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# Quantitative Mass Spectrometric Investigations of Protein Biomarkers: Serum Thymidine Kinase 1 and Human Osteopontin

Morse Faria

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# QUANTITATIVE MASS SPECTROMETRIC INVESTIGATIONS

# OF PROTEIN BIOMARKERS:

# SERUM THYMIDINE KINASE 1 AND HUMAN OSTEOPONTIN

Submitted in partial fulfillment of the requirement for

Doctor of Philosophy degree at Virginia Commonwealth University

By

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*This work is dedicated to my mother, Late Mrs. Margaret Joanita Faria, whose loss to cancer has been a key motivation for my research work.*

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I would like to dedicate this thesis to my mother who we lost to cancer 25 years ago. My research was focused around cancer biomarkers and the loss of my mother was a constant driving force for my research. I hope that with the continuous dedicated efforts of thousands of scientists around the world, we will one day have cure for cancer.

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| | |
|---|---|
| %CV | Percent coefficient of variation |
| %DFN | Percent deviation from nominal concentration |
| %RSD | Percent relative standard deviation |
| API | Atmospheric pressure ionization |
| AQUA | Absolute Quantification |
| ATCC | American Type Culture Collection |
| ATP | Adenosine 5'-triphosphate |
| AZT | 3'-azido-3'-deoxythymidine |
| BEH | Ethylene Bridged Hybrid |
| BLAST | Basic Local Alignment Search Tool |
| BrdU | 5-bromodeoxyuridine |
| BrdUMP | Bromodeoxyuridine monophosphate |
| CE | Collision Energy |
| CSE | Cigarette smoke extract |
| CTP | Center for Tobacco Products |
| CXP | Collision cell exit potential |
| DMEM | Dulbecco's Modified Eagle's Medium |

| | |
|---|---|
| DMSO | Dimethyl Sulfoxide |
| DP | Declustering potential |
| DTE | Ddithioerythritol |
| dThd | Deoxythymidine |
| DTT | Dithiothreitol |
| dUrd | Deoxyuridine |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme Linked Immuno Sorbent Assay |
| EP | Entrance potential |
| ER | estrogen receptor |
| ESI | Electrospray ionization |
| ESP | Enhanced Signature Peptide Predictor |
| FAIMS | Field asymmetric waveform ion mobility spectrometry |
| FBS | Fetal bovine serum |
| FLT- d3 | 3'-Deoxy-3'fluorothymidine-5'-d3 |
| FLT | 3'deoxy-3'-fluorothymidine |
| FLT-MP | 3'deoxy-3'-fluorothymidine monophosphate |
| FLT-MP-d3 | 3'-Deoxy-3'fluorothymidine-5'-monophosphate-d3 |
| FWHM | Full width at half maximum |
| HCC | Hepatocellular carcinoma |
| HCl | Hydrochloric Acid |
| Her2/*neu* | Human epidermal growth factor receptor 2 |
| HILIC | Hydrophilic interaction liquid chromatography |

| | |
|---|---|
| hOPN | Human osteopontin |
| HPLC | High performance liquid chromatography |
| HRMS | High Resolution Mass Spectrometry |
| HUVECs | Human Umbilical Vein Endothelial Cells |
| IA | Immunoaffinity |
| IBS | Institute for Systems biology |
| IC | Immunocapture |
| IEC | Ion-exchange chromatography |
| IS | Internal Standard |
| LBA | Ligand binding assays |
| LC-MS | Liquid chromatography coupled with mass spectrometry |
| LC-MS/MS | Liquid chromatography coupled with tandem mass spectrometry |
| LIT | Linear ion trap |
| LLOQ | Lower limit of quantification |
| LOQ | Limit of Quantification |
| MALDI | Matrix assisted laser desorption ionization |
| MFLC | Microflow Liquid Chromatography |
| $MgCl_2$ | Magnesium chloride |
| MMP-3 | Metalloproteinase-3 |
| MRM | Multiple reaction monitoring |
| MS | Mass spectrometry |
| MS/MS | Tandem mass spectrometry |
| $MS^2$ | Tandem mass spectrometry |

| MS3 | Three stage tandem mass spectrometry |
|---|---|
| NIH | National Institute of Health |
| NIST | National Institute of Standards and Technology |
| OPN-SP | OPN Signature Peptide |
| PBS | Phosphate buffered saline |
| pDI QC | Plasma dilution quality control |
| pHQC | Plasma high quality control |
| pLLOQ | Plasma lower limit of quantification |
| pLQC | Plasma low quality control |
| pMQC | Plasma medium quality control |
| PPD | Pharmaceutical Product Development |
| PR | progesterone receptor |
| PSAQ | Protein Standard Absolute Quantification |
| PTM | Post translational modification |
| QC | Quality Control |
| QonCAT | Quantification concatemer |
| QqQ | Triple quadropole (QqQ) |
| QTrap | Quadrapole Ion Trap (QTrap) |
| rhOPN | Recombinant Human osteopontin |
| RPLC | Reverse phase liquid chromatography |
| SD | Standard deviation |
| SIL | Stable isotope labeled |
| SILAC | Stable isotope labeling by amino acids in cell culture |

| | |
|---|---|
| SIL-IS | Stable isotope labeled internal standard |
| SISCAPA | Stable Isotope Standards with Capture by Anti-Peptide Antibodies |
| SPE | Solid phase extraction |
| SRM | Selected reaction monitoring |
| ST | Smokeless Tobacco |
| TFA | Trifluoroacetic acid |
| TK1 | Thymidine kinase 1 |
| TK-REA | Thymidine kinase radioenzymatic assay |
| TOF | Time of flight |
| UMP | Uridine 5'-monophosphate |
| US FDA | United States Food and Drug Administration |

**ABSTRACT**


**QUANTITATIVE MASS SPECTROMETRIC INVESTIGATIONS OF PROTEIN**

**BIOMARKERS: SERUM THYMIDINE KINASE 1 AND HUMAN OSTEOPONTIN**

By Morse Faria

A dissertation submitted in partial fulfillment of the requirement for Doctor of Philosophy degree

at Virginia Commonwealth University


Virginia Commonwealth University, 2014


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

Mass spectrometry is being increasingly used in biomarker research mainly due to its ability to

achieve high selectivity coupled with high sensitivity. This dissertation focuses on quantitative

mass spectrometric investigations of two protein biomarkers i.e. serum thymidine kinase 1 (TK1)

and human osteopontin (hOPN).

A liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) method for measuring the activity of TK1 in serum by monitoring the conversion of a TK1 specific exogenous substrate, 3'-deoxy-3'-fluorothymidine (FLT), to its mono-phosphorylated form 3'-deoxy-3'-fluorothymidine monophosphate (FLT-MP). A method to quantify FLT-MP on LC-MS/MS was developed and validated. The method was linear over the range of 2.5-2000 ng/mL with a mean correlation coefficient of 0.9935. The applicability of the developed method was demonstrated by measuring TK1 activity in serum from hepatocellular carcinoma (HCC) patients and age-matched controls.

Another method was developed and validated for quantifying hOPN from plasma using immunoaffinity isolations coupled with microflow LC-MS/MS. A biologically relevant tryptic peptide 'GDSVVYGLR' was used as a signature peptide. The method was validated over a range of 25-600 ng/mL. A stable isotope labeled (SIL) peptide GDSVVYGLR* and an extended SIL peptide TYDGRGDSVV*YGLRSKSKKF' were evaluated as internal standards (IS) to account for digestion variability. In the digestion variability studies, the use of extended SIL peptide as internal standard limited the total variability within ±30% in comparison to ±70% when the SIL-peptide was used. The applicability of the validated method was demonstrated by analyzing plasma samples obtained from 10 healthy individuals and 10 breast cancer patients.

In a proof of concept investigation, a SIL-peptide was evaluated as an internal standard to compensate for immunocapture variability during quantification of hOPN by immunoaffinity coupled LC-MS/MS. Immunocapture variability was induced by varying the antibody amount per well. The use of SIL-peptide reduced the immunocapture variability from ±81% to ±37% immunocapture variability.

In addition, an attempt was made to develop a cell based system to evaluate tobacco products for cardiovascular risk based on the LC-MS/MS measurement of secreted osteopontin and MMP-3 cleaved osteopontin fragments. However, in our preliminary investigations did not yield detectable levels of the secreted osteopontin concentrations in the cell culture studies and hence this study was terminated.

**CHAPTER 1**

# 1    MASS SPECTROMETRY BASED PROTEIN BIOMARKER QUANTIFICATION

## 1.1    INTRODUCTION

Biomarkers are used clinically for diagnosis, patient stratification, and therapy selection and for determination of a surrogate end point in clinical trials (Fleming*, et al.* 2012, Goodsaid*, et al.* 2007, Pletcher*, et al.* 2011). The pharmaceutical industry is optimistic that increased biomarker applications will enable faster and more successful drug development (Kola*, et al.* 2004, Lee*, et al.* 2009). However, one of the limitations in the use of biomarkers in clinical settings and in drug development, has been the lack of good quantitative assays (Drucker*, et al.* 2013, Makawita*, et al.* 2010). Traditionally, immunoassays have been used for targeted quantification of large molecule biomarkers, but issues with selectivity, robustness and long development time associated with these assays have led to the search for alternative methods for measurement of biomarkers. (Hoofnagle*, et al.* 2009, Makawita*, et al.* 2010, Pan*, et al.* 2009, Rauh 2012, Wang*, et al.* 2009). Over the last decade, there has been a tremendous increase in the use of mass spectrometry for targeted quantification of biomarkers in clinical settings (Lee*, et al.* 2009, Makawita*, et al.* 2010, Pan*, et al.* 2009, Ramanathan*, et al.* 2011, Rauh 2012, Wang*, et al.* 2009).

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In this chapter, we will review the current practices for development and validation of protein biomarkers from biological matrices using mass spectrometry based methods.

### 1.1.1 Biomarkers – Definition and Classification

Biomarker is a combination of the words 'biological' and 'marker'. Over the last two decades, many definitions have been attributed to the word biomarker. The official National Institute of Health (NIH) definition is: 'a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Atkinsons, *et al.* 2001). Biomarkers have further been classified into three types: prognostic, predictive and surrogate end biomarkers (Strimbu, *et al.* 2010, Weigel, *et al.* 2010). Prognostic biomarkers are used to forecast the natural history of a disease irrespective of treatment. They are used for diagnosis and patient stratification. Predictive biomarkers are used to suggest the population which is likely to benefit from a particular treatment. For example, expression of the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (Her2/*neu*) are the three most commonly used prognostic and predictive biomarkers in breast cancer patients (Strimbu, *et al.* 2010, Weigel, *et al.* 2010). Over expression of ER and/or PR in breast cancer patients is used as a predictive biomarker in therapy selection since these patients would have a higher likelihood of benefit from endocrine based therapy such as tamoxifen (Strimbu, *et al.* 2010, Weigel, *et al.* 2010). Some biomarkers like Her2/neu can have both prognostic and predictive value (Strimbu, *et al.* 2010, Weigel, *et al.* 2010). Her2/*neu* overexpression is associated with poor prognosis and is indicative of tumor relapse and shorter overall survival in breast cancer patients. Her2/*neu* is the target of the anti-cancer therapeutic monoclonal antibody trastuzumab (Herceptin), and thus, Her2/neu status can be used to predict the effectiveness of trastuzumab. Thus, Her2/*neu* status can also be used for

selection of therapy. In conclusion, prognostic and predictive biomarkers are used for diagnosis, patient stratification and therapy selection (Buyse, *et al.* 2010, Strimbu, *et al.* 2010, Weigel, *et al.* 2010)

Surrogate endpoint biomarkers provide early prediction of a clinical endpoint and/or the effect of treatment on the endpoint. Surrogate endpoint markers are useful when the primary end point is undesirable (death) or when the number of events is so small that one cannot obtain statistically significant numbers in a clinical trial. A classic example of a surrogate end point marker is blood cholesterol concentration (Psaty, *et al.* 1999). Drugs such as simvastatin can use 'reduction in blood cholesterol' as a surrogate endpoint to approve a clinical trial as the primary endpoint (i.e. stroke, heart attack or death) is undesirable.

### 1.1.2 Quantification of Protein Biomarkers

Biological or pathological status of a system can be evaluated by measuring the concentrations of biomolecules such as lipids, proteins and nucleic acids. These biomolecules are involved in signaling pathways and thus altered expression or activity of these biomolecules can have an impact on biological status. Hence, these molecules are extensively used as biomarkers. Proteins are ultimate functional units for various signaling pathways in cells. Protein levels can be predicted by quantifying mRNA levels but it has a number of limitations (Dhingra, *et al.* 2005, Rogers, *et al.* 2008). The mRNA levels may not correlate with the protein level due to inefficient translation or to the degradation of mRNA. Additionally, the proteins may not be functionally active, because its activity may be regulated by post-translational modifications. Proteins may also be susceptible to degradation or complexation. Hence, measuring protein concentration or its activity is preferred (Dhingra, *et al.* 2005, Rogers, *et al.* 2008).

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Protein biomarkers can be measured from tissue or blood (plasma/serum). Protein biomarkers found in plasma/serum are preferred over tissue biomarkers as it is comparatively less invasive to obtain a blood sample than a biopsy sample of a solid tissue. As a result of ease of availability, plasma/serum biomarkers can be effectively used for screening the general population for early disease detection, patient stratification and therapy monitoring.

Quantification of protein biomarkers is traditionally carried out using immunoassays. In particular, Enzyme Linked Immuno Sorbent Assay (ELISA), has been the standard for protein quantification from biological fluids (Makawita, *et al.* 2010). Due to high sensitivity, high throughput and cost effectiveness, sandwich ELISAs have been the primary choice for protein quantification. ELISA requires a pair of well characterized antibodies having high specificity for the protein biomarker. One of the major drawbacks of ELISA has been its long development time (1-2 years) and the huge development cost (100,000's-1,000,000's) (Wang, *et al.* 2009). This has resulted in restricting the number of biomarker candidates that can advance from the biomarker discovery phase to the biomarker validation phase. Immunoassays have additional intrinsic challenges arising from antibody cross-reactivity, the presence of post-translational modifications on the analyte protein, interferences from autoantibodies and anti-reagent antibodies, and the high-dose hook effect (Hoofnagle, *et al.* 2009). This can lead to variable and inaccurate measurements, which may result in misleading clinical interpretations and decisions (Hoofnagle, *et al.* 2009).

Mass spectrometry, as a quantitative tool, was largely restricted to the evaluation of small molecules untill the 1990's. "This was due to the lack of good soft ionization techniques that are required for large molecule quantification. The development of soft ionization techniques such as electrospray ionization (ESI) and matrix assisted laser desorption ionization (MALDI) facilitated

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the use of mass spectrometry for analysis of peptides and proteins (Makawita, *et al.* 2010, Ramanathan*, et al.* 2011, Rauh 2012).  In the last decade, mass spectrometry based bioanalysis has played a major role in biomarker discovery and validation. Advancements in mass spectrometric instrumentation and the better quantification strategies has resulted in a shift of biomarker analysis from immunoassays to mass spectrometry (Makawita*, et al.* 2010, Ramanathan*, et al.* 2011, Rauh 2012).

Tandem mass spectrometry using the selected reaction monitoring (SRM) mode, has been well established for quantification of small molecules, primarily, due to its high specificity and sensitivity (Rauh 2012). In the SRM mode, analyte precursor ions and product ions, obtained after fragmentation of the precursor ion, are selected based on their mass to charge ratio in the mass analyzer. The SRM mode's high specificity is due to the low probability of two molecules having the same mass to charge ratio and fragmenting under standardized conditions to form fragments of the same mass to charge ratio. Thus, mass spectrometry based quantification using the SRM mode provides high selectivity and specificity. In addition, it has the ability to measure absolute levels of post-translational modifications or protein isoforms. These assays involve lower costs and development time in comparison to ELISA (Makawita, *et al.* 2010).  In addition, these assays allow easy multiplexing to measure multiple analytes in a single measurement. The multiplexing ability provides the possibility of evaluating multiple biomarker lead molecules during the validation phase, allowing a relief in the bottleneck in biomarker discovery. Another advantage that mass spectrometry provides is the ease of transferability of methods between matrices and species in comparison to immunoassays. A comparison of immunoassay and LC-MS/MS for peptide and protein quantification is given Table 1-1.

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*Table 1-1: Comparison of immunoassay and LC-MS/MS for peptide and protein quantification*

| | LC-MS/MS | Immunoassay |
|---|---|---|
| Antibody Requirement | Not Required unless method involves immunoaffinity enrichment | A well characterized pair of antibodies required for ELISA |
| Development Time | 2 Weeks – 2 Months | Months -2 years |
| Development Cost | Moderate | High |
| Specificity | High | Moderate |
| Dynamic Range | $10^3$-$10^5$ | $10^2$ |
| Variability | Low | High |
| Multiplex Capability | High | Low |
| Ability to measure PTMs | High | Low |
| Sensitivity | ng/ml | pg/ml |
| Throughput Capability | Moderate | High |

## 1.2   MASS SPECTROMETRIC BASED PROTEIN QUANTIFICATION

Mass spectrometry based protein quantification is normally carried out using a stable isotope dilution approach (Barr, *et al.* 1996). Most of the current targeted mass spectrometry based quantification involves digestion of the analyte protein to yield one or more unique tryptic peptides commonly referred to as signature peptides. The signature peptide is then used as a surrogate analyte and quantified using the mass spectrometry platform. This approach of digesting the protein into peptides prior to its instrumental analysis is referred to as bottom-up quantification. A stable isotope labeled (SIL) form of the signature peptide/protein may be added during sample processing for internal standardization. These peptides are commonly referred to as SIL internal standards (SIL-IS) (Li, *et al.* 2012, Makawita, *et al.* 2010, Rauh 2012). SIL-IS peptides are synthetically obtained by incorporating a stable isotope of $^{13}$C and $^{15}$N on one selected amino acid of the chosen signature peptide sequence (Li, *et al.* 2012, Makawita, *et al.* 2010, Rauh 2012). The resulting SIL-IS will have physicochemical properties similar to the analyte molecule, thus resulting in similar chromatographic separation, ionization efficiency and fragmentation pattern. As the isotope label on the SIL-IS peptide results in a difference in its mass with respect to the signature peptide, these peptides can be differentially detected using mass spectrometry. The ratio of analyte intensity and internal standard intensity is considered as the final response. Any variation, due to sample complexity or analytical conditions, affecting the intensity of the analyte will have a proportional effect on the internal standard, thus ensuring that the final response ratio remains constant. This response ratio evaluation is the fundamental principle of isotope dilution quantifications (Zhang, *et al.* 2014). This protein quantification strategy that relies on the use of a synthetic stable isotope labeled internal standard peptide that can mimic the signature peptide produced during proteolysis wherein the   final response is

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measured as the response ratio between the signature peptide and SIL-IS peptide, is referred to as the Absolute Quantification (AQUA) strategy. An AQUA strategy was first illustrated by Gerber et al. in 2003 wherein the absolute levels of low abundance protein and a phosphorylated protein were quantified from whole cell lysates after an initial separation using SDS/PAGE (Gerber, *et al.* 2003). Since then there have been a number of alternate strategies for absolute quantifications which are discussed in detailed further in the text under internal standardization.

