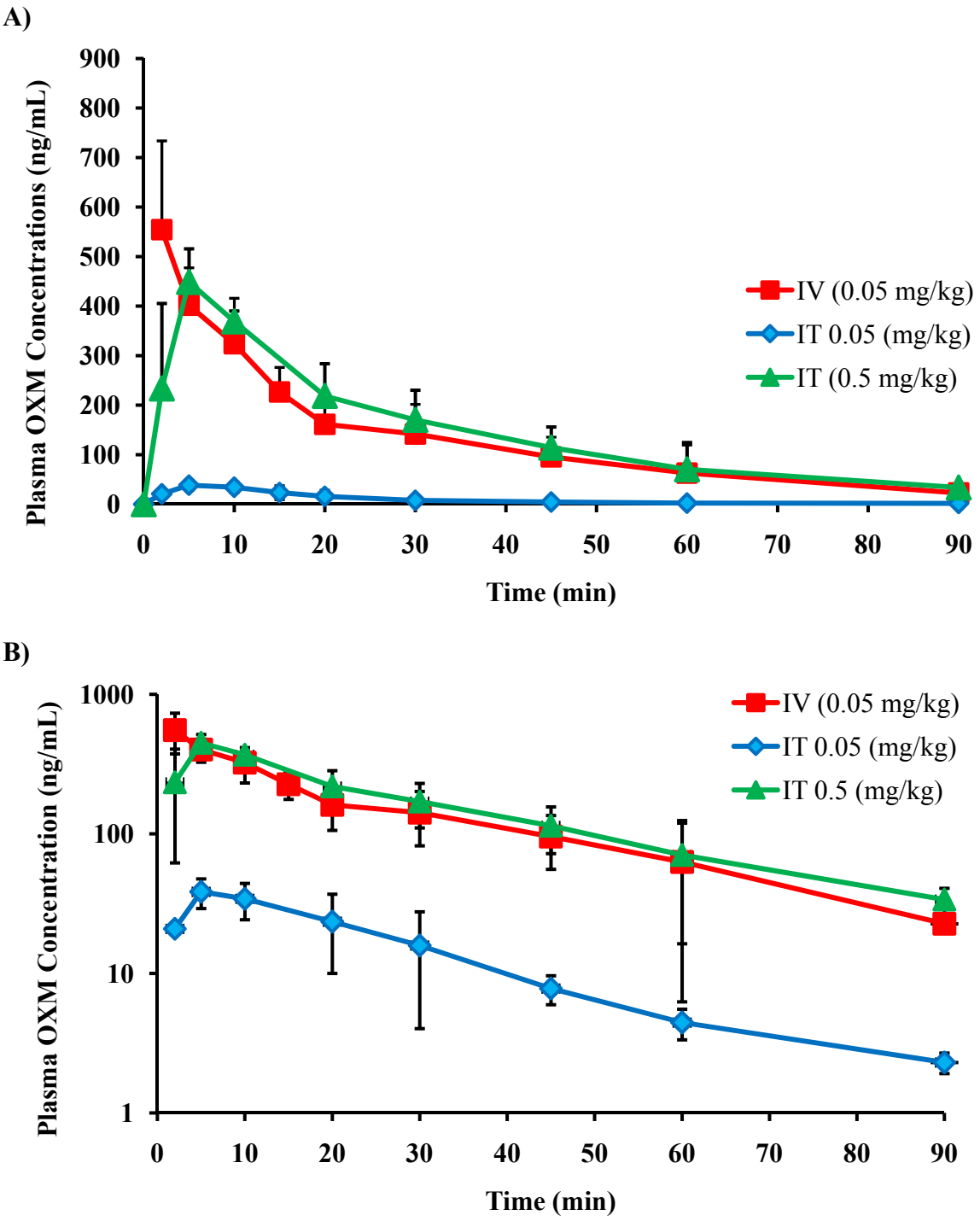


Figure 5.3 Oxyntomodulin (OXM) concentration in plasma vs. time profiles following intravenous bolus injection (IV) at 0.05 mg/kg, and orotracheal instillation (IT) at 0.05 mg/kg and 0.50 mg/kg in rats on A) linear and B) semi-log scales. The solid lines are plotted by linear interpolation. Note: Only two rats had results at 90 minutes for IV and IT dose (0.5 mg/kg) Data represent mean±SD (n=4).

5.D. DISCUSSION

5.D.1 PHARMACOKINETIC SUMMARY

The validated LC-MS/MS method for the analysis of OXM in rat plasma as described in Chapter 4 was used to determine plasma the plasma concentration-time profiles, using non-compartmental pharmacokinetic analysis. The plasma concentration-time profile was shown to be a biexponential plot following intravenous bolus injection. The terminal half-life was 20±3.3 minutes and clearance (CL) was 1.4±0.5 mL/min, suggesting that OXM was eliminated by the kidneys . The pulmonary administration of OXM yielded half-lives similar to that for intravenous injection. Additionally, the $AUC_{0\text{-}inf}$ and C_{max} were dose-proportional, suggesting that linear kinetics max exist between the dose range of 0.05 and 0.5 mg/kg. This dose-proportional pharmacokinetics most likely lent a support to lung absorption via diffusion and showed non flip-flop kinetics as previously concluded with a similar peptide, $PYY_{3\text{-}36}$ (Nadkarni, 2009). The bioavailability of OXM with pulmonary administration remained low at 13% and OXM disappeared from systemic circulation within 90 minutes, which was likely the reason for 4-6 hours of food intake suppression in rats (Nadkarni, 2009).

5.D.2 ANALYTICAL EFFECTS ON PHARMACOKINETICS OF OXYNTOMODULIN WITH DIFFERENT SPECIES

Pharmacokinetic studies of oxyntomodulin have been reported following intravenous infusion (IV-inf), subcutaneous, or IV bolus routes of administration with radioimmunoassays. Pharmacokinetic parameters for C_{max}, area under the curve ($AUC_{0\text{-}inf}$), half-life ($t_{1/2}$), clearance (CL), and volume of distribution (Vd_{cc}) have been determined based on traditional non-

compartmental methods and are shown in Table 5.4. Terminal half-life ($t_{1/2}$) was similar in the present study compared as to a study in pigs (Baldissera et al., 1988), in which $t_{1/2}$ was approximately 20 minutes for both species; however, pigs were subjected in IV bolus and multiple infusions with oxyntomodulin as compared to the intravenous (IV bolus) administration in rats. In addition, metabolism between species can be different. A human study also reported $t_{1/2}$, which was found to be 12±1minute following an IV-infusion rate of 100 and 400 $ng/kg^{-1}/h^{-1}$ (Schjoldager et al., 1988). Studies performed in humans with infusions of OXM at different doses were comparable, where $AUC_{0\text{-}inf}$/dose was calculated for Schjoldager et al. and Cohen et al resulting in 364.8 versus 349.3 kg x min/ml, respectively. Cross evaluation of species and route of administration indicates differences in reported pharmacokinetic parameters but these differences may be effects related to the species themselves, the route of administration, and also possibly the analytical technique employed. Radioimmunoassay's (RIA) have been used traditionally for the pharmacokinetics of peptides such as oxyntomodulin. Some disadvantages to these techniques such are: the need for radioactive disposal, normally require two antibodies, limited linearity is normally one to two orders of magnitude, and non-specific binding, which can affect precision and accuracy (Hoofnagle et al., 2008). In table 5.4, three of the pharmacokinetic studies were obtained by an in-house radioimmunoassay (Cohen et al., 2003; Schjoldager et al., 1988; Wynne et al., 2005). These results were determined based estimating human enteroglucagon (later named OXM) from subtraction of RIA values obtained with a glucagon antiserum that is C-terminally reacting (RCS5) from those acquired with an N-terminal-to midmolecule-reacting antiserum (R59). However, these results could be inaccurate due to non-specific binding from anti-reagent antibodies, which may result in false positives (Hoofnagle et al., 2009; Whiteaker et al., 2007). Immunoassays have also shown inter-laboratory variability