### 1.2.1  Selection of Signature Peptides

In bottom-up protein quantification, selection of a signature peptide (s) is a critical part of method development. Some important characteristics of a signature peptide, as summarized by van den Broek et al (2013), are as follows: (1) It should be unique and specific to the target protein (2) It should be formed reproducibility during enzymatic digestion (3) It should be stable throughout the entire analytical procedure (4) It should be sufficiently resolved from background interferences during chromatographic separations (5) It should be easily ionized and dissociated and have sufficient sensitivity during MS/MS analysis. Trypsin is the most commonly used enzyme to carry out proteolytic digestion due to its selective cleavage on the c-terminus of the positively charged amino acid residues lysine or arginine (Evnin, *et al.* 1990, Olsen, *et al.* 2004). A number of criteria are used during selection of the signature peptides. Tryptic peptides having a chain length between 5-25 amino acids are preferred to reduce charge state distribution and provide adequate retention and MS/MS fragmentation. Tryptic peptides containing amino acid residues with potential post-translational modification (PTM) sites are usually avoided due to a potential change in peptide mass that would affect reproducible quantification. However, if the intended purpose is to quantify a post-translational modification, a tryptic peptide containing the specific PTM is selected (Gerber, *et al.* 2003, Kirkpatrick, *et al.* 2005, Liu, *et al.* 2013). Tryptic

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peptides containing amino acids susceptible to oxidation such as methionine and tryptophan are avoided as chemical modifications of these molecules can result in a change in its mass and thus can affect method reproducibility. Usually tryptic peptides containing cysteine residues are avoided as they undergo iodoacetamidation (Switzar, *et al.* 2013). However, methods using signature peptide containing a cysteine residue have been reported after accounting for any mass change occurring prior to mass spectrometric detection. (Enjalbert, *et al.* 2013, Palandra, *et al.* 2013, Zhang, *et al.* 2011). Additionally, peptides containing ragged ends or dibasic ends next to each other (such as in Arg-Arg, Lys-Lys or Arg-Lys) should be avoided as they are known to result in missed cleavages (Brownridge, *et al.* 2011a, Lawless, *et al.* 2012, Switzar, *et al.* 2013, Wu, *et al.* 2011) . This could be due to the inability of trypsin to act as a dipeptidyl peptidase (Brownridge, *et al.* 2011a, Lawless, *et al.* 2012). Various commercially available software and databases that can perform *in-silico* digestion, verify uniqueness of the tryptic peptides and determine mass spectrometric parameters are well summarized by Colangelo et al. and are listed in Table 1-2 (Colangelo, *et al.* 2013). After in-silico digestion studies, multiple tryptic peptides are selected as potential signature peptides and evaluated for digestion reproducibility, ionization intensity and process stability. Digestion optimizations are carried out with recombinant protein. Chromatographic and mass spectrometric optimization are done using synthetically obtained peptides. Chromatographic optimization during signature peptide selection ensure adequate retention and resolution from matrix components. Mass spectrometric optimizations will involve selection of the mass transitions that are specific to the analyte along with sufficient signal response. In the case of the unavailability of a recombinant protein, digestion optimization may be carried out with biologic samples containing the analyte protein. It is advisable to establish the

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selectivity of the signature peptide in the desired matrix using the optimized LC and mass spectrometric conditions during method development.

*Table 1-2: List of software used for signature peptide selection*

| Software Use | Name | Vendor |
|---|---|---|
| Database search | Basic Local Alignment Search Tool (BLAST) | National Center for Biotechnology Information |
| | PeptideAtlas | Institute for Systems biology (IBS) |
| | Protein Prospector | University of California, San Francisco |
| | SRMAtlas | IBS |
| | PRIDE | Martens,  European Molecular Biology Laboratory |
| | GPMdb | Global Proteome Machine Database |
| | NIST peptide mass Spectral libraries | National Institute of Standards and Technology (NIST) |
| | PABST | Seattle Proteome Center |
| In-Silico Digestion and Signature Peptide Transition Selection | Skyline | MacCoss Lab, University of Washington |
| | MRMPilot | ABSciex |
| | Pinpoint | Thermo Scientific |
| | MRMaid | Bessant, Cranfield University |
| | Enhanced Signature Peptide Predictor (ESP) | Broad Institue |
| | MaRiMba | Seattle Proteome Center |

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### 1.2.2 Internal Standardization

The selection of an internal standard is an important aspect of the method development process for LC-MS/MS quantification of proteins. Internal standards compensate for variation during sample processing and instrumental response fluctuations. A number of IS strategies for mass spectrometry based protein quantification have been proposed, previously and reported in several review articles (Bronsema*, et al.* 2012, Brun*, et al.* 2009, Pailleux*, et al.* 2012, van den Broek*, et al.* 2013a).

### 1.2.3 SIL-Protein IS

A stable isotope labeled (SIL) form of the analyte protein is the most ideal IS for absolute quantification of proteins since it will have the same physicohemical behavior throughout the analytical procedure. SIL-proteins can be obtained by incorporating stable isotope labeled amino acids into the target protein, or PSAQ (Protein Standard Absolute Quantification) standard (Ong 2002, Picard*, et al.* 2012). This can be achieved using metabolic labeling by incubating cells in a medium containing stable isotope labeled amino acids popularly known as Stable isotope labeling by amino acids in cell culture (SILAC) (Ong 2002). Alternatively, SIL-proteins can also be made by *in vitro* protein synthesis in a cell free system (Brun*, et al.* 2007). If the analyte protein is small it can be chemically synthesized (Jian*, et al.* 2013). SIL-protein standards can compensate for immunoaffinity isolation, enzymatic digestions, pre-analytical treatments and final LC-MS/MS analysis. Hence, analysis carried out with SIL-proteins will be reproducible and robust. A major restriction in the use of SIL-proteins as ISs for biomarker quantification is their commercial unavailability or the high cost of production.

Alternatively, a structural protein analogue can also be used as an internal standard (Halquist, *et al.* 2011a, Li, *et al.* 2011a). It would compensate partially for any digestion variability, however its major drawback would be its limited ability to compensate for LC-MS/MS variability.

### 1.2.4 SIL-peptide IS

A SIL form of the signature peptide (SIL-peptide) is the most commonly used internal standard during protein quantification. A SIL-peptide IS contains amino acids labeled with the stable isotopes of $^{13}$C or $^{15}$N thus resulting in peptide analogues which are physiochemically identical to the signature peptide but can be easily distinguished due to the mass difference. SIL-peptide ISs can efficiently compensate for extraction recovery, peptide instability and LC-MS/MS variability. However, unlike a SIL-protein it does not account for proteolytic digestion variability or any immunoaffinity based purification processes (Brun, *et al.* 2009, Li, *et al.* 2012). The major advantage of using a SIL-peptide is that these can be synthetized at relatively low cost. In fact, custom synthesized SIL-peptides can be easily obtained from various commercial sources.

A SIL-peptide IS is usually added before enzymatic digestion as it can account for peptide stability and extraction recovery. However, if a recombinant form of the biomarker is not available, protein concentrations are calculated stiochometrically, solely based on the known molar concentration of the SIL-peptide used, in such instances, the IS is added post digestion to obtain reproducible peptide quantification (Domanski, *et al.* 2012, Keshishian, *et al.* 2007).

### 1.2.5 Extended SIL-peptide

Variation in digestion efficiency can be accounted for with the use of an extended SIL-peptide which have cleavable groups flanking either side of side of a SIL-peptide (Barnidge, *et al.* 2004, Neubert, *et al.* 2013, Ocana, *et al.* 2010). Generally, the cleavable groups consist of three to six amino acids residues from the original protein sequence at both the N- and C- terminus (Kushnir,

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*et al.* 2013, Neubert, *et al.* 2013, Ocana, *et al.* 2010). The addition of an extended SIL-peptide IS prior to digestion provides a more cost effective alternative to compensate for variability in digestion efficiency, extraction recovery, LC-MS/MS analysis and peptide stability.

Beynon et al. developed the QonCAT (Quantification concatemer) approach to produce recombinant proteins that have multiple SIL signature peptides linked together by using an synthetically obtained artificial gene (Beynon, *et al.* 2005). This allows application to a large group of proteins and is useful for multiplexed methods. However, its ability to compensate for digestion efficiency may be limited as differences may exist between the QconCAT polypeptide and the analyte proteins with respect to the accessibility of the proteolytic site and digestion rates (Brownridge, *et al.* 2011b).

### 1.2.6  Sample Preparation

Plasma and serum are highly complex biological fluids. In fact, in serum, 20 of proteins comprise 99% of total protein content and the concentrations of all proteins demonstrate  more than 12 orders of magnitude (Makawita, *et al.* 2010). Analysis of direct digests of serum or plasma would be ideal as it would involve minimal sample preparation. However, matrix effects and interferences from other highly abundant proteins would adversely affect the limit of quantification and specificity of these mass spectrometric methods. Thus, sample clean-up prior to enzymatic digestion is an essential part of protein quantification.

### 1.2.6.1  Protein Purification

#### 1.2.6.1.1  *Non-antibody-based protein purification*

For proteins smaller than 15 kDa, various strategies which are not specific for protein purification can employed. Partial protein precipitation using organic solvents along with surfactants is used as a simple sample purification technique used for smaller proteins or peptides

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(Halquist, *et al.* 2011a, Murao, *et al.* 2007, Sleczka, *et al.* 2012, Wu, *et al.* 2011). Protein precipitation could have low recovery due to losses as a result co-precipitation, which would be a drawback. Solid phase extraction (SPE) is another purification technique that is employed solely or along with other purification techniques for sample clean up wherein the analyte is a smaller protein or peptide (Barton, *et al.* 2010, Halquist, *et al.* 2012, Jian, *et al.* 2013, Sleczka, *et al.* 2012, Yang, *et al.* 2009).

### *1.2.6.1.2 Abundant Protein Depletion*

The plasma proteome is comprised of a number of proteins having a concentration range with more than 10 orders of magnitude (Anderson, *et al.* 2002). Albumin is a protein that covers approximately 50% of the total protein content (Anderson, *et al.* 2002). In fact, it is estimated that 99% of the serum's total protein mass is due to the top 20 most abundant proteins. Several commercial kits are available which use immunoaffinity depletion to selectively remove serum albumin, immunoglobulins and other high abundant proteins (Anderson, *et al.* 2006, Echan, *et al.* 2005, Keshishian, *et al.* 2009, Polaskova, *et al.* 2010). These kits have shown to reduce protein content by up to 85% (Echan, *et al.* 2005). The high costs of these kits and recovery issues have been the major drawback of this approach (Fortin, *et al.* 2009, Keshishian, *et al.* 2007). Abundant protein depletion has been used in a number of biomarker quantification methods (Fortin, *et al.* 2009, Kuhn, *et al.* 2004, Yu, *et al.* 2012). Recently, Lui et al. showed that isopropanol with 1.0% trichloroacetic acid was effective in removing 95% of the total albumin in human plasma samples while retaining 60-100% of the three analyte proteins that were evaluated. The recovery using this approach was found to be better than commercially available albumin depletion kits (Liu, *et al.* 2014).

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### *1.2.6.1.3 Immuno-capture enrichment*

Use of specific antibodies directed toward the analyte protein can be used for isolating the analyte molecule from a complex biological matrix. These enrichments may be carried out with single or multiple antibodies depending on the availability of analyte specific antibodies and the amount of selectivity required. Although this technique requires resources and development time to produce the necessary antibodies, it provides sufficient purification to achieve quantification of low abundance proteins from plasma (Berna*, et al.* 2007, Callipo*, et al.* 2010, Keshishian*, et al.* 2007, Ocana*, et al.* 2010, Winther*, et al.* 2009). Low recoveries and a lack of specificity are some of the issues seen during immune-capture enrichment (Adrait*, et al.* 2012, Dubois*, et al.* 2007). Another drawback is that the internal standardization of the immuno-capture step requires the use of a SIL-protein (Bronsema*, et al.* 2012, Pailleux*, et al.* 2012) .

Anderson et al. introduced a Stable Isotope Standards with Capture by Anti-Peptide Antibodies (SISCAPA) strategy wherein immuno-capture enrichment is directed towards a signature peptide after digestion using anti-peptide antibodies (Anderson*, et al.* 2004). This technique allowed quantification of low abundance proteins from plasma, however required the production of antibodies specific to the signature peptide. Some methods have employed this type of immuno-capture online using specialized columns containing analyte specific antibodies (Berna*, et al.* 2006, Dufield*, et al.* 2012, Neubert*, et al.* 2010).

### 1.2.6.2  Enzymatic Digestion

Most protein quantitative LC-MS/MS methods involve enzymatic digestion of the proteins to yield smaller peptides which can be easily quantified by commercially available quantitative mass spectrometers. Some smaller proteins or polypetides may be analyzed without enzymatic digestions (Becher*, et al.* 2006, Chambers*, et al.* 2013, Dubois*, et al.* 2007, Wang*, et al.* 2012a).

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A typical protein digestion procedure involves denaturation, reduction and alkylation followed by proteolysis. Denaturation is carried out to unfold the protein so that it can be easily accessible to the proteolytic enzyme. Urea is the most commonly used for denaturation during protein quantification. Alternatively, denaturation has been achieved using other chaotropic agents such as guanidine HCl, surfactants such as sodium deoxycholate, organic solvents such as methanol and heat (95ºC) (Hoofnagle, *et al.* 2008, Switzar, *et al.* 2013, Wu, *et al.* 2011, Yang, *et al.* 2009, Yu, *et al.* 2012). Reduction of the protein is carried out using dithiothreitol to break the disulfide linkages between cysteine residues. The resulting free thiol groups are then derivatized using an alkylating agent such as iodoacetamide in order prevent formation of disulfide linkages.

Trypsin is the most commonly used enzyme for protein digestions primarily as tryptic peptides a c-terminal basic residue that favors ionization. In addition, average tryptic peptides have lengths suitable for detection on commonly used quantitative mass spectrometers (Zhang, *et al.* 2014). However, other enzymes such Lys-C, Arg-C, pepsin, chymotrypsin can be used when a specific cleavage is required (Cingoz, *et al.* 2010, Liu, *et al.* 2011, Lu, *et al.* 2009, van den Broek, *et al.* 2007). Sequencing or proteomics grade trypsin is normally used as it has higher digestion efficiency. Additionally, it is treated to prevent autolysis that can result in unwanted cleavage. To improve digestion efficiency, different approaches have been illustrated including high temperature, microwave assisted digestion and use of organic solvents (Berna, *et al.* 2009, Li, *et al.* 2009). The 'pellet digestion' method is a simplified method in which the proteins are precipitated using an organic solevent like acetonitrile to form a pellet and the supernatant containing interfering molecules such as phospholipids are discarded. This method provides an easy, efficient way of performing a fast clean-up and has resulted in improved digestion

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efficiency in comparison to direct digestion (Duan*, et al.* 2009, Ouyang*, et al.* 2012, Yuan*, et al.* 2012, Yuan*, et al.* 2013).

### 1.2.6.3 S*tr*ategies for LC-MS/MS Sample Preparation

A number of reviews have compiled various strategies used for protein quantification using LC-MS/MS (Ackermann*, et al.* 2007, Anderson*, et al.* 2006, Brun*, et al.* 2009, Gucinski*, et al.* , Kirkpatrick*, et al.* 2005, Li*, et al.* 2012, Makawita*, et al.* 2010, Rauh 2012, Ruan*, et al.* 2011, van den Broek*, et al.* 2013a, Wang*, et al.* 2009, Zhang*, et al.* 2014). Figure 1-1 illustrates a decision tree to choose an appropriate sample preparation strategy. For smaller proteins simple techniques such as protein precipitation and solid phase extraction would be the method of choice.  Protein purification methods are chosen depending on the analyte concentration, availability of purification tools (such as analyte specific antibodies) and the matrix. Depending on the analyte concentration, protein purification methods are selected. Many methods use a 'double clean-up' procedure wherein protein purification method is employed prior to digestion combined with a peptide purification step after digestion (Ahn*, et al.* 2009, Kushnir*, et al.* 2013, Neubert*, et al.* 2013).
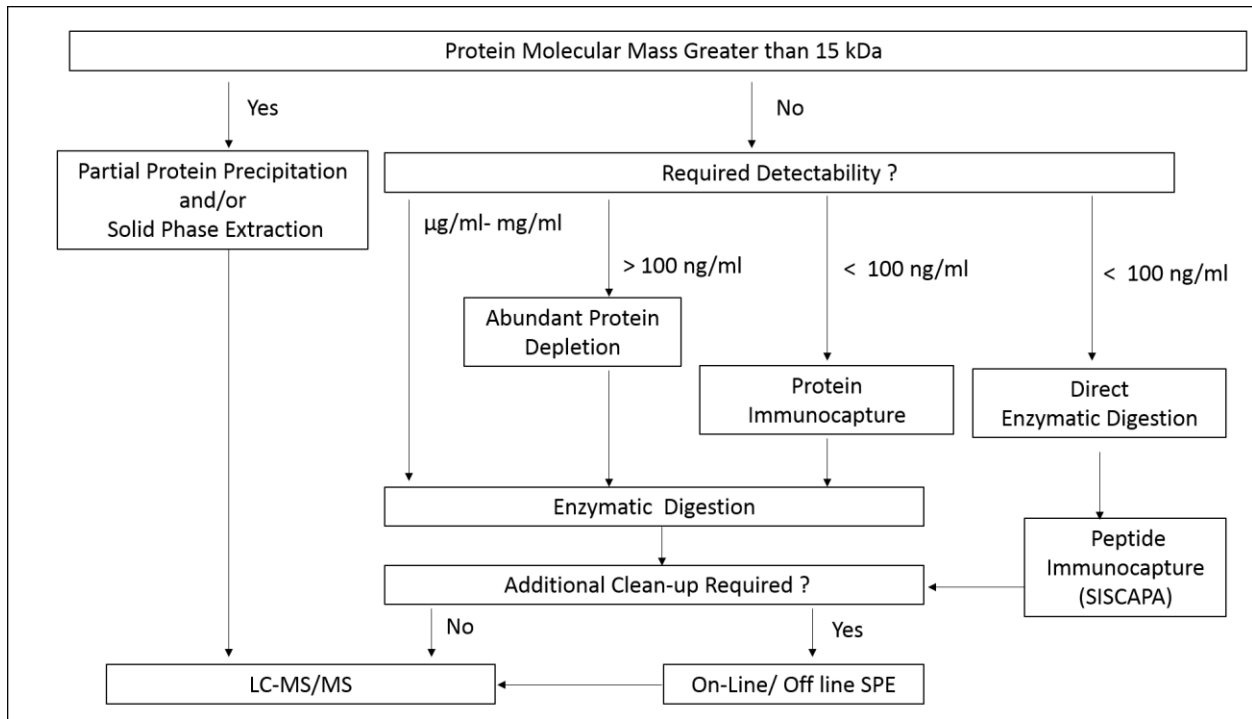
*Figure 1-1: Decision tree for sample preparation for protein quantification using LC-MS/MS*

### 1.2.7  Mass Spectrometric Instrumentation

### 1.2.7.1  LC-MS/MS

Mass spectrometry based protein quantification is primarily carried out on a high pressure liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) system. A list of recently published methods is summarized in various reviews (Ackermann*, et al.* 2007, van den Broek*, et al.* 2013a, Whiteaker*, et al.* 2010, Zhang*, et al.* 2014). The most widely used mass analyzers for quantification are triple quadropole (QqQ) and Quadrapole Ion Trap (QTrap using the multiple reaction monitoring (MRM) mode. In the MRM mode, analyte precursor ions and product ions, obtained after fragmentation of the precursor ion, are selected based on their mass to charge ratio in the mass analyzer. The MRM mode's high specificity is due to the low probability of two molecules having the same mass to charge ratio and fragmenting under standardized conditions to form fragments of same mass to charge ratio. The non-scanning nature of the MRM mode allows increased sensitivity up to two orders of magnitude in comparison to the 'full scan' modes. MRM mode analysis can be used for polypetides and smaller proteins which have a mass less than 15 kda without digesting the protein.  Some low molecular weight proteins which have difficulty fragmenting have been analyzed using Pseudo-MRM i.e. monitoring the parent ion to parent ion transition using very low collision energy (Ji*, et al.* 2003, Zhu*, et al.* 2014). Unlike the MRM mode that relies on isolations based on parent ion and fragment ion mass to charge ratio, the pseudo-MRM approach relies on a single isolation, which can result in loss of specificity. Advances in mass spectroscopic sensitivity have been achieved mainly by increasing ionization efficiency and reducing losses during transmission from the ion source to the MS detector (Shi*, et al.* 2012, van den Broek*, et al.* 2013a).  Enhanced ionization efficiency has been achieved by using nano and micro flow rates and by using higher ion source temperatures. Ion transmission

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efficiency has been improved by using guides or ion funnels (Shi, *et al.* 2012, van den Broek, *et al.* 2013a).

Improved selectivity has been achieved using orthogonal separation modes such as Field asymmetric waveform ion mobility spectrometry (FAIMS) and differential mobility spectrometry (Arnold, *et al.* 2013, Shi, *et al.* 2012). These ion mobility differentiation systems separate gas-phase ions based on their mobility differences in high and low electric fields primarily occurring due to the differences in size and shape of the ions. This orthogonal separation allows lowering matrix noise and in turn provides higher selectivity and sensitivity. Additional fragmentation modes measuring $MS^3$ instead of $MS^2$ fragment ions have been used in some methods to improve MS selectivity. A three to five fold lower LOQ has been achieved using the $MS^3$ mode in comparison to the $MS^2$ mode, especially, in cases where the limitation was due to matrix interference (Jeudy, *et al.* 2014, Lemoine, *et al.* 2012, Shi, *et al.* 2012).

### 1.2.7.2 Full Scan Liquid Chromatography- High Resolution Mass Spectrometry (LC-HRMS)

Time of flight (TOF) and orbitrap mass analyzers are currently used to carry out HRMS quantification. (Gordon, *et al.* 1999, Gucinski, *et al.* , Liu, *et al.* 2011, Rochat, *et al.* 2013, Ruan, *et al.* 2011). These mass analyzers have high resolving power (greater than 30K Full width at half maximum (FWHM)) and better than 3 parts per million mass accuracy. The high resolving power and mass accuracy allows resolution between analyte and background signals and thus can be used for analysis of intact protein molecules. Quantification is done based on peak areas using narrow mass windows (less than ± 0.05 Da) for the extracted ion chromatogram. Full scan LC-HRMS quantification is carried out usually for smaller proteins with molecular weights less than 15 kda (Zhang, *et al.* 2014). A typical protein spectrum containing multiple charge states

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each having a distribution of its multiple isotopic peaks is seen in a full HRMS scan. High mass spectrometric resolution allows reproducible detection of individual peaks. The charge state and isotopic peak with the highest intensity and least background interference is chosen. Occasionally multiple peaks may be used to improve reproducibility and signal intensity. (Zhang, *et al.* 2014). LC-HRMS simplifies sample processing as one can avoid enzymatic digestions. A comparison of LC-MS/MS and LC-HRMS platforms for protein quantification was carried out by Dillen et al. (Dillen, *et al.* 2012). The study indicated that LC-HRMS had a lower sensitivity than an LC-MS/MS system but provided higher specificity. LC-HRMS platforms can also be used to carryout quantitative and qualitative measurement simultaneously, thus providing the possibility of concurrent identification and quantification (L, *et al.* 2013, Ramanathan, *et al.* 2011).

### 1.2.7.3 Chromatographic System

Liquid chromatography is used for separation of the protein or peptide mixture prior to mass spectrometric detection. Usually, reversed phase liquid chromatography (RPLC) is used to achieve separation of the peptide mixture (Ahn, *et al.* 2009, Barton, *et al.* 2010, Berna, *et al.* 2009, Callipo, *et al.* 2010, Fortin, *et al.* 2009, Kuhn, *et al.* 2004, Neubert, *et al.* 2013, Ocana, *et al.* 2010, Whiteaker, *et al.* 2010, Winther, *et al.* 2009, Yu, *et al.* 2012), however, hydrophilic interaction liquid chromatography (HILIC) has been used to separate hydrophilic peptides. Columns used for peptide separation have particle size and dimensions similar to those used for small molecule quantification (Arsene, *et al.* 2010, Barnidge, *et al.* 2004, Barton, *et al.* 2010, Kirsch, *et al.* 2007, Kumar, *et al.* 2010, Yu, *et al.* 2012). However, specialized columns having pore size around 300 Å have been used especially wherein low molecular weight proteins need to be separated. In addition, a number of quantification approaches involving nano and micro

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flow LC systems have been carried out using capillary columns having small inner diameters (I.D.) such as 75 and 150 µm (Ahn*, et al.* 2009, Keshishian*, et al.* 2007, Kuhn*, et al.* 2004, Neubert*, et al.* 2013, Whiteaker*, et al.* 2010). Recent developments in chromatographic hardware, mainly precise delivery pumps for micro and nano volumes, tighter system integration to reduce dead volumes, smaller diameter columns and newer LC-MS interfaces such as the Waters ionkey/MS separation device cartridge that holds a capillary column and a nanospray needle, have enabled use of nano and micro LC-MS/MS systems (Arnold*, et al.* 2013). The nano and micro-LC systems have better sensitivity due to higher electrospray efficiency obtained by the use of low flow rates, In addition, these systems utilize less solvent and thus help in cost reduction (Ahn*, et al.* 2009, Keshishian*, et al.* 2007, Kuhn*, et al.* 2004, Neubert*, et al.* 2013, Whiteaker*, et al.* 2010).

Two-dimensional chromatographic separations, such as ion-exchange chromatography (IEC)-RPLC or RPLC-HILIC, has been utilized to fractionate and clean up samples, thus improving sensitivity of detection (Gilar*, et al.* 2005, Keshishian*, et al.* 2007, Liu*, et al.* 2009, van den Broek*, et al.* 2013a, Zhang*, et al.* 2014). Additionally, most of the currently published methods have used column trapping prior to analytical separation to remove salts and highly hydrophilic peptides (Ahn*, et al.* 2009, Berna*, et al.* 2009, Callipo*, et al.* 2010, Kuhn*, et al.* 2004, Ocana*, et al.* 2010, Whiteaker*, et al.* 2010, Winther*, et al.* 2009).

Most peptide LC-MS/MS quantifications are carried out using gradient mobile phase systems containing additives such as formic acid and trifluroacetic acid (TFA). Though TFA improves peak shape by reducing the interaction between peptides and the stationary phase, it is known to suppress electrospray ionization intensity (Apffel*, et al.* 1995). Ionization suppression by TFA can be circumvented by addition of acetic acid (0.5%) or propionic acid (1%), or by post-column

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infusion of propionic acid and isopropanol. The large excess of weak acid protonates TFA anions back to neutral TFA molecules, thus freeing up the analyte ions from being paired with TFA anions (Apffel, *et al.* 1995, Shou, *et al.* 2005). Studies have shown that addition of 5% DMSO as a cosolvent can enhance ionization intensity through charge state coalescence (Meyer, *et al.* 2012).

## 1.3    ANALYTICAL VALIDATION OF BIOMARKER ASSAYS

With the increasing use of biomarkers for diagnosis, patient stratification in clinical settings and higher acceptance of surrogate markers in clinical trials by regulatory bodies, there is a need for a greater focus on validation of quantitative biomarker assays (Buyse, *et al.* 2010, Lee 2009, Lee, *et al.* 2006). The objective of validating a method is to demonstrate that a particular method is reliable for the intended purpose. The intended use of biomarker data is an important consideration to determine the rigor of method validation. Thus, a 'fit-for-purpose' approach is most suitable for biomarker validation (Lee 2009, Lee, *et al.* 2006). In the last few years, there has been some consensus regarding LC-MS/MS method validation for biomarkers. Currently, biomarker assay validations using LC-MS/MS are being carried out using USFDA bioanalytical guidelines with intermediate acceptance criteria i.e. criteria ranging between those used for ligand binding assays (LBA) and small molecule bioanalytical assays (Bower, *et al.* 2014, Lee 2009, Lee, *et al.* 2006, Stevenson, *et al.* 2013).  Some aspects that need to be considered prior to undertaking a 'biomarker analytical validation' are discussed below.

### 1.3.1    Reference Standards

 A reference should have high purity and should be well characterized and should represent the biologically relevant form of the analyte protein. Reference standards of smaller proteins like insulin and steroid hormones are commercially available in highly purified and well

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characterized form (Lee, *et al.* 2006). However, most biomarkers are large proteins and their commercially available standards are often impure, poorly characterized and may not fully representative of the biologically relevant form. Additionally, there may be inconsistency in the purity and form between lots and manufacturers (Lee, *et al.* 2009). If the reference standards are obtained as recombinant proteins from non-eukaryotic cells, they may differ from the endogenous form as it will not have the necessary PTMs. If there exists no reference standard or if the reference standard is not a complete representative of the endogenous form, the method is referred to as quasi-quantitative (Lee, *et al.* 2006).

### 1.3.2   Standard Calibrator matrix

Biomarkers are endogenous molecules and selection of a blank matrix for preparation of calibrators and quality control samples is an important step in biomarker method validation (Lee, *et al.* 2006, Lee, *et al.* 2007, Lee, *et al.* 2009).  A simplified way is to pool together lots containing low endogenous concentrations. However, this would result in measureable concentrations of biomarker in the background and limit the evaluation of lower limits (van de Merbel 2008). A second option is to use a surrogate matrix such as stripped plasma wherein the endogenous levels have been removed using affinity adsorption, activated charcoal or some other means of removal (van de Merbel 2008). A third option is to use a substitute matrix such as a biological matrix from another species which does not produce the biomarker protein. Another option is to use a buffer as a surrogate matrix. Use of a surrogate matrix or substituted matrix avoids the need to continuously screen lots to identify low biomarker concentration matrices. A disadvantage is that additional studies will need to be carried out to demonstrate the absence of matrix effects. When using calibrators spiked with a surrogate matrix, it is essential to compare recovery to demonstrate that the concentration-response relationships are similar. Additionally, a

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parallelism experiment is required to demonstrate that the endogenous biomarker behaves in a similar immunochemical manner to the reference standards (Lee*, et al.* 2006, Lee*, et al.* 2007, Lee*, et al.* 2009).

### 1.3.3   Selectivity

Selectivity is the ability of the method to determine the analyte unequivocally in the presence of components that may be expected to be present in the sample. During method development, selectivity is built into the method by choosing a unique signature peptide and choosing a SRM transition that has the least interference. As biomarkers are endogenous molecules, selectivity is evaluated by spiked recovery. To evaluate the selectivity, the basal level in individual lots is initially screened against a calibration standard curve obtained using calibrators prepared in a surrogate buffer matrix. The reference standard is then spiked in these lots to obtain a lower limit of quantification (LLOQ) concentration. At least 6 lots for each population are evaluated to account for inter-subject variability. Spiked recovery for each lot is estimated after accounting for baseline concentrations from the measured concentrations. The spiked recovery will have to be within a criterion set a-priori.

### 1.3.4   Parallelism

Parallelism is a dilutional linearity test of an authentic sample. This study demonstrates that the endogenous analyte in the unknown sample behaves similarly regardless of dilution by the standard matrix. Several individual samples, with high concentrations are chosen and analyzed undiluted and with a dilution factor of 3 or 4. The ratio of the calculated results (observed concentration x dilution factor) divided by the mean of the results are plotted against the inverse of the dilution factor. Parallelism is demonstrated if the ratio is not affected by dilution. If parallelism is cannot be established the method is only quasi-quantitative (Lee*, et al.* 2009).

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### 1.3.5  Specificity

Specificity is the ability of the method to distinguish between the analyte and other structurally similar molecules (Lee, *et al.* 2009). Cross-reactivity during immunoaffinity isolations during sample preparation, may result in a negative bias. If metabolites or isoforms of the biomarker protein are known and available as standards, a study can be designed to ensure that their presence does not have an  impact on protein biomarker  measurement.

### 1.3.6  Accuracy, precision & assay range

Many biomarker assays are developed to differentiate between healthy and diseased populations. A 'fit-for-purpose' assay range that covers baseline healthy individual concentrations as well as patient populations, is used. Additionally, dilution integrity should be established to enable analysis of unknown samples whose concentration falls above this assay range.

Validation samples (calibration curve standards and quality control samples) are prepared using reference standards and used for measuring intra- and inter- assay accuracy and precision. Accuracy (expressed as percent deviation from normal (% DFN)) is the agreement between the measured result and its theoretical true value. Precision is a quantitative measure (usually expressed as %RSD and %CV) of the random variation between a series of measurements from the same homogenous sample. Usually different concentrations covering the assay range are prepared and used for validation studies.  Traditionally five levels are used : 1) LLOQ (2) lower quality control (LQC) which is approximately three times of LLOQ 3) Mid quality control (MQC) 4) High quality control (HQC) which is within 90% of the higher end of the assay range 5) Dilution quality control (DQC) which is approximately 1.5 times the highest calibration standard. Accuracy and precision are assessed in multiple (at least 3) validation runs processed over different days. Each validation run is comprised of two sets of calibration curve standards

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and six replicates of quality control samples. A predefined criterion is used for acceptance of accuracy and precision of the validation run.  The criteria used often range between those used for USFDA bioanalytical guidelines for small molecules and ligand binding assays (LBA) (Bower*, et al.* 2014, Lee 2009, Lee*, et al.* 2006, Stevenson*, et al.* 2013)

### 1.3.7  Stability

Stability of the analyte in stock solutions and the biological matrix should be demonstrated during validation. The analyte protein may undergo proteolytic cleavage or chemical (oxidation or aggregation) during storage. Usually freeze-thaw stability, bench-top stability, short and long term storage stability is established during validation. Incurred sample analysis may be performed to establish long term stability of patient samples. During sample collection, relevant inhibitors (protease inhibitor or platelet activation inhibitors) should be added, if degradation of the analyte protein or conversion of precursors is known (Ahmed 2009, Lee*, et al.* 2009, Zhu*, et al.* 2010).

### 1.4  CONCLUDING REMARKS

Immunoassays, primarily ELISAs, have dominated protein quantification over the last four decades. However, due to development in mass spectrometry over the last decade, this trend has started to shift. Mass spectrometry provides a universal platform for analysis of biomarker proteins. In addition to providing greater selectivity, ease of multiplexing and lower development cost, mass spectrometry based quantification of protein biomarkers has decreased biomarker assay development time, thus, enabling acceleration of the timeline between biomarker discovery and biomarker validation.  The use of sample preparation techniques such as 'immunoaffinity isolations' and 'double extraction' along with advancements in LC-MS/MS instrumentation has resulted in achieving detection limits comparable to those of ELISA.

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Method development of mass spectrometry based biomarker assays involves a number of challenges including selection of signature peptides, selection and optimization of protein purification and digestion techniques and internal standardization. In recent years, there has been a lot of effort from regulatory authorities and industry to build consensus on development and validation of mass spectrometry based assays for proteins (Bower, *et al.* 2014, Sailstad, *et al.* 2014, Stevenson*, et al.* 2013, van de Merbel*, et al.* 2014). With further advancements in mass spectrometric instrumentation, protein analysis software and sample preparation technology, biomarker bioanalysis will be more reliable, enabling more utility in clinical settings. Accurate and precise biomarker assays enable faster clinical decisions with regard to diagnosis and therapy selection. More reliable biomarker analysis will also promote higher acceptance of surrogate endpoint markers by regulatory authorities, allowing shorter clinical trials and shorter drug development time.

# CHAPTER 2

## 2    DISSERTATION OBJECTIVES

Mass spectrometry is being increasingly used in biomarker research mainly due its ability to achieve high selectivity coupled with high sensitivity (Makawita, *et al.* 2010, Ramanathan, *et al.* 2011, Rauh 2012). This research proposal focuses on quantitative mass spectrometric investigations on protein biomarkers i.e. serum thymidine kinase 1 and human osteopontin (OPN).

The first objective of this dissertation research will be to develop and validate a liquid chromatography and tandem mass spectrometry (LC-MS/MS) method to quantitatively measure the activity of thymidine kinase 1(TK1), a tumor biomarker,  in human serum. Thymidine kinase 1 activity will be measured by monitoring the conversion of an exogenous TK1 substrate 3'-fluorothymidine (FLT) to its phosphorylated product i.e. 3'deoxy-3'-fluorothymidine monophosphate (FLT-MP), using LC-MS/MS. The developed analytical method will be validated as per US FDA guidelines (Center for Drug Evaluation and Research (U.S.), *et al.* 2001). Application of validated method will then be demonstrated by distinguishing TK1 activity in serum samples obtained from hepatocellular carcinoma patients along with matched controls.

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Human osteopontin is secreted plasma protein which is elevated in various cancers and is indicative of poor prognosis (Rodrigues, *et al.* 2007). The second objective of this dissertation will involve development and validation of a capillary microflow LC-MS/MS method to quantify human osteopontin (hOPN) from plasma using a biologically relevant signature peptide. This method will use an immunopurification for isolation of hOPN from plasma. The isolated protein will then be digested with trypsin in the presence of an internal standard peptide to generate a biologically relevant signature peptide i.e. GDSVVYGLR. Preliminary studies showed that the peptide yields decreased with increased digestion time indicating signature peptide instability. A specific aim of this part of research would be the evaluation of a SIL peptide and an extended SIL peptide as internal standards to track digestion variability.

The third research objective will be to develop an *in vitro* method for evaluating the cardiovascular risk potential of tobacco products by measuring the levels of secreted osteopontin and its metalloproteinase-3 (MMP-3) cleaved N-terminal fragment in the media of tobacco extract exposed endothelial cells. In order to achieve this objective, a method will be developed to simultaneously measure human osteopontin and its metalloproteinase-3 (MMP-3) cleaved N-terminal fragment from cell culture media.

The fourth objective of this dissertation will be to evaluate the use of peptide standards as internal standards to compensate for the immunocapture variability during LC-MS/MS quantification of a biomarker protein. We hypothesized that an extended SIL-IS peptide could be used to compensate for immunocapture variability during protein quantification provided that immunocapture is carried out with an antibody that binds to a common epitope present on both the protein and SIL-IS peptide. In this proof of concept investigation, we evaluate the ability of an extended SIL-IS peptide to compensate for immunocapture variability using a hOPN specific

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antibody (MAB222P) that has an epitope in the signature peptide region (DSVVYG) of the protein.