(Rawlins et al., 2004), where groups demonstrated sample data variations with six immunoassay platforms for thyroid stimulating hormone. Pharmacokinetic parameters can be affected by the lack of selectivity or variability. Parameters affected by variations in plasma concentration-time profile data include: $AUC_{0\text{-}inf}$, clearance, and bioavailability.

LC-MS/MS is a viable alternative for pharmacokinetics studies due to its selectivity and ability to quantify multiple analytes in one sample simultaneously, if necessary. Minimal studies have been performed investigating inter-laboratory variability when employing LC-MS/MS for the analysis of polypeptides. However, a recent multi-site assessment between eight laboratories and simultaneous quantification of seven proteins fortified in human plasma indicated good inter-laboratory precision (<20% RSD) for six of the seven proteins evaluated (Addona et al., 2009b). Leptin was the only exception, which had a larger inter-laboratory variation (>20% RSD). Although new immunoassay technologies have similar capabilities detecting multiple analytes, they lack the ability to selectively quantify metabolites or derivatives of similar peptides.

Table 5.4 Pharmacokinetic parameters of oxyntomodulin in various species

Species	Route	Dose (μg/kg)	Analytical Method	C_{max} (pmol/L or ng/mL)	$AUC_{0\text{-}inf}$ (ng*min/mL)	$t_{1/2}$ (min)± SD	Reference
human	IV-inf	0.40	RIA	264, 1175	145,947	12±1	(Schjoldager et al., 1988)
human	IV-inf	1.4	RIA	907, 4035	489,079	59	(Cohen et al., 2003)
human	SC	18.9	RIA	972, 4325	536,719	90	(Wynne et al., 2005)
pig	IV/IV-inf	#	RIA	300, 1335	57,002	20±4	(Baldissera et al., 1988)
rat	IV	50.0	LC-MS/MS	124, 554	13,008	20±3.3	present study

Notes: Standard deviations are shown when available for half-life as published or with the present study. $AUC_{0\text{-}inf}$ is based on estimated plasma concentration-time profiles digitized from the plasma concentration vs. time profiles presented in the literature or present data. #Dose for pig study was not calculated based on μg/kg due to variable dosing regimen throughout study with IV and IV-inf doses. RIA = radioimmunoassay, LC-MS/MS = liquid chromatography tandem mass spectrometry.

5.D.3 COMPARISON OF OXYNTOMODULIN TO OTHER PEPTIDES FOLLOWING PULMONARY ADMINISTRATION IN RATS

Pulmonary administration has been evaluated in rats for similar sized peptides (Gedulin et al., 2008; Nadkarni, 2009). Table 5.5 presents some physical characteristics along with bioavailability F(in %) following pulmonary administration in rats. The molecular weight (MW), number of amino acid residues (number of AA), isoelectric point (pI), percent hydrophobic amino acids (%H, AA), percent acidic amino acids (%A, AA), percent basic amino acids (%B, AA), percent neutral amino acid (%N, AA), and bioavailability (%F) were compared to determine if similar peptide physical and chemical characteristics would be expected to yield

similar low bioavailability. Similar bioavailability results were obtained with OXM, exenatide, and PYY_{3-36} in rats, as molecular weights, number of amino acids, and percent hydrophobic amino acid residues (%H) were comparable. This implies that these characteristics may result in similar bioavailability for pulmonary administration. However, lung absorption and non-absorption (other than absorption) rates may be different for these peptides.

Table 5.5 Comparison of similar peptide physical characteristics that have employed pulmonary delivery in rats

Peptide	MW (g/mol)	number of AA	pI	%H AA	%A AA	%B AA	%N AA	F (%)	Analytical Method	Reference
OXM	4449	37	9.9	32.43	8.11	18.92	40.54	12.7	LC-MS/MS	Present study
PYY_{3-36}	4149	34	9.3	38.24	14.71	20.59	26.47	13.0	ELISA	(Nadkarni, 2009)
Exenatide	4187	39	4.5	28.21	15.38	10.26	46.14	13.6	ELISA	(Gedulin et al., 2008)

Note: AA = amino acids. pI = isoelectric point. %H = percent hydrophobic AA, %A = percent acidic AA, %B = percent basic AA, %N = percent neutral AA, F = bioavailability in percent. ELISA = enzymed linked immunosorbent assay.

5.E. CONCLUSIONS

Liquid chromatography tandem mass spectrometry (LC-MS/MS) was employed to successfully determine the in vivo pharmacokinetics of oxyntomodulin (OXM) following intravenous injection and intratracheal instillation in rats. The validated method was used to obtain plasma concentration-time profiles of OXM, which were then characterized with noncompartmental analysis. Intravenous administration of OXM had profiles that appeared to be biexponential, yielding the terminal $t_{1/2}$ of 20.0±3.3 min. Its Vd_{pss} and CL were 130.3±41.3 ml/kg and 3.81±1.32 ml/min/kg, respectively. These results suggest a narrow distribution of OXM within the body and that OXM was most likely eliminated by the kidneys. Pulmonary administration for both doses (0.05 and 0.5 mg/kg) indicated dose-proportional C_{max} and AUC_{0-inf}, and was also confirmed by CL/dose. The terminal slopes were consistent with that observed in the intravenous profile. This also indicates that lung absorption kinetics were linear. Furthermore, the lung absorption was not rate-determined in its overall pharmacokinetics, i.e., non- "flip-flop". The low bioavailability was shown to be only 13 %, and the peptide was not detected after 90minutes.