# 3 LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY METHOD FOR QUANTIFICATION OF THYMIDINE KINASE ACTIVITY IN HUMAN SERUM BY MONITORING THE CONVERSION OF 3'DEOXY-3'-FLUOROTHYMIDINE TO 3'DEOXY-3'-FLUOROTHYMIDINE MONOPHOSPHATE.

*Drawn from published paper (Faria, et al. 2012)*

## 3.1 INTRODUCTION

Thymidine kinase 1 (TK1, ATP; Thymidine 5'-phosphotransferase; EC.2.7.1.21) is a key cellular

enzyme in DNA synthesis which catalyzes the one step pyrimidine salvage pathway (Bradshaw*,

et al.* 1984). Specifically, TK1 catalyzes the transfer of terminal phosphate from ATP to the 5'

hydroxyl group of deoxythymidine (dThd) to produce deoxythymidine monophosphate (dTMP).

Increased activity of serum or plasma TK1 has been reported in diseases involving DNA

metabolism, e.g., viral infections (Gronowitz*, et al.* 1984, Gronowitz*, et al.* 1986), vitamin $B_{12}$

deficiency (Gronowitz*, et al.* 1984) and in a variety of malignant diseases including acute and

chronic leukemia(Hagberg*, et al.* 1985), Hodgkin's disease (Eriksson*, et al.* 1985), non-

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Hodgkin's lymphoma (Hallek, *et al.* 1992, Topolcan, *et al.* 2008) , lung cancer (Topolcan, *et al.* 2008) and ovarian cancer (Hallek, *et al.* 1997, Topolcan, *et al.* 2008). TK1 also phosphorylates derivatives of thymidine or deoxyuridine (dUrd). In particular, TK1 can phosphorylate nucleotide analogs with modifications at the 5'-position of the pyrimidine ring, and the 3'-position of the ribose including 5-fluoro-2'-dideoxythymidine and 3'-azido-3'-deoxythymidine (Zidovudine) (Eriksson, *et al.* 1991, Furman, *et al.* 1986). These nucleotide analogs can therefore be used to monitor the activity of TK1 (Gronowitz, *et al.* 1984, Ohrvik, *et al.* 2004, Topolcan, *et al.* 2008). For example, $^{18}$F-3'deoxy-3'-fluorothymidine ($^{18}$F-FLT), has been used to monitor tumor proliferation via positron emission tomography (Rasey, *et al.* 2002, Salskov, *et al.* 2007, Toyohara, *et al.* 2002). Phosphorylation of FLT by TK1 leads to cellular trapping and accumulation of FLT-monophosphate (FLT-MP) without incorporation into DNA. Since FLT has low affinity for TK2 in comparison with FLT-MP, it acts as a selective substrate to monitor TK1 activity (Eriksson, *et al.* 1991, Munch-Petersen, *et al.* 1991).

Serum TK1 activity can reflect tumor burden and proliferation, and is most commonly measured using a commercially available thymidine kinase radioenzymatic assay (TK-REA), which uses $^{125}$I-deoxyuridine as a substrate to measure thymidine kinase 1 activity (Gronowitz, et al. 1984). This assay is time-consuming and requires radioactive waste management. A competitive enzyme-linked immunosorbent assay (ELISA) has also been used for measuring serum TK1 activity for measuring phosphorylation of the selective TK1 substrate 3'-azido-2'-deoxythymidine (AZT) (Ohrvik, et al. 2004). Alternatively, 5-bromodeoxyuridine (BrdU) incorporation into DNA can be monitored by ELISA (Gronowitz 2007). After phosphorylation by TK1, bromodeoxyuridine monophosphate (BrdUMP) is processed to bromodeoxyuridine triphosphate (BrdUTP) by yeast enzymes, and the BrdUTP is immobilized by incorporation into

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an immobilized DNA strand using a recombinant reverse transcriptase. Further, the amount of BrdUTP incorporated in DNA is estimated using an ELISA.  This assay is more sensitive than the assay using AZT as a substrate (Gronowitz 2007). However, complex sample processing, reagent expense, and selectivity issues associated with ELISA methods are limitations to its application.

Quantification of nucleotide analogues can be performed using high performance liquid chromatography (HPLC) with ultraviolet or fluorescence detection (Jancita, *et al.* 1980, Serve, *et al.* 2010). In the last decade, mass spectrometric (MS) detection has increasingly been used for quantification of nucleoside and nucleotide analogs (Banoub, *et al.* 2005, Cohen, *et al.* 2009). Mass spectrometry allows extremely specific and sensitive quantification of nucleotide analogs from biological matrices including serum. Thus, LC-MS/MS can be used as a tool to measure the conversion of FLT to FLT-MP in serum.

In our previous work, a method was developed to quantify the intracellular conversion of FLT to FLT-MP in cell lysates using LC-MS/MS (Li, *et al.* 2011b). Separation of the analytes was achieved using C18 column and detection using Applied Biosystems SCIEX API 4000 QTrap mass spectrometer. This method was able to monitor proliferating cell TK1 activity in as few as 500 cells per well in LNCaP prostate cancer cells. In the present report, we describe the development and analytical validation of a suitable non-isotopic, non-immunologically based assay for quantitative monitoring of FLT to FLT-MP in human serum (See Figure 3-1). This method uses a labeled isotope internal standard, and column trapping to improve assay ruggedness.

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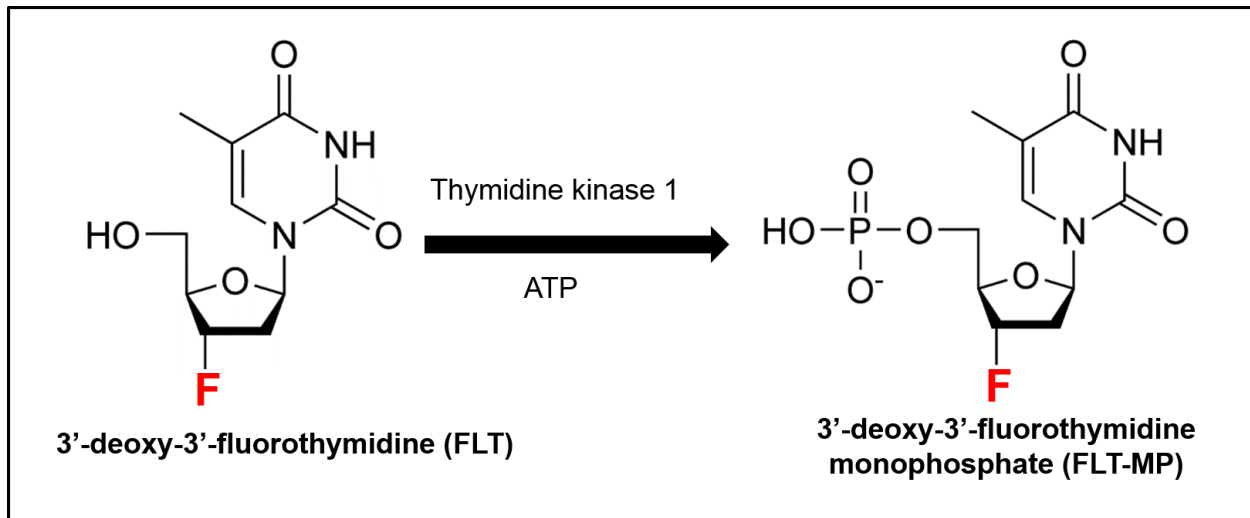
*Figure 3-1: Illustration showing the conversion of FLT to FLT-MP by thymidine kinase 1.*

## 3.2    MATERIALS AND METHODS

### 3.2.1    Chemicals and reagents

HPLC grade acetonitrile and methanol were purchased from Honeywell Burdick & Jackson (Muskegon, MI, USA). 3'-Deoxy-3'fluorothymidine was purchased from Sigma Aldrich (St. Louis, MO, USA). 3'-Deoxy-3'fluorothymidine-5'-monophosphate disodium salt (FLT-MP), 3'-Deoxy-3'fluorothymidine-d3 (FLT -d3) and 3'-Deoxy-3'fluorothymidine-5'-monophosphate-d3 (FLT-MP-d3) were purchased from Toronto Research Chemicals Inc. (TRC, Toronto, ON, Canada). Citric acid, formic Acid, sodium acetate, adenosine 5'-triphosphate (ATP), uridine 5'-monophosphate (UMP) , magnesium chloride ($MgCl_2$) and dithioerythritol (DTE) were obtained from Sigma-Aldrich (St. Louis, MO, USA).  High purity water was obtained in-house using a NANOpure Diamond Life Science ultrapure water System from Barnstead International (Dubuque, IA, USA). Nitrogen was obtained from a Parker Balston Tri Gas Generator LCMS-5000 (Haverhill, MA, USA). Microcentrifuge tubes (1.5ml) and disposable glass centrifuge (10ml) were purchased from VWR International (Westchester, PA, USA). Human serum was obtained from Biochemed Services, Inc. (Winchester, VA, USA).

### 3.2.2    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) and a Waters Acquity UPLC® system (Waters Corporation, Milford, USA).  Phenomenex Security Guard column (Gemini C18, $4 \times$ 2.0mm, 5 μm) from Phenomenex (Torrance, CA, USA) was used as the loading column and an Aquasil C18 column (100mm$\times$2.1mm I.D., 5 μm) from Thermo Scientific (Waltham, MA, USA) was used as the analytical column. The analytical column was maintained at 40°C.

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Sample loading was achieved using a Waters Acquity UPLC® system. Pumps A and B delivered loading mobile phase A (0.1% formic acid) and loading mobile phase B (0.1% formic acid in acetonitrile), respectively. Sample loading was carried out with 100% loading mobile phase A maintained at a flow rate of 0.300 ml/min. Following an initial loading time of 1.5 min, the diverter valve was switched to position B and the elution initiated. A 10-port Cheminert switching valve and a microelectric actuator obtained from Valco Instruments Co. Inc. (Houston, TX, USA) was used to divert flow to the analytical column in position B. After a running time of 5 minutes, the diverter valve position was switched back to allow flushing of the loading column. The flushing of the loading column was carried out at 0.400ml/min with 50% loading mobile phase B. After flushing for one minute, the loading mobile phase composition was changed back to 100% loading mobile phase A pumped at a flow rate of 0.300 ml/min. Elution was carried out under gradient conditions using two Shimadzu pumps which were operated with a Shimadzu system controller. The elution mobile phases consisted of 0.1 % formic acid in water (A) and 0.1 % formic acid in acetonitrile (B). The flow rate was set to 0.300 ml/min. Gradient conditions were as follows: 0.0 –2.0 min, isocratic 5% B; 2.00 –4.0 min, linear from 5% to 75% B; 4.1 –7 minutes, isocratic 5% B. The total running time was 7 minutes and the injection volume was 25 µL. The column switching LC-MS/MS design is shown in Figure 3-2.

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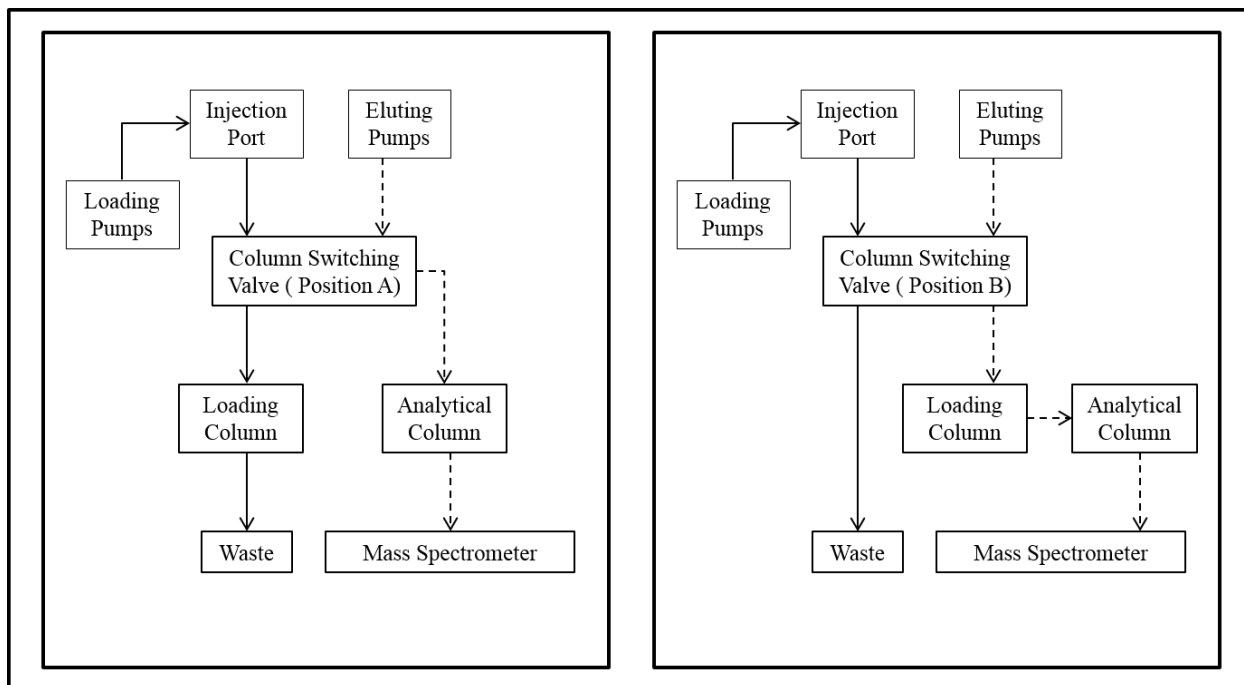
*Figure 3-2: Column switching LC-MS/MS design*

### 3.2.3 Mass Spectrometry conditions

Mass spectra were obtained using an Applied Biosystems SCIEX API 4000 QTrap mass spectrometer operated in positive electrospray ionization mode (ESI). Tuning and optimization of the mass spectrometer parameters were performed for the analytes and internal standard (IS, see below) by direct infusion of a 1 µg/mL standard solution at a flow rate of 12 µL/min. The multiple reaction monitoring (MRM) transitions, declustering potential (DP) entrance potential, collision energy (CE) and collision cell exit potential (CXP) for all the compounds are listed in Table 3-1. The mass spectrometric parameters were as follows: ion source temperature (TEM=450°C), ion transfer voltage (IS=5500 V), collision gas (CAD=high), curtain gas (CUR=20), ion source gas 1 (GS1=55) and ion source gas 2 (GS2=45). The units for gases are arbitrary. The data were acquired with Analyst software, Version 1.5.

### 3.2.4 Preparation of stock solutions, standards and quality control samples

Stock solutions were prepared by dissolving FLT and FLT-MP in methanol to yield 12.5 and 50 µg/mL solutions, respectively. Intermediate stock solutions were prepared by further diluting the stock solution in methanol to prepare spiked serum samples. Internal standard stock solutions were prepared by dissolving FLT-d3 and FLT-MP-d3 in methanol to yield a concentration of 500 µg/mL. A working IS solution (final concentration of 200 ng/mL) was subsequently prepared by diluting the stock solutions of each compound in methanol. Stock solutions, QC solutions and IS solutions were stored at -20°C.

### 3.2.5 Preparation of calibration standards and quality control samples in human serum

Calibration standards and quality control samples were freshly prepared daily by spiking working solutions and quality control solutions in pooled human serum. The concentration range of the calibration standards was FLT (0.500-500 ng/mL) and FLT-MP (2.5-2000 ng/mL). The

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quality control samples (Lower limit of quantification (LLOQ), Low quality control (LQC), Medium quality control (MQC), High quality control (HQC) and dilution quality control ) had the following concentrations: FLT (0.50, 2.00, 40, 375 and 2000 ng/mL) and FLT-MP (2.50, 6.00, 60, 1800 and 5000 ng/mL).

### 3.2.6 Preparation of Phosphorylation Buffer and Reconstitution Solution

Phosphorylation buffer was prepared fresh prior to sample analysis. The buffer comprised 0.1 M sodium acetate, 7.8 mM ATP, 2 mM UMP, 12mM DTE and 30 mM $MgCl_2$ in HPLC grade water. Reconstitution solution was prepared by dissolving 19.2 mg of citric acid and 37.2 mg of EDTA in 100 ml HPLC grade water to obtain a mixture of 1mM Citric acid and 0.5 mM EDTA. This was used for reconstituting the samples prior to injection on to LC-MS/MS system.

### 3.2.7 Sample preparation

A 50 µl aliquot of serum (samples, calibration and quality control samples) was added to a1.5 ml microcentrifuge tube containing 50 µl of phosphorylation buffer solution. 10 µl of freshly prepared FLT (10 µg/ml), in phosphate buffered saline (PBS), was transferred to the microcentrifuge tubes containing serum samples, whereas 10 µl of PBS alone was transferred to the microcentrifuge tubes containing calibration standards and quality controls. The samples were incubated for 2 hours at 37ºC in Labline Orbit Shaker Bath model 3540 (Melrose Park, ILIL, USA) at 125 rpm. The reaction was terminated by addition of 300 µl of methanol to each tube. The calibration standards and quality control samples were not incubated; instead the reaction was terminated immediately by addition of 300 µl of methanol to each tube. To each tube, 50 µL of internal standard was added and the tube was vortex mixed for 2 minutes and centrifuged for 10 min. at 4ºC/10,000 rpm using an Eppendorf centrifuge model 5805 (Hamburg, Germany). Approximately, 400 µL of supernatant was transferred into a 10 ml disposable glass

centrifuge tube and evaporated to dryness under nitrogen gas at 50°C for ~15minutes. The samples were reconstituted with 50 μL of a reconstitution solution. The reconstituted samples were transferred to a 1 mL 96-well plate and analyzed by LC-MS/MS.

### 3.2.8 Matrix effects

In order to evaluate matrix effects, a post-column infusion study was conducted. A 100 ng/mL solution (FLT and FLT-MP) in methanol was prepared and continuously infused at 20 μL/min post HPLC column directly into the mass spectrometer using a "tee" connection. After stabilization of the baseline, a blank sample was injected and the profiles were investigated for suppression or enhancement at the retention time of FLT and FLT-MP.

### 3.2.9 Validation procedures

Validation of the assay was performed according to the FDA guidelines for Bioanalytical Method Validation (Center for Drug Evaluation and Research (U.S.), *et al.* 2001). Validations runs containing duplicate calibration standards, blank samples, and blank sample spiked with internal standard and replicates of QC samples were run on three separate days.

### 3.2.9.1 Selectivity

Human serum samples from six different sources were analyzed in duplicate along with spiked LLOQ serum calibration standards to evaluate selectivity of the analytical method. Each individual lot was extracted according to the sample preparation procedure for calibration standards and quality control samples given above. Selectivity requirements were that any peak area co-eluting at the retention time of analytes (FLT/FLT-MP) must be less than 20% of the peak area of the average of LLOQ samples for all six lots of blank serum samples. Additionally, any peak area co-eluting at the retention time of internal standards (FLT-d3 and FLT-MP-d3)

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must be less than 5% of the average peak area of the internal standard concentration for all six lots of blank serum samples.

### 3.2.9.2 Linearity and LLOQ

Eleven calibration standards were extracted in duplicate and analyzed in 3 independent runs. Calibration curves were fitted using linear regression of the ratio of the peak area response of the analyte and the internal standard versus concentration. A weighting factor of $1/x^2$ was used for both the analytes (FLT and FLT-MP). The acceptance criteria followed FDA guidelines for bioanalytical method validation [22].