CHAPTER 6

SUMMARY AND OVERALL CONCLUSIONS

Targeted protein and polypeptide quantification have increasingly become important to the validation of biomarker candidates, their quantitative analysis and the development of protein therapeutics. Peptide or protein therapeutic treatment requires pharmacokinetic studies performed with reliable analytical methodologies. Since the 1950's, ligand binding assays have been the standard of practice for measuring protein and peptides in clinical samples (Hoofnagle et al., 2009). The platform for immunoassays has evolved from radioimmunoassay to primarily enzyme linked immunosorbent assays (ELISA), and microfluidic point of care testing immunoassays (Hoofnagle et al., 2009). Despite these advancements in technology for immunoassays, they suffer from the same limitations, which is that the selectivity of the assay is limited by the antibody used. This lack of selectivity is especially a concern with structurally related compounds such as drug metabolites which may be found in biological samples. The inaccuracy from this lack of selectivity in human or animal samples can lead to falsely elevated results that would affect the pharmacokinetic data, and interpretation of the appropriate dose. As a serious example of this problem, five different immunoassay platforms where evaluated for the determination of a tumor marker for pancreatic cancer (cancer antigen 19-9, CA 19-9). Unfortunately, the study revealed the potential for a patient to be misdiagnosed with a recurrence of cancer in one immunoassay, only to find out with another method they were still in remission (La'ulu et al., 2007). These inadequacies associated with immunoassays support alternative methods for quantitative analysis is desirable. This led us and others to investigate alternative methods for quantitative analysis of large molecules (i.e., proteins and peptides) in plasma using the small molecule gold standard, liquid chromatography tandem mass spectrometry.

In the first chapter, strategies for quantitative analysis of large molecules using liquid chromatography tandem mass spectrometry were discussed. Technological advances over the last twenty years in biochemistry and mass spectrometry have allowed the use of peptides as surrogates to quantify enzyme digested proteins using triple quadrupole mass spectrometers. Multiple sample preparation processes may be incorporated to achieve quantification of target proteins using these signature peptides. Employing signature peptides as quantitative surrogates of the target protein may be considered a viable alternative to immunoassays. This multi-step process includes 1) *In Silico* signature peptide prediction and modeling 2) *In silico* MRM peptide transition modeling coupled with real mass spectra 3) protein purification 4) enzymatic digestion 5) signature peptide purification 6) incorporation of an isotope labeled internal standard peptide or protein 7) quantitative LC-MS-MS using MRM transitions for signature peptide(s) and internal standard(s).

Employing this multi-step signature peptide approach for quantitative analysis of a proteins was demonstrated in chapter 3. Specifically, quantitative analysis of a therapeutic protein through use of surrogate proteotypic peptides was evaluated for the measurement of Amevive (Alefacept) in human plasma using liquid chromatography tandem mass spectrometry. Signature peptides were obtained through *in silico* and iterative tuning processes to represent Alefacept for quantification. Horse heart myoglobin was chosen as a protein analogue internal standard to compensate for errors associated with matrix effects and to track recovery throughout the entire sample pretreatment process. The myoglobin signature peptide was chosen due its similar retention time to the alefacept signature peptide (3.6 versus 4.2 minutes). Therefore,

protein analogue internal standards can be an effective way of tracking sample pretreatment, digestion, and ultimately correcting for matrix effects when a similar retention time is obtained. Samples were prepared for analysis by selective precipitation of the target proteins with pH controlled at 5.1 and heat denaturation at 45°C followed by enzymatic digestion, dilution, and filtration. This partial precipitation extraction requires multiple combinations of pH and temperatures to fully optimize this sample purification step. However, by applying the optimization studies discussed in Chapter 3, other proteins with isoelectric points well away from human serum albumin may use this approach for purification prior to digestion. This will allow an antibody-free purification step in plasma. Following the partial protein precipitation, an on –line extraction of the signature peptides was carried out which allowed for multiple injections with only changing the guard column. The same analytical column was used for the entire validation, with over 400 injections and counting. Therefore, on-line sample purification preceded by a partial protein precipitation can be combined as an effective and robust analytical method for analysis of plasma samples. Tandem mass spectrometric detection was performed on a hybrid triple quadrupole linear ion trap equipped with electrospray ionization to positively ionize signature peptides for Alefacept and myoglobin. The method was linear for Alefacept (protein) concentrations between 250 and 10,000 ng/mL. Precision and accuracy for inter and intra-assay for the lower limit of quantification was less than 20% (16.2 and 10.3, respectively). The method was validated according to current FDA guidelines for bioanalytical method validation and no antibody purification step was needed to quantify therapeutic levels.

In chapter 4, intact polypeptide quantification was investigated as a second alternative approach to immunoassays. Biomolecules can exist as intact ions, with

multiple charges using electrospray ionization (Fenn et al., 1990). Using these principles, a polypeptide was chosen for intact quantification by using its multiple charge state form. The multiple charges allowed for the m/z to be reduced to within the triple quadrupole mass range (2800 m/z) for analysis of oxyntomodulin (4449 Da) in rat plasma. In this assay, a modified solid phase extraction (SPE) method coupled with two-dimensional reversed phase ion pair chromatography tandem mass spectrometry was used for the validation and analysis of oxyntomodulin in rat plasma. Modified SPE, two-dimensional liquid chromatography coupled with 3-nitrobenzyl alcohol as a mobile phase additive, along with monitoring of multiply charged SRM transitions (+7 charge state) of OXM were found to be necessary to achieve a lower limit of quantification of 1 ng/mL. The method was validated within range of 1 to 1000 ng/mL, and met current FDA guidelines. This linear range was appropriate for obtaining the plasma concentration time-profiles presented in chapter 5. Following a complete validation, the method was applied to a pharmacokinetic study using rat plasma samples that were intravenously (IV bolus) dosed or received pulmonary administration of oxyntomodulin.

Alternative approaches to immunoassays were established and validated in chapters 3 and 4. A critical part of the evaluation of any methodology however is application to real samples. Real samples provide a better argument using alternative methodologies. Therefore, the validated oxyntomodulin method described in chapter 4 was applied for the pharmacokinetic evaluation of rats dosed with intravenous and oratracheal instillation (IT) routes of administration at doses of 0.05 mg/kg for IV and IT, and 0.5 mg/kg IT. The method allowed for the accurate determination of plasma concentration-time profiles which exhibited a biexponential profile; therefore, simultaneous distribution and

elimination was postulated. The terminal half-life for intravenously dosed rats was 20.0±3.3 min. Pulmonary administration (using IT) profiles were dose-proportional at 0.05 and 0.5 mg/kg and indicated linear kinetics. Furthermore, the terminal half-lives were similar to the intravenous injection, which suggested non-"flip-flop" pharmacokinetics for oxyntomodulin in rats. The absolute bioavailability (%F) was low at ~13%, which may suggests non-absorptive loss of oxyntomodulin from the lung.

Overall this dissertation successfully provided useful approaches for quantitative analysis of large molecules in plasma. In chapter 3, partial protein precipitation (selective precipitation) was demonstrated as an effective sample purification for proteins that possess an isoelectric point away from major plasma proteins such as albumin (pI = 4.7). This approach combined with on-line sample purification could be applied to other proteins with similar pI's following optimization of pH and temperature. Combined with these purification steps was the employment of a protein analogue internal standard, myoglobin. Even though progress has been made recently with use of extended peptide internal standards (Plumb et al., 2012), using a protein analogue internal standard is an economical approach to compensating for matrix effects and tracking the entire sample pretreatment process. Retention matching signature peptides may be applied to other similar proteins.

In chapter 4, taking advantage of the multiple charges on an intact biomolecule proved to be another alternative to immunoassays. Combining mass to charge (m/z) ratio reduction with optimizing the charge state distribution and product ion formation allowed for a large polypeptide (OXM: 4449 Da) to be quantified with a triple quadrupole mass spectrometer. This is unique in itself given that most large polypeptides have not been

quantified using a multiple charge state and selected reaction monitoring (Van Den Broek et al., 2008). Using 3-nitrobenzyl alcohol (3-NBA) was shown to increase charge states and intensity in the peptides evaluated. Although this has been shown with cytochrome c previously (Lavarone et al., 2001), chapter 4 demonstrated the novelty with 3-NBA as an ion pair for the first time using it coupled with reversed phase chromatography for separation of OXM and enhancement of sensitivity.

LC-MS/MS was shown to precisely and accurately quantify a signature peptide as a surrogate for a protein therapeutic, Alefacept and quantify a multiply charged intact polypeptide, oxyntomodulin, in plasma. These approaches show great promise as alternatives to immunoassays and are applicable to other large molecules.

Technological advances will improve these alternative approaches and eventually become a new tradition of for quantification of proteins and peptides. Advances with using extended peptide internal standards or even stable isotope labeled proteins will allow for sufficient tracking of sample purification and compensate for matrix effects. Using purification techniques such as immunoaffinity may improve sensitivity of protein quantification with LC-MS/MS as recently demonstrated (Xu et al., 2010), which may compete with immunoassay detection limits. Detection limits are still a limiting factor with LC-MS/MS compared to immunoassays, but with superior selectivity and technological advancement, it is only a matter of time when quantitative analysis of samples in plasma will use LC-MS/MS as the gold standard for small and large molecules.

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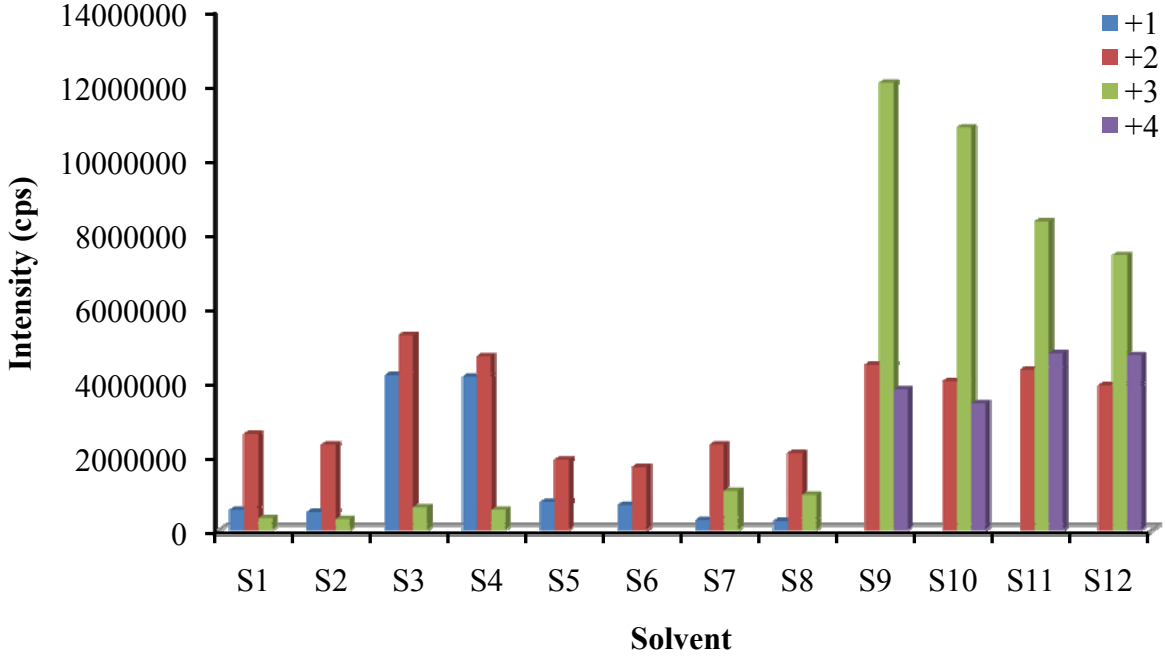
APPENDIX 1

CHARGED STATE DISTRIBUTION INTENSITY OF POLYPEPTIDES IN TWELVE SOLVENT MIXTURES

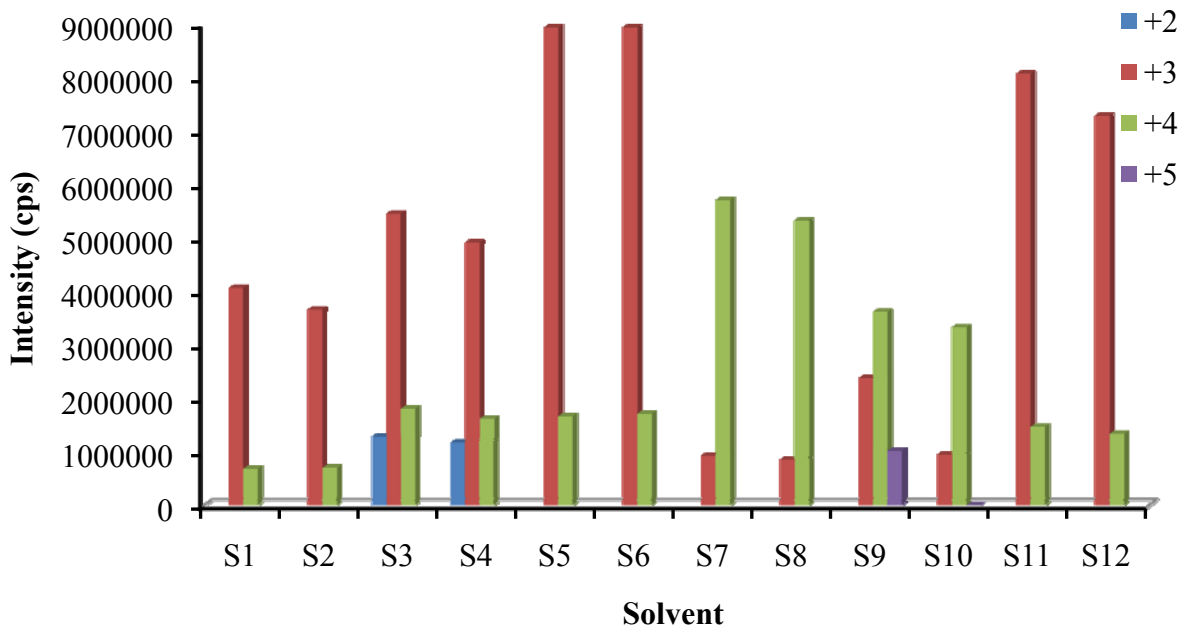
Solvents	Description
S1	25:75 ACN:H2O
S2	25:75 MeOH:H2O
S3	25:74:1 ACN:H2O:3-NBA
S4	25:74:1 MeOH:H2O:3-NBA
S5	25:74:1 ACN:H2O:glycerol
S6	25:74:1 MeOH:H2O:glycerol
S7	25:74:1 ACN:H2O:formic acid
S8	25:74:1 MeOH:H2O:formic acid
S9	24:74:1:1 ACN:H2O:3-NBA:formic acid
S10	24:74:1:1 MeOH:H2O:3-NBA:formic acid
S11	24:74:1:1 ACN:H2O:glycerol:formic acid
S12	24:74:1:1 MeOH:H2O:glycerol:formic acid