### 3.2.9.3 Accuracy and precision

Accuracy and precision were determined from QC samples (LQC, MQC, HQC, dilution QC) in three independent runs. A criterion of ±15% of the nominal concentration was used to assess accuracy and precision was expressed as %RSD, which should not exceed ±15%. Intra-assay precision and accuracy were determined from 6 replicates of each QC sample on a single assay. Inter-assay precision and accuracy were determined by analyzing three different validation runs.

### 3.2.9.4 Recovery and carryover

Analyte recovery of the extraction procedure was determined by comparing peak areas. Blank serum was spiked with FLT and FLT-MP at two levels (LQC and HQC). These samples were compared to samples spiked after extraction with the same final concentrations of FLT and FLT-MP to compensate for variations in instrument response. The criterion for acceptance in this recovery experiment was that recovery was consistent over the two QC levels. Carry over was assessed by injecting LQC immediately after each of the highest calibration standards in an analytical run. The acceptance criterion for this experiment was that the LQC must be accurate to within 15% of the nominal concentrations.

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### 3.2.9.5 Stability Studies

Post-preparative stability was assessed using blank serum samples spiked with FLT and FLT-MP at two levels LQC and HQC. These quality control samples were processed and maintained at 5°C in the autosampler. After 48 hours, these samples were analyzed against a freshly spiked calibration standard curve. A criterion of ±20% of the nominal concentration was used to assess for 48 hours post-preparative stability. Additionally, the intermediate stability of samples, i.e. supernatant, was assessed. Spiked serum LQC and HQC samples of FLT and FLT-MP were processed as described above. The supernatant (400 μl), obtained after protein precipitation and vortex mixing, was transferred to a 10 ml disposable glass centrifuge tube, then stored at 4°C. After 48 hours, the supernatant was processed and analyzed against a freshly spiked calibration standard curve. A criterion of ±20% of the nominal concentration was used for intermediate stability.

### 3.2.9.6 Precision of the FLT Phosphorylation reaction

In order to assess the precision of the phosphorylation procedure, a serum sample having high TK1 activity (serum H) was diluted with a serum sample having low TK1 activity (serum L) to obtain six different control concentrations as follows : 1) Undiluted serum H 2) Serum H diluted 2 times with serum L 3) Serum H diluted 5 times with serum L 4) Serum H diluted 10 times with serum L 5) Serum H diluted 25 times with serum L 6) Undiluted serum L. Six replicates were analyzed and assay precision was expressed as %RSD for each control concentration. The serum sample with high TK1 activity (serum H) was defined arbitrarily to have an activity equivalent to 1 while the serum sample having low TK1 activity (serum L) was defined as an activity equivalent to 0. Activity units for the six levels were calculated based on the ratio of the activity

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of serum H to serum L.  These activity units were used to correlate TK1 with the amount of FLT-MP generated at the end of 2 hour incubation at 37°C

### 3.2.10  Application of the validated method

The validated method was used for determination of TK1 activity in 19 serum samples obtained from hepatocellular carcinoma patients and 40 healthy, age-matched controls (20 male and 20 female; Bioreclamation Inc., Westbury, NY, USA).

### 3.3    RESULTS AND DISCUSSION

This serum TK1 activity assay monitors the phosphorylation of FLT, a thymidine analog and selective TK1 substrate, to its metabolite FLT-MP. The reaction is performed at 37°C in a 0.1M sodium acetate phosphorylation buffer supplemented with enzyme co-factors and stabilizing reagents (Karlstrom*, et al.* 1990, Ohrvik*, et al.* 2004). ATP serves as a phosphate donor in the reaction. The reducing agent DTE liberates TK1 from inhibitory serum protein complexes, and UMP serves as a FLT-MP degradation inhibitor [11]. The FLT phosphorylation FLT phosphorylation reaction is terminated by methanol extraction. Calibration and quality control standards were freshly prepared to avoid analyte degradation in human serum that can occur after extended storage [11]. Since recombinantly produced TK1, and TK1 generated from cell lysates may not be biochemically representative of TK1, serum with high endogenous TK1 activity was diluted with low-activity serum to serve as positive controls (Rasey*, et al.* 2002, Salskov*, et al.* 2007). Target analytes were extracted using protein precipitation, and chromatographically separated using a column switching strategy.  Specifically, samples were loaded onto a C18 Phenomenex security guard column, which retains the analytes while impurities were eluted to waste. After column switching, analytes were separated on an Aquasil C18 analytical column

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and detected using the QTrap in MRM mode.  Without column switching, a build-up of matrix on the column after multiple injections was observed, which resulted in a loss of sensitivity (data not shown).

LC-MS/MS conditions were optimized for the analytes (FLT, FLT-MP, FLT-d3 and FLT-MP-d3), allowing their quantification with a total run time of 7 min.  Retention times and mass transitions are shown in Table 3-1. Representative chromatograms of blank samples for FLT and FLT-MP are shown in Figure 3-3 and 3-4, respectively. Representative chromatograms of FLT and FLT-MP at the LLOQ are shown in Figure 3-5 and 3-6, respectively.  Matrix effects were evaluated with post-column infusion of a 100 ng/mL FLT and FLT-MP solution. No suppression or enhancement was seen at retention time of analyte peaks.

*Table 3-1:Mass Spectrometer Parameters and Approximate Retention Times*

| Analyte | Retention Time (mins) | Multiple Reaction Monitoring Transitions (Parent ion → Product ion) | DP ( Volts) | EP ( Volts) | CE ( Volts) | CXP ( Volts) |
|---|---|---|---|---|---|---|
| FLT | 3.95 | 245.1 → 127.1 | 40 | 10 | 20 | 10 |
| FLT-d3 | 3.95 | 248.0 → 130.2 | 40 | 10 | 20 | 10 |
| FLT-MP | 3.53 | 325.3 → 81.2 | 50 | 10 | 28 | 10 |
| FLT-MP-d3 | 3.53 | 328.1 → 81.2 | 50 | 10 | 28 | 10 |

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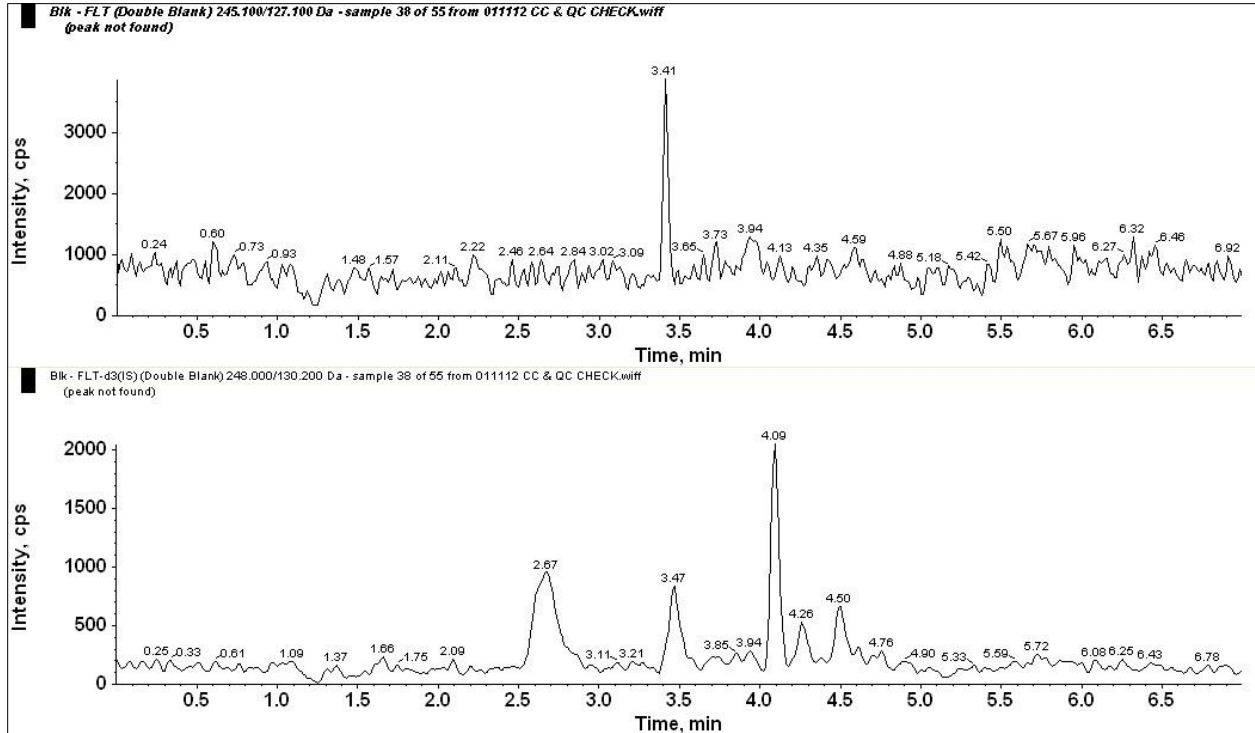
*Figure 3-3: Representative chromatogram of serum blank (FLT and FLT-d3)*
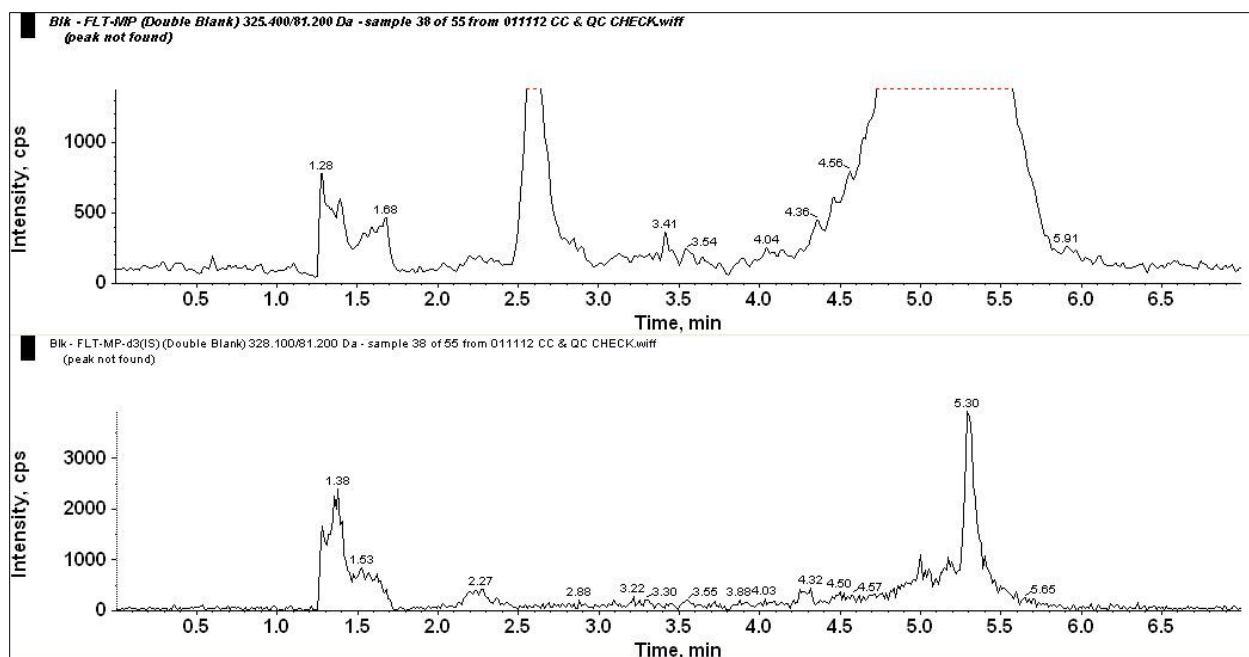
*Figure 3-4: Representative chromatogram of serum blank (FLT-MP and FLT-MP-d3).*
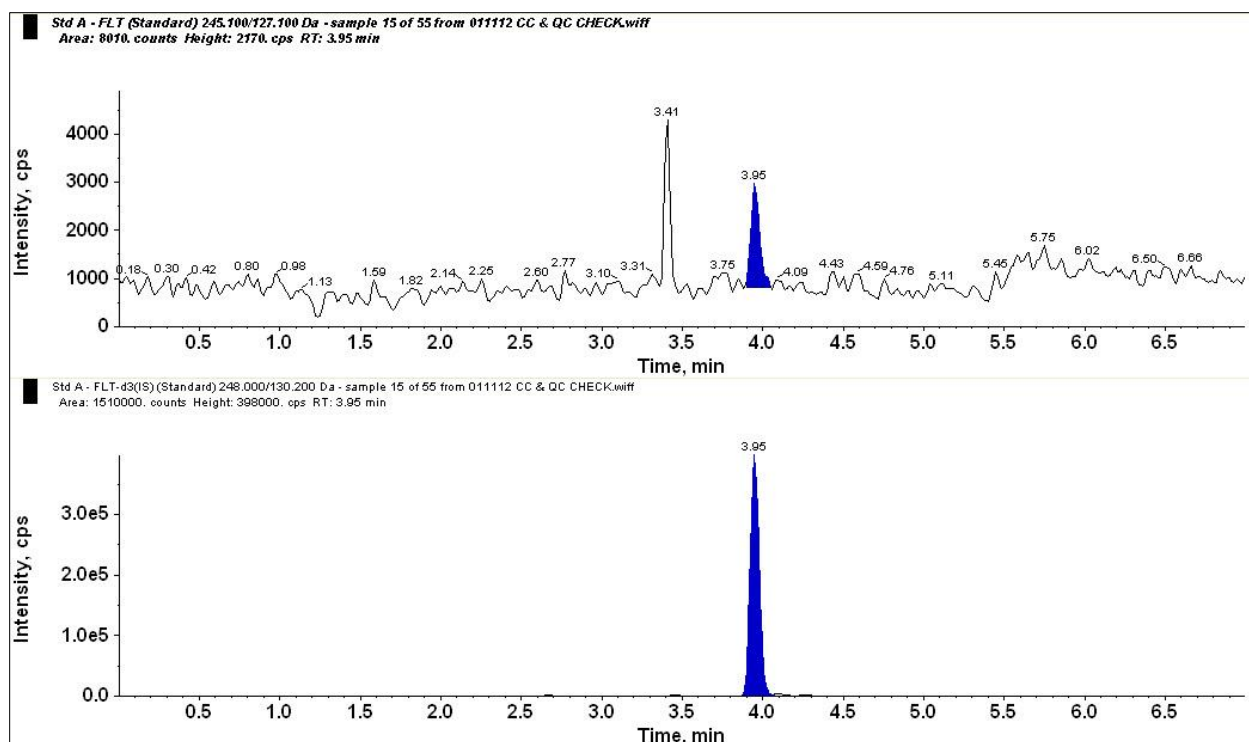
*Figure 3-5 : Representative chromatogram of Lower limit of Quantification(LLOQ) of FLT and FLT-d3.*
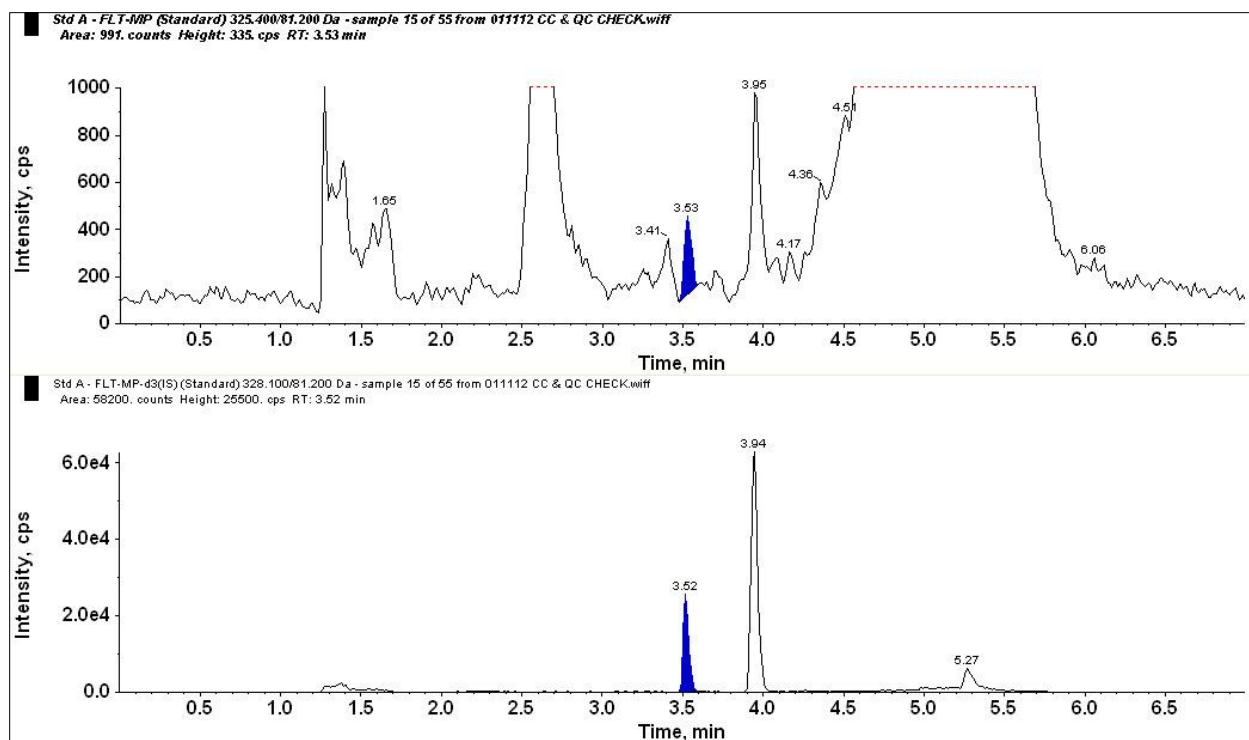
*Figure 3-6 : Representative chromatogram of Lower limit of Quantification(LLOQ)  of FLT-MP (LLOQ) and FLT-MP-d3*

### 3.3.1 Validation of the analytical method

### 3.3.1.1 Selectivity

The peak area at the retention time for all the analytes in the six human serum lots was found to be less than 10% of the respective LLOQ serum calibration standard. Thus, indicating that the method was selective estimating FLT and FLT-MP from human serum samples.

### 3.3.1.2 Linearity

The peak area ratio of FLT to FLT-d3 and FLT-MP to FLT-MP-d3 were linear over the range 0.5-500 ng/mL and 2.5-2000 ng/mL, respectively. The calibration curves yielded a mean correlation coefficient of 0.9964 and 0.9935 for FLT and FLT-MP, respectively (n=3). A weighting factor of $1/x^2$ was used for both analytes (FLT and FLT-MP). The back calculated concentrations of the calibration curve standards in the validation runs are summarized in Table 3-2 and 3-3. The percent deviation from nominal (%DFN) for the mean back-calculated values of the calibration standards were between -14.7% and 11.7% for FLT and -4.3% to 7.5% for FLT-MP. Precision of the calibration standards, measured as the percent relative standard deviation for the mean back-calculated values, ranged between 6.0% to 13.4% for FLT and 3.5% to 14.1% for FLT-MP.

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*Table 3-2: Summary of back calculated concentrations of FLT calibration curve standards*

| RUN | FLT (ng/mL) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.50 | 1.00 | 2.50 | 5.00 | 10.0 | 25.0 | 50.0 | 100 | 250 | 400 | 500 |
| Validation Run 1 | 0.402 | A | 1.85 | 4.96 | 12.5 | 27.2 | 60.8 | 101 | 226 | 386 | 431 |
| | A | 0.85 | 2.58 | 4.86 | 10.3 | 23.9 | 50.4 | 92 | 246 | 434 | A |
| Validation Run 2 | 0.459 | 0.96 | 2.15 | 4.75 | 10.3 | 27.3 | 53.2 | 89 | 217 | 317 | 399 |
| | 0.443 | 0.86 | 2.45 | 5.71 | 10.9 | 27.5 | 52.7 | 107 | 273 | 413 | 451 |
| Validation Run 3 | A | 0.718 | 2.37 | 4.85 | 11.5 | 24.4 | 50.7 | 89 | 230 | A | 485 |
| | 0.42 | 0.875 | 2.73 | 5.60 | 11.5 | 27.3 | 55.7 | 113 | A | 384 | 523 |
| Mean | 0.43 | 0.85 | 2.36 | 5.12 | 11.2 | 26 | 54 | 98 | 238 | 387 | 458 |
| StdDev | 0.03 | 0.09 | 0.32 | 0.42 | 0.8 | 1.7 | 3.9 | 10.2 | 22 | 44 | 47.99 |
| %RSD | 6.0 | 10.3 | 13.4 | 8.2 | 7.6 | 6.3 | 7.2 | 10.4 | 9.2 | 11.4 | 10.5 |
| %DFN | -14.0 | -14.7 | -5.8 | 2.4 | 11.7 | 5.1 | 7.8 | -1.6 | -4.6 | -3.3 | -8.4 |

A = deleted from calculations per SOP criteria of ± 15% (20% for LOQ)

*Table 3-3: Summary of back calculated concentrations of FLT-MP calibration curve standards*

| RUN | FLT-MP(ng/mL) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2.50 | 5.00 | 10.0 | 25.0 | 50.0 | 100 | 250 | 500 | 1000 | 1850 | 2000 |
| Validation Run 1 | 2.38 | 4.32 | 9.05 | 21.8 | 52.2 | 93.4 | 265 | 499 | 973 | 1810 | 1990 |
| | A | 5.45 | 10.5 | 27.0 | 49.5 | 109 | 246 | 552 | 1070 | 1890 | 1900 |
| Validation Run 2 | 2.62 | 4.34 | 8 | 22.9 | 51.5 | 107 | 267 | 564 | 1040 | 1900 | 2010 |
| | 2.60 | 4.94 | 11.1 | 26 | 52.4 | 109 | 252 | 561 | 937 | 1730 | 1920 |
| Validation Run 3 | 2.96 | 5.05 | 9.13 | 24.9 | 41.5 | 87.9 | 241 | 494 | 1360 | 1610 | 1830 |
| | 2.88 | 5.07 | 10.5 | 25.4 | 51.4 | 88.1 | 232 | 466 | 1030 | 1680 | 1890 |
| Mean | 2.69 | 4.86 | 9.7 | 24.7 | 49.8 | 99 | 251 | 523 | 1068 | 1770 | 1923 |
| StdDev | 0.23 | 0.45 | 1.2 | 2.0 | 4.2 | 10.4 | 14 | 42 | 151 | 117 | 67 |
| %RSD | 8.7 | 9.2 | 12.1 | 7.9 | 8.4 | 10.5 | 5.5 | 7.9 | 14.1 | 6.6 | 3.5 |
| %DFN | 7.5 | -2.8 | -2.9 | -1.3 | -0.5 | -0.9 | 0.2 | 4.5 | 4.5 | -4.3 | -4.3 |

A = deleted from calculations per SOP criteria of ± 15% (20% for LOQ)

### 3.3.1.3 Accuracy and Precision

The lower limit of quantification (LLOQ) was established at 0.5 ng/mL for FLT with a precision of 8.8%. The LLOQ for FLT-MP was set at 2.5 ng/mL with a precision of 11.5%. Accuracy at the LLOQ was -9.2% and -3.8% for FLT and FLT-MP, respectively. Inter- and intra-assay precision and accuracy for FLT and FLT-MP quality control samples are shown in Table 3-4.

### 3.3.1.4 Extraction recovery and Carryover studies

The mean extraction recovery for FLT in human serum, determined at the LQC and HQC levels, was 103.5% and 100.3%, respectively. FLT-MP mean extraction recovery was 57.0% and 56.9% for LQC and HQC, respectively.

All the LQCs were within 15% of the nominal concentrations when injected after a high calibration standard for both analytes (FLT and FLT-MP). Thus, illustrating that carryover is not significant for this method.

*Table 3-4: Inter-assay and Intra-assay precision and accuracy for FLT and FLT-MP.*

| | Nominal Concentration (ng/mL) | | | |
|---|---|---|---|---|
| | **2.0** | **40.0** | **375** | **2000** |
| **Observed FLT Concentration (ng/mL)** | | | | |
| Inter-assay mean ± SD | 2.0±0.2 | 40.0±5.9 | 377.0±39.0 | 1830.0±84.0 |
| Inter-assay precision (%RSD) | 9.4 | 14.9 | 10.4 | 4.6 |
| Inter-assay accuracy (%DFN) | -0.2 | -1.1 | 0.4 | -8.5 |
| | | | | |
| Intra-assay mean ± SD (n= 6) | 2.1±0.2 | 45.0±2.6 | 378.0±14.0 | 1797.0±50.0 |
| Intra-assay precision (%RSD) | 7.7 | 5.9 | 3.6 | 2.8 |
| Intra-assay accuracy (%DFN) | 3.5 | 11.6 | 0.9 | -10.2 |
| | **6.0** | **60.0** | **1800.0** | **5000.0** |
| **Observed FLT-MP Concentration (ng/mL)** | | | | |
| Inter-assay mean ± SD | 6.6±1.0 | 65.0±5.7 | 1939.0±95.0 | 5159.0±342.0 |
| Inter-assay precision (%RSD) | 14.6 | 8.8 | 4.9 | 6.6 |
| Inter-assay accuracy (%DFN) | 9.4 | 7.8 | 7.7 | 3.2 |
| | | | | |
| Intra-assay mean ± SD (n =6) | 6.7±0.3 | 66.0±2.5 | 1958.0±64.0 | 5275.0±304.0 |
| Intra-assay precision (%RSD) | 4.4 | 3.7 | 3.3 | 5.8 |
| Intra-assay accuracy (%DFN) | 11.8 | 10.4 | 8.8 | 5.5 |

SD = Standard Deviation, %DFN = percent deviation from nominal value, %RSD = percent relative standard deviation. Samples were diluted 10-fold for the FLT (2000 ng/mL) and FLT-MP (5000 ng/mL) controls.

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### 3.3.1.5   Post preparative stability

For FLT, %DFN for the mean back-calculated values of the quality control standards were between -20.0% and -10.2% for LQC and -10.5% to -5.5% for HQC.  For FLT-MP, %DFN for the mean back-calculated values of the quality control standards were between -9.3% and -6.3% for LQC and 1.5% to 2.8% for HQC. Thus, post-preparative stability was found to be within the acceptance criteria.

### 3.3.1.6   Intermediate processing stability

For FLT, %DFN for the mean back-calculated values of the quality control standards were between -14.6% and -13.2% for LQC and -2.0% to -3.0% for HQC.  For FLT-MP, %DFN for the mean back-calculated values of the quality control standards were between -13.1% and -8.6% for LQC and 19.0% to 9.0% for HQC.  Thus, the supernatant obtained during processing was considered stable within specifications when stored at 4°C for 48 hours.

### 3.3.1.7   Precision of the FLT phosphorylation reaction

Phosphorylation reaction precision (% RSD) was within 15% at all concentrations except the defined zero level (Table 3-5). Additionally, the average concentration of FLT-MP in these samples and TK1 activity were found to have a positive linear correlation with a correlation coefficient of 0.9862 (See Figure 3-7).

*Table 3-5: Precision of the FLT phosphorylation reaction.*

|  | Enzyme Level-1 | Enzyme Level-2 | Enzyme Level-3 | Enzyme Level-4 | Enzyme Level-5 | Enzyme Level-6 |
|---|---|---|---|---|---|---|
| TK1 Activity Units* | 1.00 | 0.50 | 0.20 | 0.10 | 0.04 | 0.00 |
| Average concentration of FLT-MP (ng/ml)** | 965.33 | 599.80 | 245.80 | 131.83 | 58.05 | 5.09 |
| SD | 34.915 | 60.726 | 32.980 | 14.662 | 4.972 | 1.273 |
| %CV | 3.62 | 10.12 | 13.42 | 11.12 | 8.56 | 25.03 |

**\*** The serum sample with high TK1 activity (serum H) was defined arbitrarily to have an activity equivalent to 1 while the serum sample having low TK1 activity (serum L) was defined as an activity equivalent to 0. Activity units for the six levels were calculated based on the ratio of the activity of serum H to serum L. ** The FLT-MP concentrations were generated under standardized conditions of 2 hour incubation at 37°C.
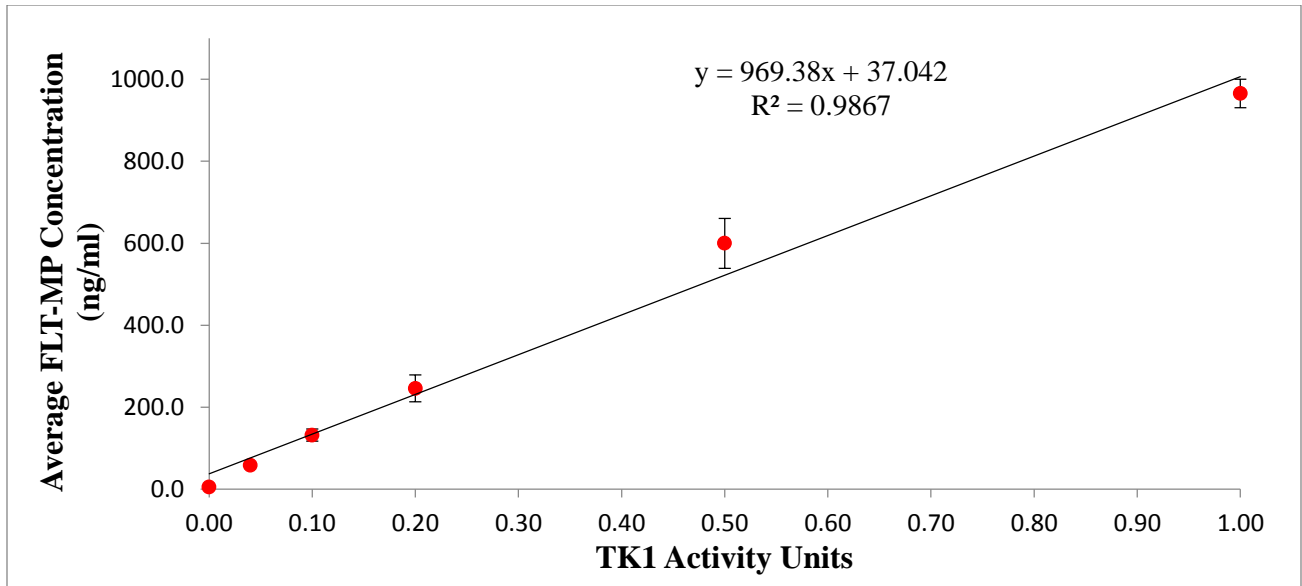
*Figure 3-7: Plot of average FLT-MP concentration formed versus TK1 activity.*

### 3.3.2   Application of the method to patient samples

Average FLT-MP generation in serum from healthy male and female volunteers aged 50 or older was $19.1 \pm 10.5$ ng/ml and $18.7 \pm 5.8$ ng/ml, respectively when analyzed using the standardized reaction conditions described here (37° C, 2 hours).

The average FLT-MP concentration in healthy individuals was $18.9 \pm 8.5$ ng/ml.   In contrast, the hepatocellular carcinoma patient serum samples showed a relatively wide variation in TK1 activity as seen by the varying of FLT-MP concentrations (11.7-1350.5 ng/ml). A comparison of FLT-MP concentrations between the hepatocellular carcinoma (HCC) patient samples and the matched controls is seen in Figure 3-8. From this figure, it can been seen that a sub-population of the HCC patient samples demonstrated almost a  20 fold increase in TK1 activity. The increased TK1 activity in the HCC sub-patient population may have been due to differences in the severity of their disease although this could not be verified since detailed information regarding these samples was not available.

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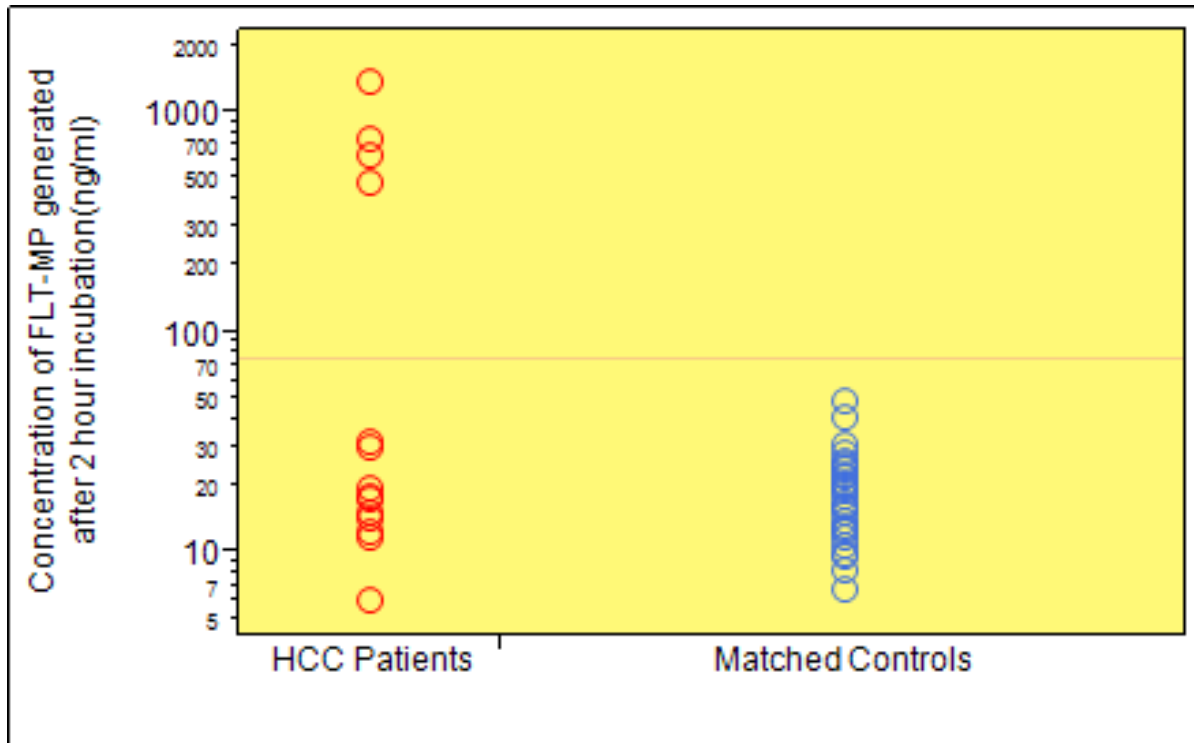
*Figure 3-8:Comparison of serum TK1 activity in hepatocellular carcinoma (HCC) patient*

*samples & matched controls.*

## 3.4 CONCLUSIONS

Serum TK1 activity is a potentially useful biomarker for monitoring haematological malignancies and solid tumors. A sensitive, non-radiometric LC-MS/MS assay for was developed and validated for monitoring the conversion of FLT to FLT-MP in human serum. Based on established, radiometric TK1 activity assays, this novel method shows good linearity and selectivity in human serum samples. Stability studies demonstrated adequate intermediate processing and post-preparative analyte stability.

The applicability of the method for measuring serum TK1 activity was demonstrated in hepatocellular carcinoma patient serum samples and age-matched control sera. Significantly higher concentrations of FLT-MP were found in 26.3% of the hepatocellular carcinoma patient samples in comparison with controls. This method is proposed as an alternative to ELISA and radio-enzymatic assays for rapid and selective determination of serum TK1 activity.

# 4 COMPARISON OF A STABLE ISOTOPE LABELED (SIL) PEPTIDE AND AN EXTENDED SIL PEPTIDE AS INTERNAL STANDARDS TO TRACK DIGESTION VARIABILITY OF AN UNSTABLE SIGNATURE PEPTIDE DURING QUANTIFICATION OF A CANCER BIOMARKER, HUMAN OSTEOPONTIN, FROM PLASMA USING CAPILLARY MICROFLOW LC-MS/MS.

## 4.1 INTRODUCTION

Human osteopontin (hOPN) is a matricellular protein that mediates diverse biological functions (Lund*, et al.* 2009). It is involved in normal physiological processes and is implicated in the pathogenesis of a variety of disease states, including atherosclerosis, cancer, and several chronic inflammatory diseases (Lund*, et al.* 2009, Rodrigues*, et al.* 2007, Waller*, et al.* 2010). It is reported to play a major role in tumor promotion mechanisms including cell survival, adhesion, migration, invasion and angiogenesis (Rodrigues*, et al.* 2007). Over expression of hOPN has been found in a variety of cancers, including breast cancer, lung cancer, colorectal cancer, stomach cancer and ovarian cancer (Rodrigues*, et al.* 2007). It is also secreted in serum, plasma,

urine and other body fluids. Elevated plasma OPN levels have been associated with poor survival in cancer patients (Rodrigues, et al. 2007). OPN primarily operates by binding to various integrins mainly through its RGD (arginine-glycine-aspartic acid) amino acid sequence. Integrins are transmembrane receptors that mediate attachment between a cell and its surroundings. The adhesive RGD domain of OPN mediates interactions via αvβ1, αvβ3, αvβ5, αvβ6, α8β1, and α5β1 integrins (Shimada, et al. 2005). OPN can be cleaved by thrombin near its RGD integrin binding domain to expose a cryptic SVVYGLR amino acid sequence that can bind to α9β1, α4β1, and α4β7 integrins (Scatena, et al. 2007). Furthermore, the cryptic site can be further cleaved by metallomatrix proteinases (MMPs) at the glycine-lysine ($^{167}$G-L) bond and by plasma carboxypeptidase B-2 (CPB-2) at the ($^{168}$L-R) bond. The impact of proteolytic modification of OPN can promote or disrupt integrin binding, leading to altered cellular responses (Scatena, et al. 2007, Shimada, et al. 2005) .

Immunoassays, in particular, enzyme linked immuno-sorbent assay (ELISA), have been the method traditionally used for analysis of protein biomarkers. However, immunoassay measurements can be variable and inaccurate due to cross-reactivity of the antibodies used, the presence of post-translational modifications, interference due to autoantibodies and anti-reagent antibodies, and the high-dose hook effect (Hoofnagle, et al. 2009). Different commercially available ELISA kits for quantification of OPN have been shown to yield variable and non-comparable absolute concentrations (Anborgh, et al. 2009, Plumer, et al. 2008, Vordermark, et al. 2006). For example, Plummer et al. compared three ELISA kits which produced >20-fold differences in measured absolute hOPN concentrations for the same sample. Such variations can lead to misleading interpretations resulting in unnecessary treatment or missed opportunities for

therapeutic intervention. We therefore decided to explore an alternative platform for measuring hOPN from plasma.