Note: ACN = acetonitrile, MeOH = methanol, 3-NBA = 3-nitrobenzyl alcohol, H_2O = deionized water

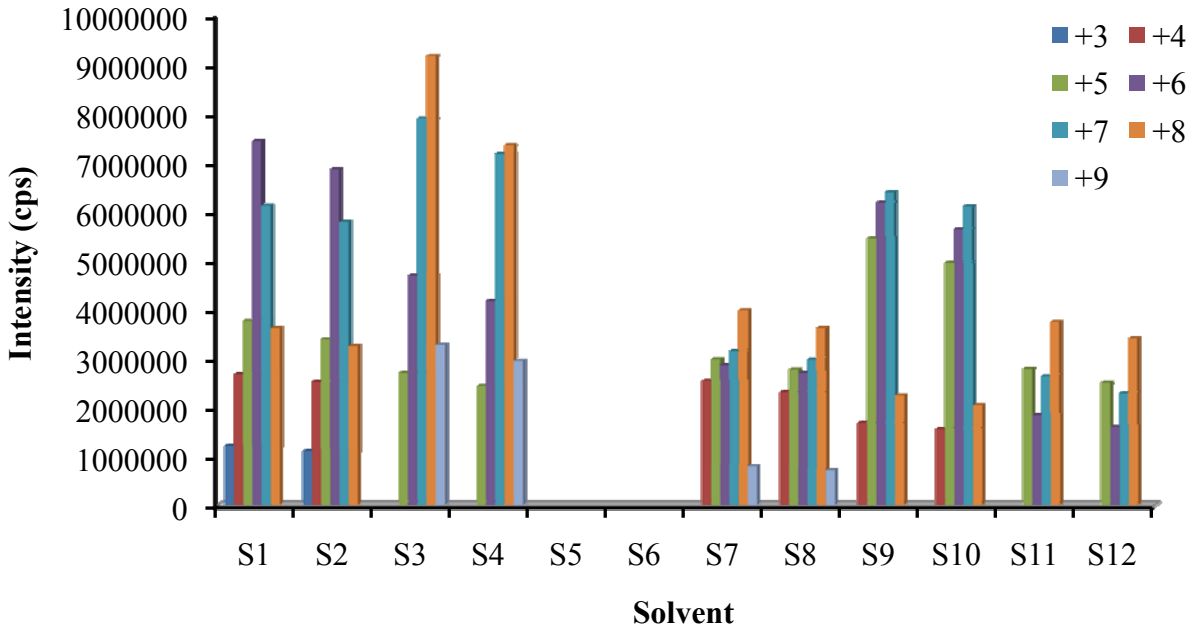
CHARGE STATE DISTRIBUTION OF ANGIOTENSIN-1