In the last decade, tandem mass spectrometry using selected reaction monitoring (SRM) is increasingly employed for analysis of biomarker proteins (Makawita*, et al.* 2010). Due to the limited mass range of most mass analyzers used for quantification, LC-MS/MS protein quantification often involves digestion of the protein to yield peptides, including unique signature peptides, which are then quantified along with an appropriate internal standard. Thus, protein quantification involves a number of challenges including signature peptide selection, protein digestion, sample enrichment and internal standardization (Makawita*, et al.* 2010).

The identification and selection of an appropriate signature peptide is an important aspect method development, as the signature peptide serves as a surrogate analyte for the protein. Utilizing the protein's peptide map or amino acid sequence, *in-silico* proteolytic digestions can be carried out using software such as Skyline software and Protein Prospector, and proteotypic peptides can be identified using *Basic* Local Alignment Search Tool (BLAST) (Becker*, et al.* 2011, Halquist*, et al.* 2011b, Luna*, et al.* 2008, Rauh 2012).

Protein biomarker measurements are usually made from tissues or blood fractions i.e. plasma or serum. Since blood measurements are less invasive, they are preferred over tissue measurements. However, plasma and serum are highly complex biological fluids. In fact, in serum, 20% of proteins comprise 99% of total protein content and the concentrations of all proteins span more than 12 orders of magnitude (Makawita*, et al.* 2010). Analysis of direct digests of serum or plasma would be ideal since it would involve minimal sample preparation. However, matrix effects and interferences from other highly abundant proteins may adversely affect the limit of quantification and selectivity of these mass spectrometric methods (Makawita*, et al.* 2010). Thus,

sample enrichment is an essential part of most protein quantification methods. Sample preparation for protein purification from a biological matrix prior to quantification using LC-MS/MS can be categorized into three major strategies i.e. abundant protein removal, immunoaffinity protein isolation and immunoaffinity signature peptide isolation after digestion (Ackermann, *et al.* 2007). Depending on the level of purification required, one of the above strategies or a combination of these could be used. For high and mid-abundance plasma proteins (>100 ng/ml), abundant protein removal may be adequate for quantification (Ackermann, *et al.* 2007). However, quantification of low abundance (< 100 ng/ml) proteins would likely require the use of immunoaffinity isolation.

Another important challenge in mass spectrometric protein quantification is the choice of an appropriate internal standard (IS). An ideal IS should mimic the properties of the analyte in all sample preparation and analysis steps. In LC-MS/MS quantifications, an IS accounts for any losses during sample preparation as well as minimizes the effects of response fluctuations on the mass spectrometer. During mass spectrometric quantification, a response ratio of the analyte and the internal standard is correlated with the concentration ratio of the analyte and the internal standard (Bronsema, *et al.* 2012).

For quantification of small molecules using LC-MS/MS, a stable-isotope labeled (SIL) form of the analyte or a structural analogue can be used as an IS. However, since protein quantification involves protein digestion, there are more options for internal standardization. A SIL form or a structural analogue of either the intact protein or the signature peptide can be used. Alternatively, an extended SIL-peptide, which can be cleaved to obtain SIL signature peptides, can be used as an IS. A SIL-protein may be an ideal IS for protein quantification. However, SIL-proteins are very difficult to obtain because they require specific folding of the linear amino acid sequence, as

well as creation of intra-molecular disulfide linkages and post-translational modification. A method known as SILAC (Stable Isotope Labeling with Amino Acids in Culture) can be used to generate SIL-proteins(Ong, *et al.* 2002). However, the production of SIL-proteins can be limited by cost and commercial availability (Bronsema, *et al.* 2012). A more commonly used internal standard for protein quantification is a SIL-peptide with the same aminoacid sequence as the signature peptide. A SIL-peptide is normally added following protein enrichment and prior to digestion. It does not compensate for digestion variability but does account for subsequent peptide analyte recovery and potential ion suppression effects (Ackermann, *et al.* 2007). An extended or "winged" SIL-peptide, however (containing additional amino acid residues on one or both ends), would be able to also track digestion, provided it is cleaved at the same rate as the signature peptide (Bronsema, *et al.* 2012). In sample preparation methods involving peptide immunoaffinity isolations, an extended SIL-peptide IS would also able to track variability and recovery during immunocapture (Ocana, *et al.*).

Recently, Wu et al (2012), quantified OPN splice variants from plasma of lung cancer patients using a combination of four OPN specific antibodies for IA protein isolation coupled with targeted mass spectrometry (Wu, *et al.* 2012). However, the validation of the method was not reported. In addition, the method used signature peptides which are reported to undergo post translational phosphorylation.

Most LC–MS/MS bioanalytical assays are performed with conventional HPLC separations, using 2.1-4.6 mm ID columns and chromatographic flow rates of 300–1000 μL/min, and mass spectrometric detection using triple quadrupole instrument operating in electrospray ionization (ESI) mode. The benefits of using microflow liquid chromatography (MFLC) were realized in the 1970s; however, reliable hardware that meets regulated bioanalysis standards were not

available (Arnold*, et al.* 2013). In recent years, accurate pumps delivering nanoflow and microflow rates, reliable lower internal diameter columns, and improvements in nanospray ion sources have facilitated the use of MFLC-MS/MS platforms for quantitative bioanalysis. Use of microflow LC-MS/MS platforms (flow rate < 3 μL/min) using columns with internal diameters less than 1 mm have resulted in increased sensitivity, reduction in sample volume and solvent consumption (Arnold*, et al.* 2013, Broccardo*, et al.* 2013, Christianson*, et al.* 2013, Dijksman*, et al.* 2012, He*, et al.* 2012, McAvoy*, et al.* 2014, Wang*, et al.* 2013, Zhou*, et al.* 2013).

In this paper, we describe the development and validation of a method to quantify hOPN from plasma using a capillary microflow LC-MS/MS system. A schematic diagram of the strategy used for osteopontin quantification with LC-MS/MS using immunoaffinity isolation is given in Figure 4-1. The method uses immunopurification for isolation of hOPN from plasma. The isolated protein is then digested with trypsin in the presence of an internal standard peptide to generate a biologically relevant signature peptide i.e. GDSVVYGLR. Peptide yields decreased with increased digestion time indicating signature peptide instability. A SIL peptide and an extended SIL peptide were evaluated as internal standards to track digestion variability. The signature peptide is quantified using a Waters nanoAcquity/TRIZAIC-Xevo-TQS LC-MS/MS system.
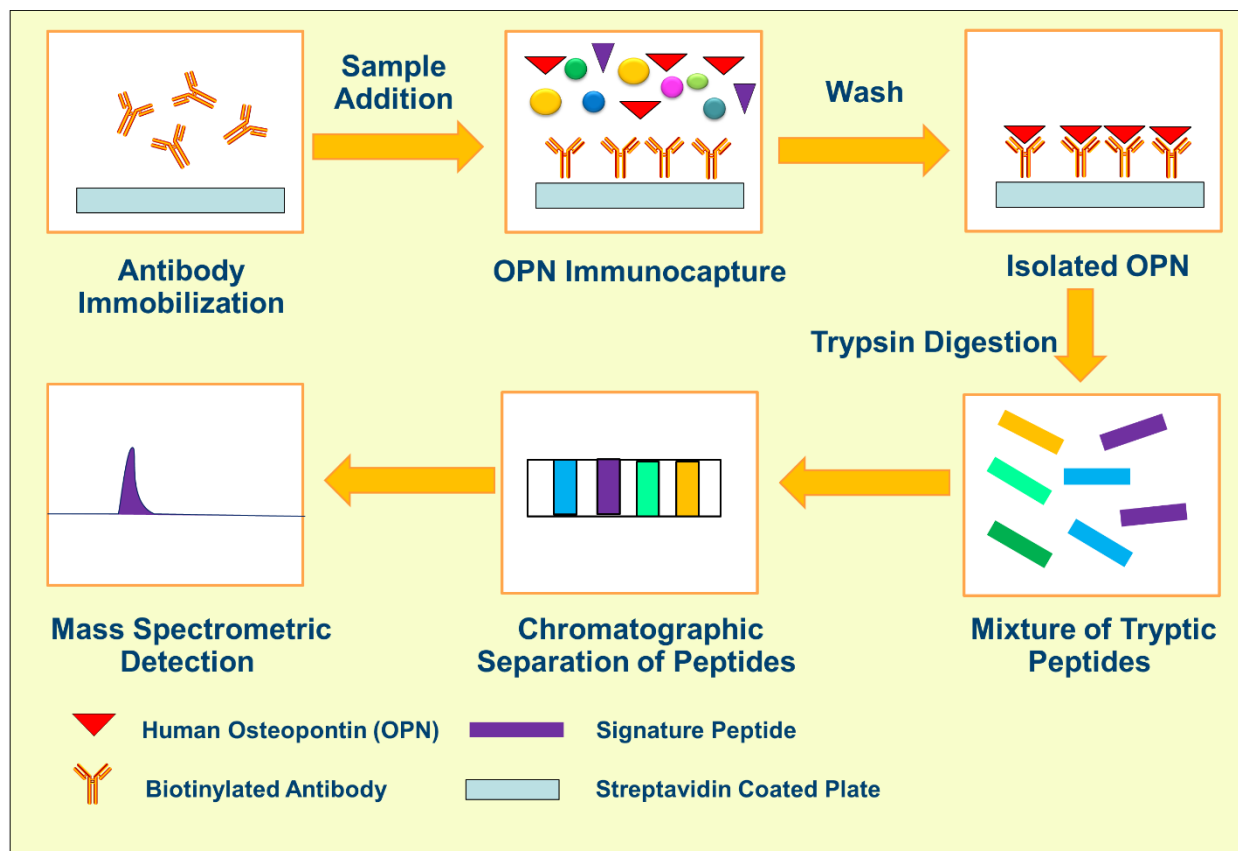
*Figure 4-1:A schematic diagram of the strategy used for osteopontin quantification using LC-MS/MS and immunoaffinity isolation.*

## 4.2   MATERIALS AND METHODS

### 4.2.1   Reference Materials

Recombinant hOPN was purchased from R&D Systems (Minneapolis, MN, USA). The OPN signature peptide (OPN-SP), having the amino acid sequence 'GDSVVYGLR', was synthesized by Elim Biopharmaceuticals (Hayward, CA, USA). A stable isotope labeled peptide having the sequence 'GDSVVYGLR*', containing an arginine residue labeled with $^{13}C$ and $^{15}N_4$ was synthesized by Thermo Scientific (Rockford, IL, USA) and used as the SIL-IS. The labeled arginine residue resulted in a mass shift of +10 Da from the mass of the OPN-SP. Another stable isotope labeled peptide having the sequence 'TYDGRGDSVV*YGLRSKSKKF', containing an valine residue labeled with $^{13}C$ and $^{15}N_4$ was synthesized by Thermo Scientific (Rockford, IL, USA) and used as an extended SIL-IS. The labeled valine residue resulted in a mass shift of +6 Da from the mass of OPN-SP.

### 4.2.2   Reagents

HPLC grade water with 0.1% formic acid and acetonitrile with 0.1% formic acid were purchased from Honeywell Burdick & Jackson (Muskegon, MI, USA). High grade water was obtained using a Milli-Q integral water purification system (Billerica, MA, USA). Bovine serum albumin, dulbecco's phosphate buffered saline, formic acid, methanol, Trizma hydrochloride (1M) and tween 20 were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Glacial acetic acid was obtained from Macron Fine Chemicals (Center Valley, PA, USA). Sodium chloride solution (5M) and EDTA solution  (0.5M) were obtained from Ambion (Foster City CA, USA). Trypsin Gold was obtained from Promega (Madison, WI, USA). Biotinylated antibodies specific to hOPN (MAB193P) were obtained as a gift from Maine Biotechnology Services (Portland, Maine, USA).  Immuno-capture (IC) buffer and IC wash buffer were prepared in-house and were

comprised of 0.1/5.0/3.0/0.2/91.7/0.1 (v//v/v/v/v/w) Tween 20/ Trizma HCl (1M)/ NaCl (5M)/EDTA (0.5M)/water/ bovine serum albumin and 5.0/3.0/0.2/91.8/0.1 (v/v/v/v) Trizma HCl (1M)/ NaCl (5M)/ EDTA (0.5M)/ water, respectively. Digestion solution, containing of 500 ng/100 µL of trypsin gold, 0.05 pg/100 µL of SIL-IS peptide and 0.68 pg/100 µL of extended SIL-IS peptide in 50 mM ammonium bicarbonate buffer, was freshly prepared prior to digestion for the validation studies.

### 4.2.3 LC-MS/MS Instrumentation

Preliminary method development studies were carried out using a conventional flow LC-MS/MS system. The method was then transferred and validated on a MFLC-MS/MS.

### 4.2.3.1 Conventional flow LC-MS/MS system

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) and a Waters Acquity UPLC$^{®}$ system (Waters Corporation, Milford, USA). ACE HPLC C4 column (50mm × 2.1 mm I.D., 5 µm) from Advanced Chromatography Technologies Ltd, (Aberdeen, Scotland) was used as the trap column and a Peptide Separation Technology (PST) XBridge BEH C18 column (100mm × 2.1mm I.D., 5 µm) from Waters (Milford, USA) was used as the analytical column. The analytical column was maintained at 50°C.

Trapping was achieved using a Waters Acquity UPLC$^{®}$ system using trap mobile phase A (HPLC grade water with 0.1% formic acid) and trap mobile phase B (acetonitrile with 0.1% formic acid), respectively. Trapping was carried out with 5% trap mobile phase B and maintained at a flow rate of 0.300 mL/min. Following an initial trapping time of 0.8 min, the diverter valve was switched to position B and the elution initiated. A 10-port Cheminert

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switching valve and a microelectric actuator obtained from Valco Instruments Co. Inc. (Houston, TX, USA) was used to divert flow to the analytical column in position B. After a running time of 4.5 minutes, the diverter valve position was switched back and flushing of the trap column was achieved at 0.300 mL/min with 5% trap mobile phase B. Elution was carried out using two Shimadzu pumps operated with the Shimadzu system controller to apply the gradient conditions and the elution mobile phases consisted of water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). The flow rate was set to 0.400 mL/min. Gradient conditions were as follows: 0.0 –1.0 min, isocratic 5% B; 2.0 –4.0 min, linear from 5% to 30% B; 4.0 –4.5 min, linear from 30% to 80% B; 4.5 –5.0 min, isocratic 5% B. The total running time was 5 min and the injection volume was 25 µL. The LC-MS/MS setup is illustrated in Figure 4-2.

Mass spectra were obtained using an Applied Biosystems SCIEX API 4000 QTrap Mass Spectrometer operated in positive electrospray ionization mode (ESI). Tuning and optimization of the mass spectrometer parameters were performed for the analytes and internal standard (IS, see below) by direct infusion of a 1 µg/mL standard solution at a flow rate of 12 µL/min. The multiple reaction monitoring (MRM) transitions, declustering potential (DP) entrance potential, collision energy (CE) and collision cell exit potential (CXP) for all the compound are listed in Table 4-1. The mass spectrometric parameters were as follows: ion source temperature (TEM=450°C), ion transfer voltage (IS=5500 V), collision gas (CAD=high), curtain gas (CUR=10), ion source gas 1 (GS1=30), ion source gas 2 (GS2=20), declustering potential (DP=60 V), entrance potential (EP= 10 V), collision cell exit potential (CXP=10 V). The data were acquired with Analyst software, Version 1.5.

*Table 4-1: LC-MS/MS parameters for API 4000 Qtrap system.*

| Peptide | Charge State | Retention time (mins) | MRM Transitions (Parent ion → Fragment ion) | CE ( Volts) |
|---|---|---|---|---|
| GDSVVYGLR | +2 | 4.2 | m/z 483.9-508.6 | 24 |
| GDSVVYGLR | +2 | 4.2 | m/z 483.9-607.8 | 22 |
| #GDSVVY | +1 | 4.0 | m/z 639.3-359.4 | 30 |

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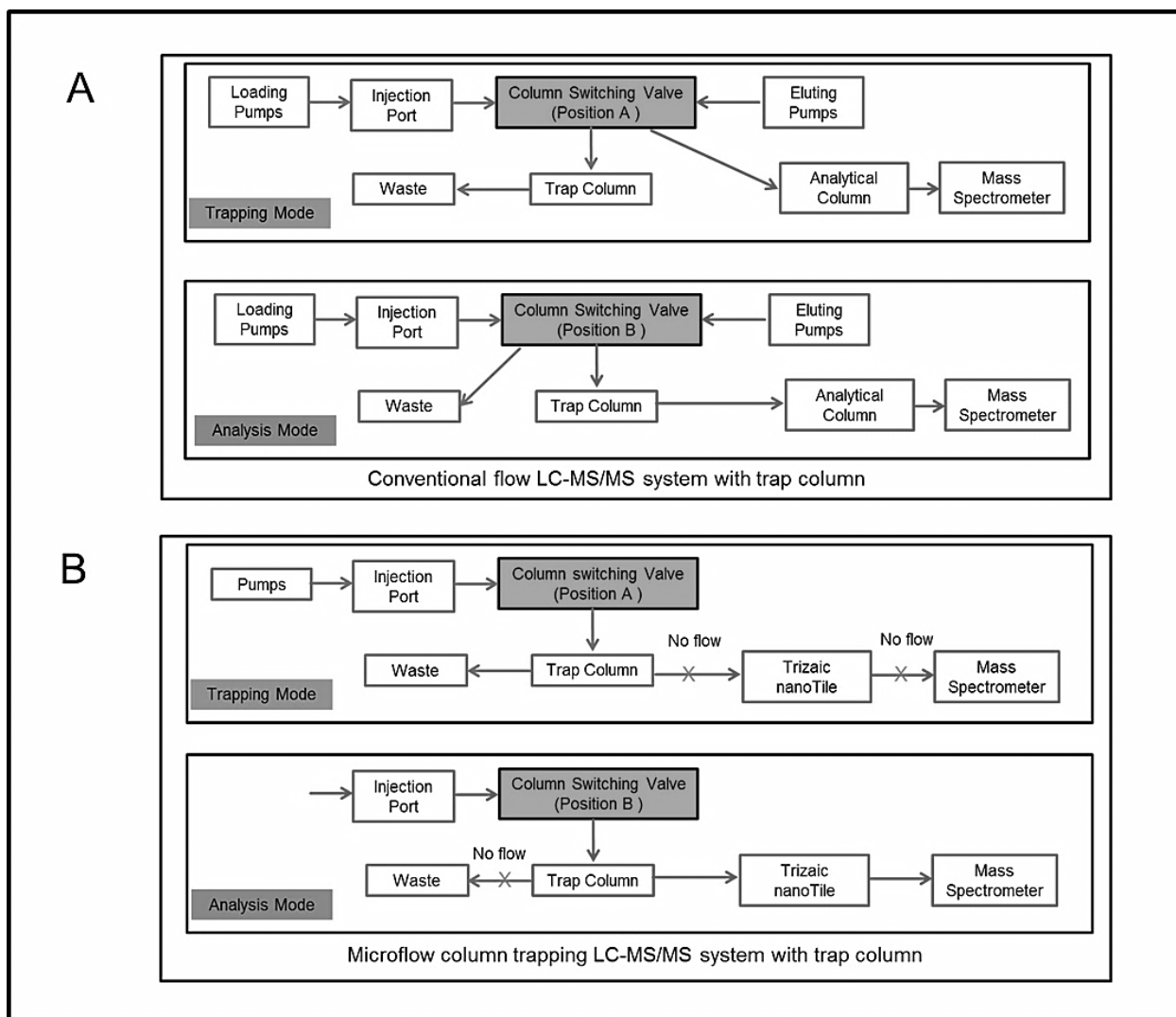
*Figure 4-2: LC-MS/MS Instrumentation. A: Block diagram of the conventional flow LC-MS/MS system. B: Block diagram of the MFLC-MS/MS system.*

### 4.2.3.2 Microflow LC-MS/MS system

The MFLC-MS/MS system consisted of a Waters NanoAcquity UPLC coupled to at Waters TQ-S mass spectrometer fitted with a prototype ion key/MS™ separation device. The separation device consisted of a compact cartridge with an in-built capillary column having an internal diameter of 150 µm and packed with 1.7 µm particles. HPLC grade water with 0.1% formic acid and acetonitrile with 0.1% formic acid were used as mobile phase A1 and B1, respectively. The LC system was operated in the trap mode using a single pump system. A Symmetry C18 Guard Column, 300 µm x 50 mm from Waters (Milford, USA) was used as the trap column. Trapping was carried out for 2 minutes at a flow rate of 20 µL/min using 99.5% mobile phase A1. Analytical separation was performed on a Waters BEH 130 C18 iKey™ Separation Device (100 mm x 150 µm I.D., 1.7 µm). Gradient conditions were as follows: 0.0–0.5 min, isocratic 5% B1; 0.5 –3.0 min, linear from 5% to 20% B; 3.0 –5 min, linear from 20% to 30% B; 5.0 –7.0 min, linear from 30% to 95% B; 7.0 –8.4 min, isocratic 95% B; 8.4 –8.5 min, linear from 95% to 5%; 8.4 –8.5 min, isocratic 5% B. The total running time was 9 min and the injection volume was 20 µL in the full loop mode. Initial flow rate from 0.0 to 0.5 min was kept at 0.5 µL/min to avoid high back pressure during switch over from trapping to analysis mode. From 0.5 to 8.5 minutes, the chromatographic separation was carried out at a flow rate of 2.5 µL/min. The flow rate was changed to 1.0 µL/min after 8.5 min. The cone voltage and collision energy were optimized for each compound using automatic tuning (Intellistart system) in the TQ-S. The parameters for each compound are listed in Table 4-2. The capillary voltage was 3.6 kV, the source temperature was 100 °C, the source offset was 60 V, and the collision gas was argon. Dwell times for all transitions were 0.044 s. The MFLC-MS/MS apparatus is diagrammatically illustrated in Figure 4-2.