CHARGE STATE DISTRIBUTION OF OBESTATIN

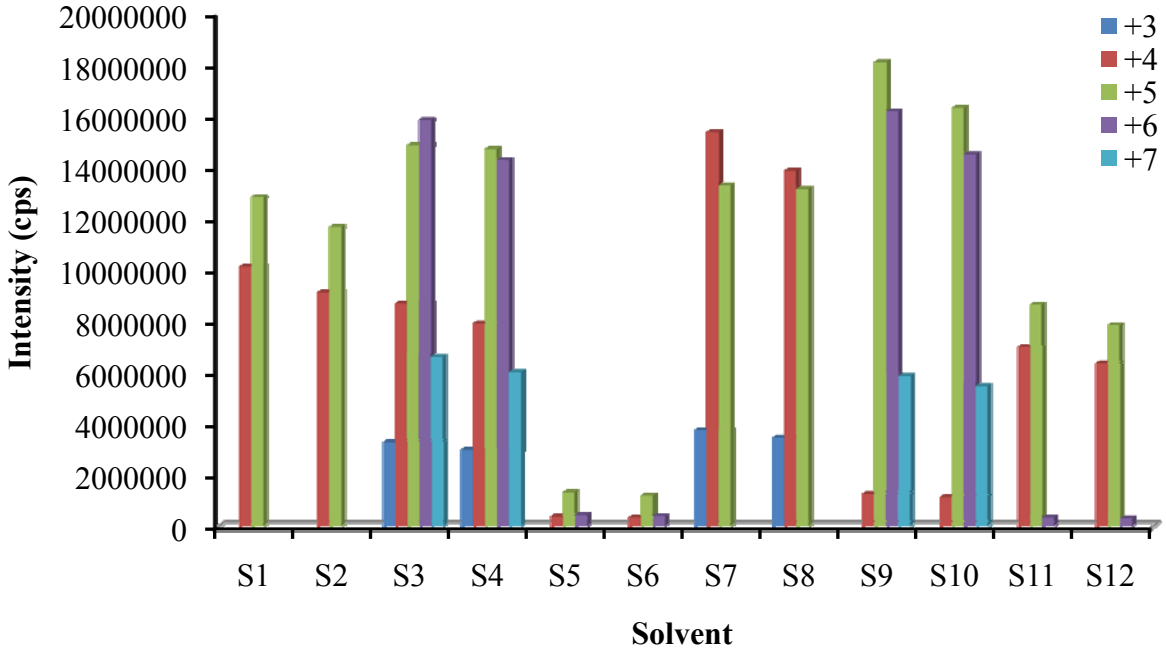


CHARGE STATE DISTRIBUTION OF GHRELIN

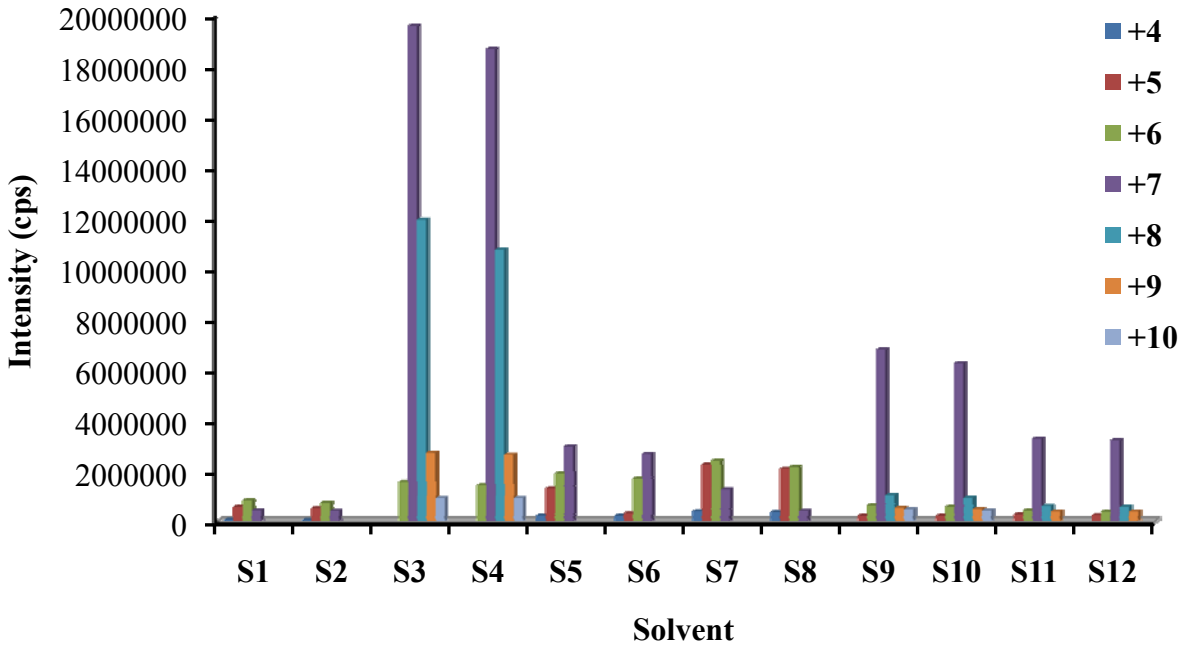


Note: no charge states were detected for solvents 5 and 6.

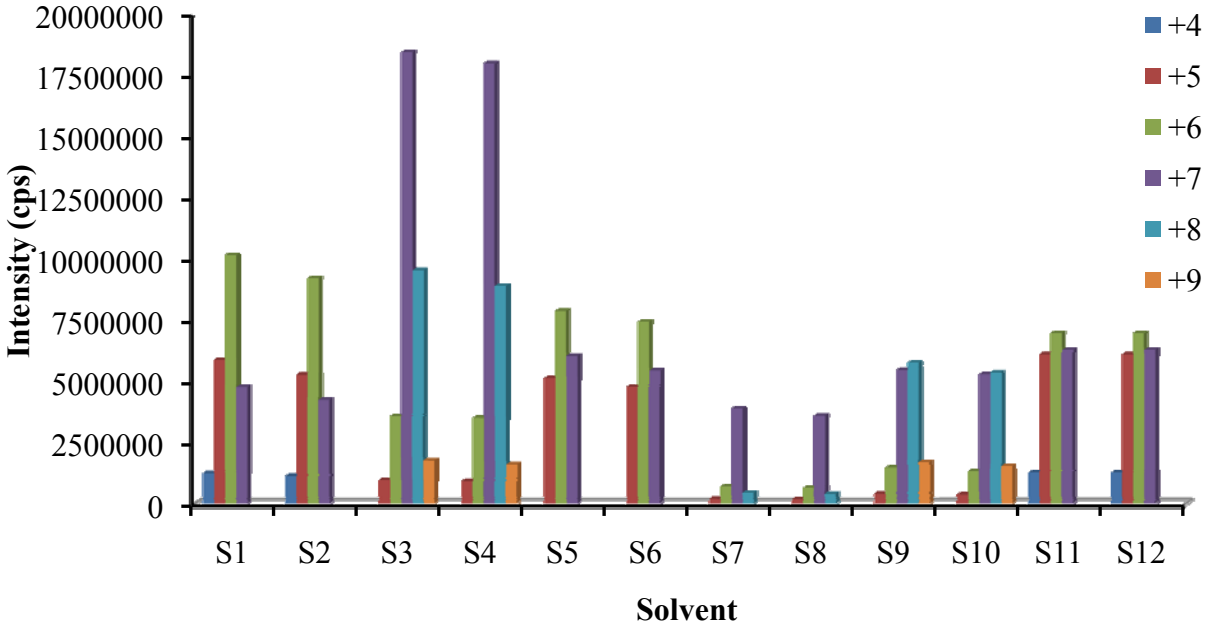
CHARGE STATE DISTRIBUTION OF GLUCAGON



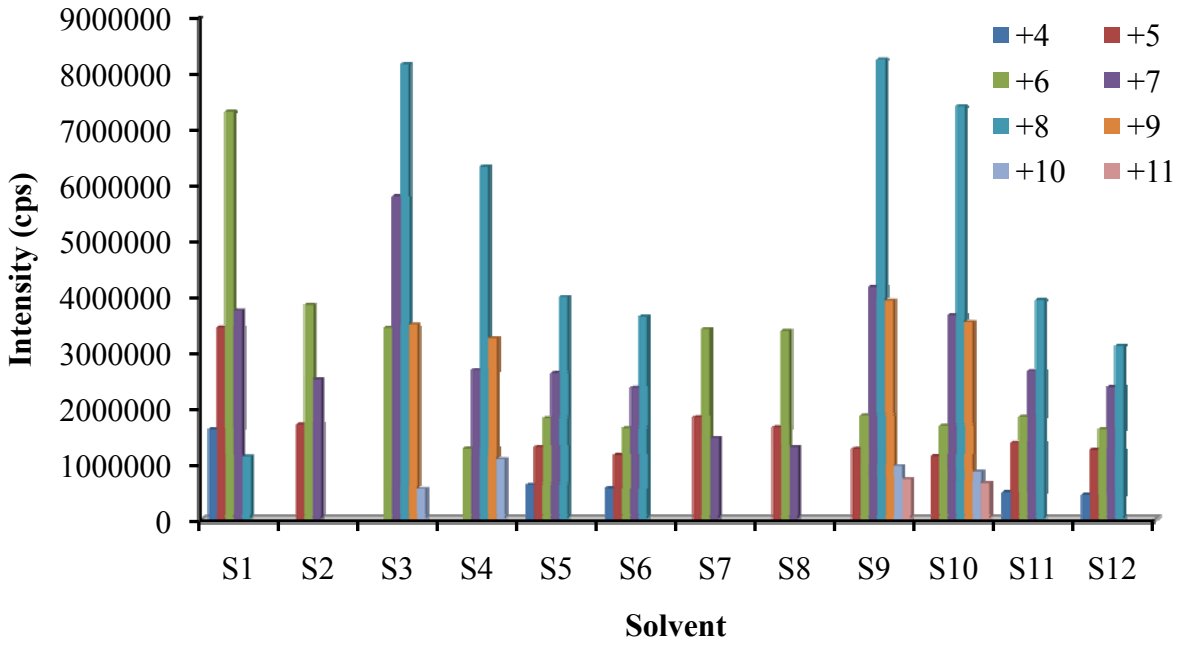
CHARGE STATE DISTRIBUTION OF PYY_{1-36}



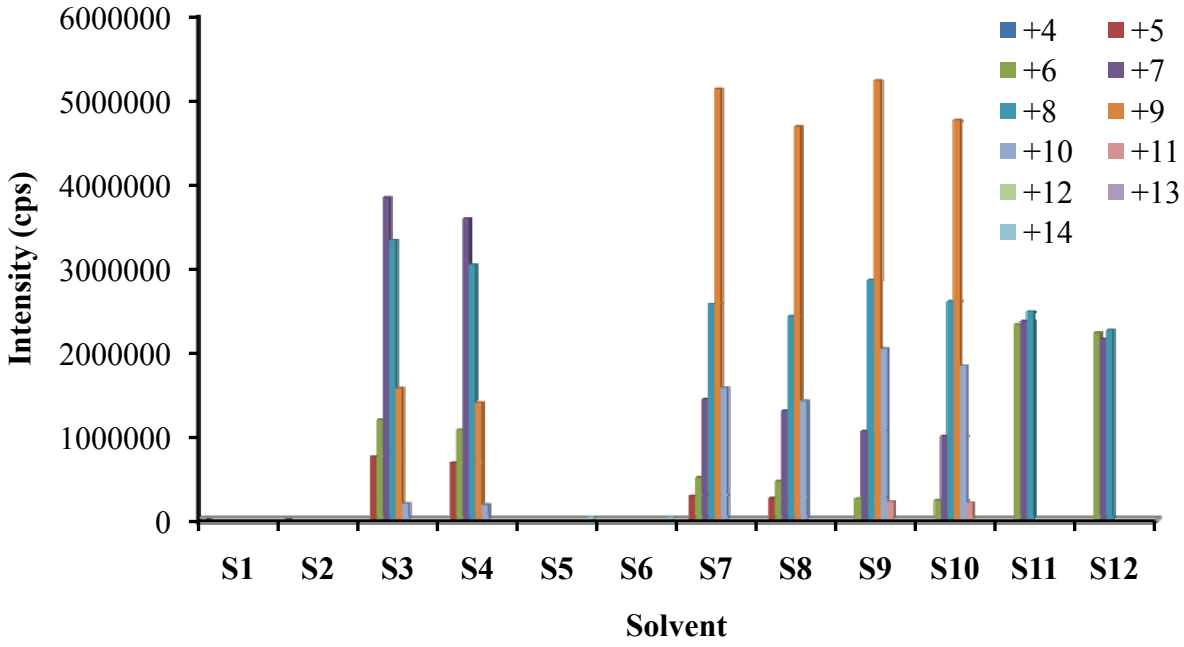
CHARGE STATE DISTRIBUTION OF PYY_{3-36}



CHARGE STATE DISTRIBUTION OF OXYNTOMODULIN

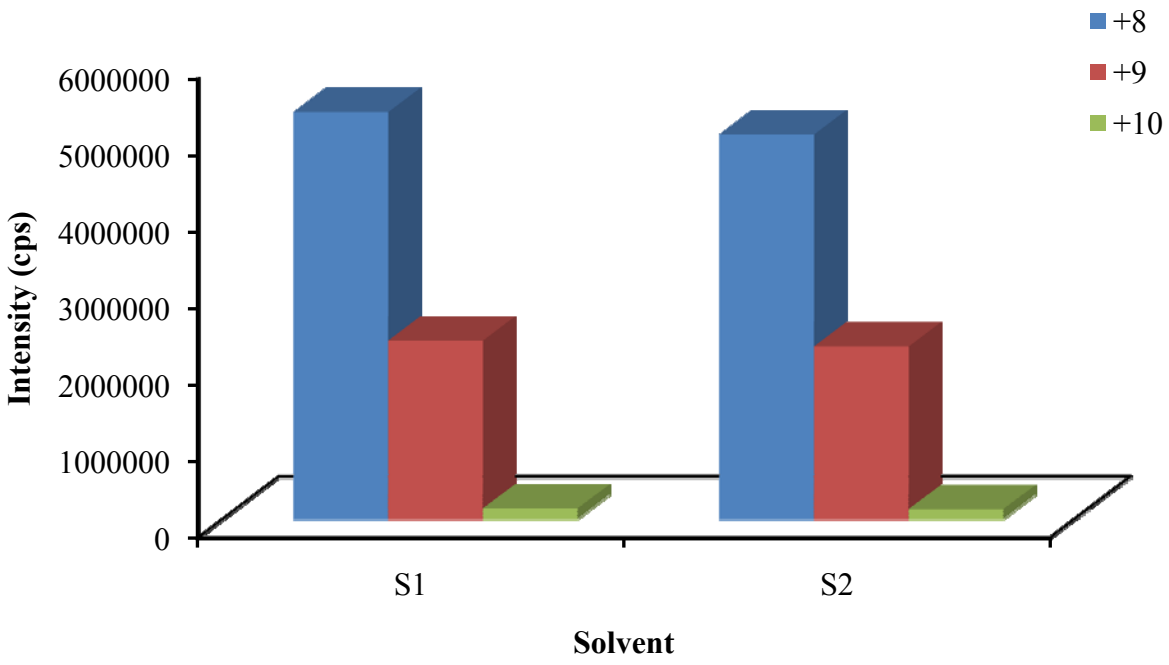


CHARGE STATE DISTRIBUTION OF INSULIN GROWTH FACTOR-1 (IGF-1)



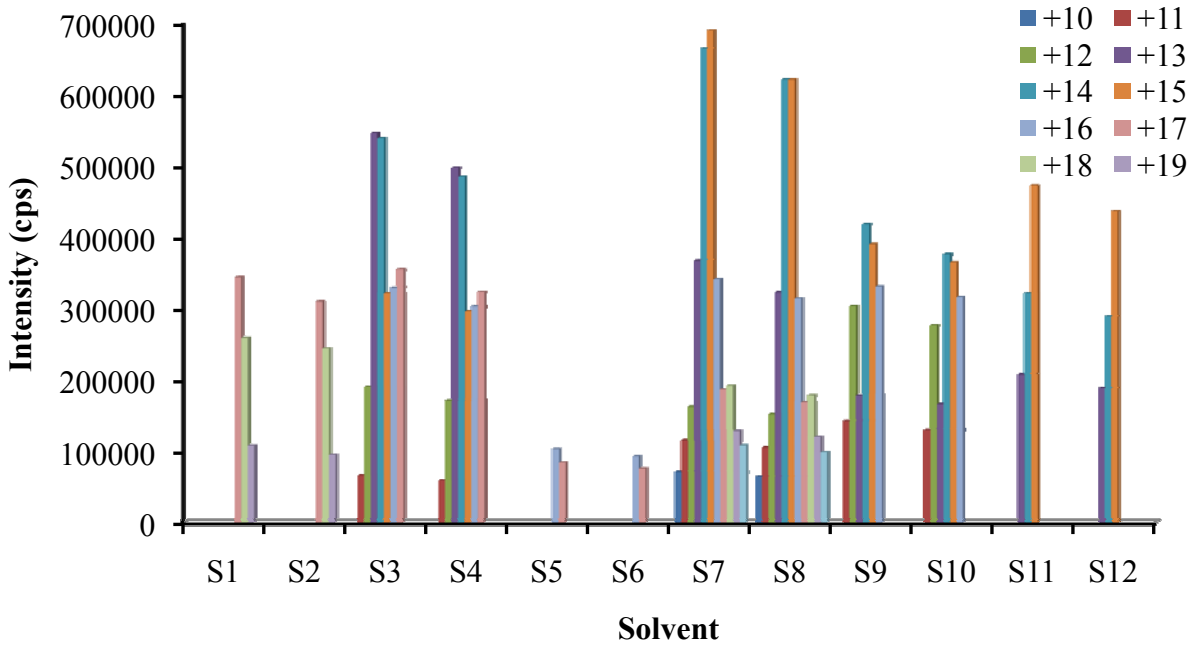
Note: no charge states were detected for solvents 1, 2, 5, and 6.

CHARGE STATE DISTRIBUTION OF PARATHYROID HORMONE (PTH)

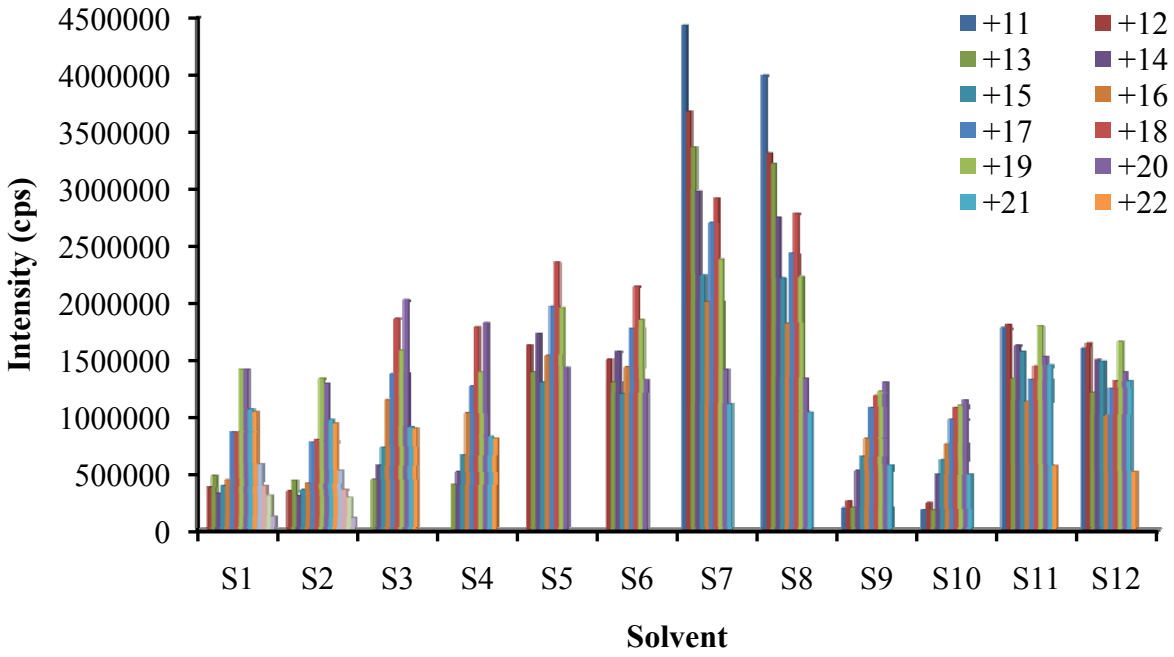


Note: only solvents 1 and 2 showed charge states for PTH. No other solvent detected any charge state for PTH.

CHARGE STATE DISTRIBUTION OF LEPTIN



CHARGE STATE DISTRIBUTION OF MYOGLOBIN



APPENDIX 2

NORMALIZED PLASMA OXM CONCENTRATION TABLES FOR RATS DOSED WITH INTRAVENOUS AND ORATRACHEAL INSTILLATION ROUTES OF ADMINISTRATION

IT 0.05 mg/kg

Time		RAT				mean	SD	SE
min	**h**	**#2**	**#8**	**#9**	**#10**			
0	0.00	**0**	**0**	**0**	**0**	0.00	0.00	0.00
2	0.03	missing	21.50	22.28	18.86	**20.88**	**1.79**	**1.03**
5	0.08	33.93	31.20	51.84	36.81	**38.45**	**9.22**	**4.61**
10	0.17	30.03	25.75	48.65	32.60	**34.26**	**10.00**	**5.00**
20	0.33	24.91	11.61	41.95	15.55	**23.50**	**13.50**	**6.75**
30	0.50	9.70	8.34	33.46	11.94	**15.86**	**11.83**	**5.91**
45	0.75	5.43	7.35	8.84	9.57	**7.80**	**1.83**	**0.91**
60	1.00	3.18	5.65	3.96	5.02	**4.45**	**1.10**	**0.55**
90	1.50	1.85	2.77	2.40	2.21	**2.30**	**0.38**	**0.19**

IT 0.5 mg/kg

Time		RAT				mean	SD	SE
min	**h**	**#1**	**#11**	**#12**	**#13**			
0	0.00	**0**	**0**	**0**	**0**	0.00	0.00	0.00
2	0.03	0.00	364.58	209.43	360.90	**233.73**	**171.77**	**99.17**
5	0.08	362.96	446.15	461.16	525.60	**448.97**	**66.90**	**33.45**
10	0.17	344.91	392.11	380.27	359.10	**369.09**	**21.13**	**10.56**
20	0.33	126.36	276.48	224.34	247.50	**218.67**	**65.13**	**32.57**
30	0.50	84.45	211.76	173.89	211.50	**170.40**	**60.00**	**30.00**
45	0.75	59.12	125.05	112.74	160.20	**114.28**	**41.91**	**20.96**
60	1.00	14.18	76.66	49.05	142.20	**70.52**	**54.19**	**27.10**
90	1.50	BLOQ	28.91	BLOQ	38.70	**33.80**	**N/A**	**N/A**

IV Bolus 0.05 mg/kg

Time		RAT				mean	SD	SE
min	**h**	**#4**	**#5**	**#6**	**#7**			
2	0.03	394.38	438.07	594.38	790.75	**554.39**	**179.45**	**89.72**
5	0.08	327.50	352.20	437.77	490.47	**401.98**	**75.58**	**37.79**
10	0.17	265.00	228.99	378.34	423.71	**324.01**	**92.02**	**46.01**
15	0.25	251.25	152.45	246.02	256.81	**226.63**	**49.65**	**24.82**
20	0.33	176.25	103.92	133.86	231.51	**161.38**	**55.37**	**27.69**
30	0.50	166.25	82.76	104.40	214.28	**141.92**	**59.82**	**29.91**
45	0.75	90.00	56.63	84.76	151.29	**95.67**	**39.87**	**19.93**
60	1.00	63.75	26.13	19.12	142.67	**62.92**	**56.66**	**40.07**
90	1.50	20.63	BLOQ	missing	24.77	**22.70**	**N/A**	**N/A**