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*Table 4-2: LC-MS/MS parameters for Xevo TQS System*

| Peptide | Charge State | Retention time (mins) | MRM Transitions (Parent ion → Fragment ion) | Cone Voltage ( V) | Collision Energy (V) |
|---|---|---|---|---|---|
| GDSVVYGLR | 2 | 5.0 | m/z 483.6-508.5 | 15 | 16 |
| GDSVVYGLR | 2 | 5.0 | m/z 483.6-607.5 | 15 | 16 |
| GDSVV*YGLR | 2 | 5.0 | m/z 486.0-508.5 | 19 | 14 |
| GDSVVYGLR* | 2 | 5.0 | m/z 488.6-518.4 | 19 | 14 |
| TYDGRGDSV*VYGLRSKSKKF | 3 | 4.6 | m/z 568.6-669.4 | 37 | 16 |

### 4.2.4   Method Development Studies

### 4.2.4.1   Signature Peptide Selection

*In-silico* digestion studies were carried out using Protein Prospector version 5.10.9 (UCSF Mass Spectrometry Facility California, USA). Trypsin was chosen as the proteolytic enzyme. Following the *in- silico* digest, the peptides were subjected to sequence homology analysis in the human plasma proteome using the Basic Local Alignment Search Tool (BLAST) software.

### 4.2.4.2   Digestion Optimization

A 50 µL aliquot of recombinant hOPN standard solution of 10 ng/mL was used for digestion studies. Digestion was carried out in 50 mM ammonium bicarbonate buffer using three different amounts of trypsin i.e. 5, 50 and 500 ng per sample. The samples were not reduced with dithiothreitol (DTT) and iodoacetamide since hOPN is devoid of cysteine di-sulfide bonds. Samples were withdrawn at various time intervals i.e. 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 10.5, 13, 20, 22, 24 hours. The reaction was terminated by adding formic acid. The samples were stored at -20°C until analysis.

### 4.2.4.3   Immunocapture method optimization

#### *4.2.4.3.1   Order of Addition*

Commercially available monoclonal antibodies 'MAB 193P' which were specific to hOPN, and having affinities for a site distant from the signature peptide i.e. '[31]QLYNKYPDAVATWLNPDPSQKQNLLAP' were used for immunoaffinity isolation. The biotinylated capture antibodies were immobilized on high binding capacity streptavidin coated 96 microwell plates and used for immunocapture. Three different strategies were evaluated for the order of assembly of the immobilized antibody/immunocapture reaction. In Strategy I, immobilization of antibody was carried out prior to OPN immunocapture.  Strategy II involved

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the formation of OPN-antibody complex prior to antibody immobilization. Strategy III allowed the antibody immobilization and immunocapture reaction to occur simultaneously. The strategy with the highest yields was used in the final method. Recombinant human osteopontin (rhOPN) was spiked in plasma to obtain a concentration of 50 ng/100 µL (not adjusted for baseline plasma OPN level). Healthy volunteer plasma was randomly chosen for this experiment. Unspiked plasma samples were processed as blanks to account for endogenous levels of OPN. JMP Pro (version 10.0.2) software was used for statistically comparing the results. The three strategies for immunocapture were as follows:

A) Strategy I (Immobilization – Immunocapture): In this biotinylated antibody (1 ug), diluted in a IC buffer, was added to streptavidin coated plates and incubated for 4 hours at room temperature on a rotary shaker. After 4 hours, the plates were washed three times with IC wash buffer and 100 µL of the sample was added and incubated for 4 or 20 hours at on a rotary shaker. The plates were then washed and the immunocaptured protein was digested.

B) Strategy II (Immunocapture – Immobilization): An aliquot of 100 µL of the sample was incubated with the antibody in 1.5 mL Eppendorf® Lobind centrifuge tubes for 4 or 20 hours on a rotary shaker. After 20 hours, the samples were transferred to streptavidin coated plates and incubated for 4 hours at room temperature on a rotary shaker. The plates were then washed and the immunocaptured protein was digested.

C) Strategy III (Immunocapture + Immobilization): An aliquot of 100 µL of the sample along with antibody was added to streptavidin coated plates and incubated for 20 hours on a rotary shaker. After 20 hours, the plates were washed and the immunocaptured protein was digested.

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### 4.2.4.3.2  *Optimization of the amount of Antibody*

The use of streptavidin coated 96-well plates as the solid support restricted the sample volume to 100 µL. On the conventional LC-MS/MS system, higher sample volumes were required to achieve lower detection limits, hence the method was modified to use streptavidin coated beads for immobilization. However, when the method was transferred to the MFLC-MS/MS system, the higher sensitivity allowed switching to the 96-well plate format. To evaluate the impact to varying the amount of antibody on the method, rhOPN was spiked in plasma having low baseline concentrations to achieve a final concentration of 600 ng/mL and analyzed as per the sample preparation procedure described under validation studies, with varying amounts of antibody. The antibody amounts used were 0.031, 0.063, 0.125, 0.250, 0.500, 1.000, 2.000 and 4.000 µg per well. Each level was analyzed in triplicate. The response was measured as the ratio of peak area of the signature peptide to that of the internal standard.

### 4.2.5   Evaluation of internal standards

### 4.2.5.1   Comparison of digestion profiles of hOPN and internal standards

Tryptic digestion of the rhOPN along with the SIL peptide 'GDSVVYGLR*and the extended SIL peptide 'TYDGRGDSVV*YGLRSKSKKF' was carried out for 0, 1, 2, 3, 4, 5, 7, 8, 10, 14 and 25 hours.

### 4.2.5.2   Evaluation of forced and inherent digestion variability of hOPN using SIL-IS and extended SIL-IS.

Recombinant protein was added to 50mM ammonium bicarbonate buffer to obtain 3 different concentration levels i.e. 80, 200 and 500 ng. These standards were digested in replicate (n=8) along with the two internal standards for 2, 7 and 14 hours. Digestion variability was induced artificially by using different amounts of trypsin i.e. 100, 500 and 900 ng to attain 20%, 100%

and 180% trypsin activity, respectively. Analyte/IS peak are ratio was used as response. Average response was calculated from standards having 100% trypsin activity. Inherent variability was expressed as the percent difference in the response obtained from standards with 100% trypsin activity from the average response, whereas forced variability was measured as the percent difference of the response of standards with 20% and 180% trypsin activity from the average response. Brown-Forsythe test of unequal variance was used to compare the variance. As the analysis involved 18 comparisons, a level of significance of $\alpha$=0.003, obtained after Bonferroni correction, was used.

### 4.2.6 Method Validation Studies

Validation was carried out using guidelines set forth in the May 2001 US FDA Guidance for Industry – Bioanalytical Method Validation (2001, USFDA 2001). Due to the lack of an internal standard control during the immunoaffinity isolation step, a wider accuracy and precision acceptance criteria (20/25%) typically used for ligand-binding assays was be applied to this validation. Both internal standards, the SIL-IS peptide and the extended SIL-IS peptide, were added to all analysis. SIL-IS peptide was used as the primary internal standard to assess the validity of the method. The extended SIL-IS was used to evaluate its impact on the precision of the method. Two SRM transitions for the signature peptide were included in the final method but only the response from fragment ion m/z 508.5 was used for quantitative analysis as it was found to show less interference from co-eluting peptides.

#### 4.2.6.1 Preparation of standards and quality control samples

The stock solution of rhOPN was prepared at 100 µg/mL in PBS and stored at -80°C as 50 µL aliquots in 0.5 mL Protein LoBind tubes (Eppendorf®). Calibration standards and quality control samples were prepared in surrogate matrix and plasma. Immuno-capture (IC) buffer was used as

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a surrogate matrix. Previously screened human plasma samples having low endogenous OPN levels were pooled and used for preparation of plasma calibration standards and quality control samples.  The calibration standards were prepared by adding appropriate amounts of stock solution or subsequent calibration standards to the surrogate matrix or plasma. Nominal concentrations in surrogate matrix standards were 25.0, 52.7, 79.0, 118.5, 177.8, 266.7, 400.0, 600.0 ng/mL of hOPN.  The same procedure was used for creation of quality control (QC) samples at three concentration levels, denoted as lower limit of quantification (LLOQ) QC, low quality control (LQC), medium quality control (MQC) and high quality control (HQC) having concentrations of 25.0, 101.0, 235.7, 550.0 ng/mL of hOPN, respectively. The nominal concentrations in plasma standards after adjusting for endogenous baseline concentrations were 60.8, 88.6, 114.9, 154.4, 213.6, 302.5, 435.8, 635.7 ng/mL of hOPN.  The plasma quality control (QC) samples, denoted as plasma lower limit of quantification (pLLOQ) QC, plasma low quality control (pLQC), plasma medium quality control (pMQC) and plasma high quality control (pHQC), had  concentrations of 60.8, 101.0, 235.7 and 550.0 ng/mL respectively after adjusting for endogenous baseline concentrations.

### 4.2.6.2  Sample Preparation

Biotinylated anti-hOPN monoclonal antibodies (0.5 µg/well) were immobilized on high capacity streptavidin coated 96 well plates (Thermo scientific, USA). Samples were diluted 40 fold with IC buffer and 100 µL of the diluted sample was added to each well. Immunocapture was carried out at room temperature for 4 hours using a constant vortex of 450 rpm. After 4 hours, the plates were washed 3 times with immunocapture wash buffer. Digestion was carried out by adding 100 µL of digestion solution to each well and incubating the plates at 37°C in a water bath for 14

hours. Digestion was terminated by adding 2 µL formic acid to each well. The digested samples were transferred a Protein LoBind 96 well plate and analyzed using LC-MS/MS.

### 4.2.6.3  Linearity and LLOQ

Calibration standards were extracted in duplicate and analyzed in 3 independent runs. Calibration curves were fitted using linear $1/x^2$ weighted regression of the ratio of the peak area response of the analyte and that of the internal standard versus concentration.

### 4.2.6.4  Accuracy and Precision

Accuracy and precision were determined from quality controls samples spiked in surrogate matrix and in three independent runs processed on two different days.  Intra-day assay precision and accuracy were determined from QC samples of two validation runs processed on the same day. Inter-day assay precision and accuracy were determined by analyzing three different validation runs processed on two separate days. Primary evaluation was carried out using SIL-IS. Extended SIL-IS was used only for comparison.

### 4.2.6.5  Dilution Integrity

An "over-the-curve" quality control sample, with hOPN concentration of 1035.5 ng/mL, was prepared and used as a plasma dilution quality control sample (pDI QC). The samples were analyzed with the sample preparation procedure after a two and four fold dilution in replicate. A criterion of ±20% of the nominal concentration was used to assess the dilution integrity.

### 4.2.6.6  Stability Studies

Stability studies were assessed using QC samples spiked in surrogate and pooled plasma at two levels LQC and HQC.  For post-preparative stability, the quality control samples were processed and maintained at 2-8°C for 9 days prior to analysis. Short term storage stability at -80 °C was assessed after 18 days.  Only plasma quality control samples were used to assess freeze-thaw

stability after subjecting the samples to four freeze thaw cycles. To assess bench top stability, the QC samples were thawed and left on the bench at room temperature for 24 hours. All stability samples were analyzed against a freshly spiked calibration standard curve. The stability studies were deemed acceptable if the coefficient of variation of the replicate determinations did not exceed 20.0% and the accuracy of the mean value is within ±20.0% of the theoretical value for that pool for both the QC levels.

### 4.2.6.7 Selectivity and Matrix Effect

Dilution of plasma samples in the surrogate matrix was used to avoid matrix inferences. Slopes of the calibrations curve obtained from standards prepared in surrogate matrix were compared with a calibration curve obtained from standards prepared in plasma. JMP Pro (version 10.0.2) software was used. Slopes of the calibration curves and confidence intervals were obtained using linear regression. Slopes were considered parallel if there was an overlap of the confidence intervals. Parallel slopes would indicate no matrix differences between the two matrices.

Four human plasma samples from healthy volunteers and four human plasma samples from breast cancer patients were analyzed in triplicate, both with and without fortification of hOPN at the LLOQ-level. Additionally, these samples fortified with only internal standards were also analyzed. Selectivity requirements were that any peak area co-eluting in the unfortified plasma samples at the retention time of the internal standards must be less than 5% of the average peak area of the individual internal standards. Spike recovery was assessed after subtraction of the basal concentrations and the percent deviation from nominal (%DFN) was evaluated using an acceptance criterion of ±25 %.

### 4.2.7 Evaluation of internal standards ability to account for digestion variability in the final method

Digestion variability was induced using different amounts of trypsin during digestion. Calibration standards prepared in IC buffer were processed as per the sample preparation procedure. Quality control samples at two levels (LQC, HQC) were prepared in IC buffer and processed as per the sample preparation procedure with varying amount of trypsin. The trypsin amounts used were 100 ng, 400 ng, 500 ng, 600 ng and 900 ng representing 20, 80, 100, 120 and 180 percent variability in trypsin activity, respectively. Six replicate samples were analyzed at each trypsin level. The OPN concentrations obtained with 100% trypsin activity were used to calculate average concentrations at each level. Plots of percent deviation from average concentrations (100% trypsin activity) versus percent trypsin activity were used to assess the impact of trypsin variability on precision. The variance was compared using Brown-Forsythe test.

### 4.2.8 Evaluation of plasma OPN levels in breast cancer patient and healthy individuals.

Samples from breast cancer patients (n=10) were purchased from Biochemed services and healthy individual plasma samples (n=10) were randomly chosen from the in-house inventory at Pharmaceutical Product Development, LLC (PPD), Richmond, USA. These samples were analyzed with the validated method.

## 4.3 RESULTS AND DISCUSSION

### 4.3.1 Method Development

#### 4.3.1.1 Signature Peptide Selection

A tryptic peptide '160GDSVVYGLR' was chosen as the signature peptide after the *in-silico* investigations. This amino acid sequence was unique for hOPN from a BLAST search of the

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Homo sapiens genome using UniProtKB/Swiss-Prot. Since this peptide (GDSVVYGLR) contains functional domains such as the integrin binding site of hOPN (RGDSVVYGLR) and the sites for proteolytic cleavage by MMPs and CPB-2, this tryptic peptide is a biologically relevant signature peptide of hOPN.
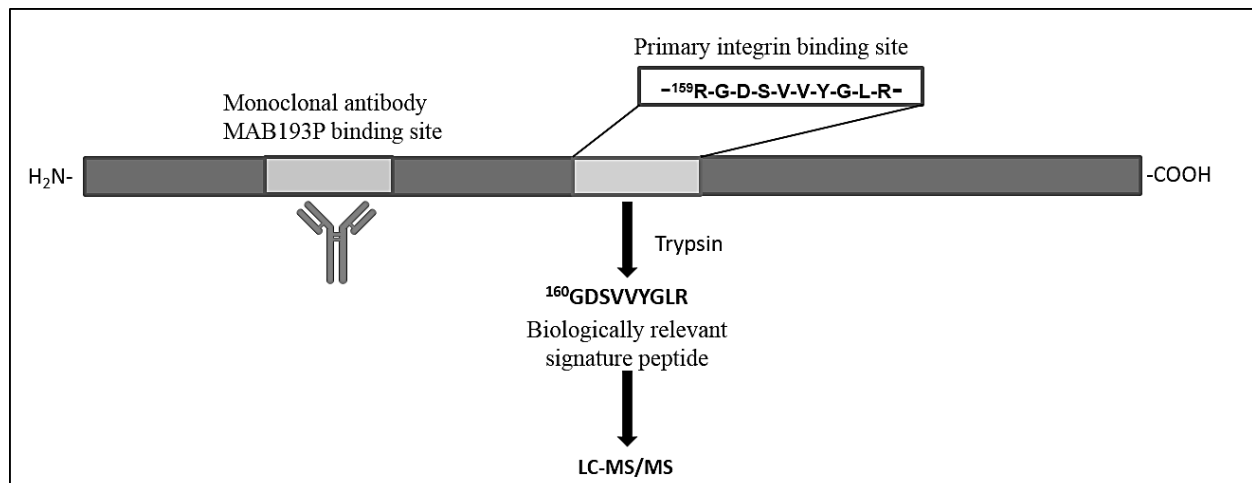


*Figure 4-3: Schematic of the method procedure.*

Additionally, this peptide did not show any post-translational phosphorylation, thus avoided an additional dephosphorylation step during sample processing. The schematic representation of method procedure is show in Figure 4-3.

### 4.3.1.2 Conventional Flow LC-MS/MS Method

Synthetically manufactured signature peptide (GDSVVYGLR) was used for development of LC-MS/MS conditions. The mass spectra showed that a peak corresponding to the +2 charge state of the peptide was the most intense and was used as the precursor ion. The selected precursor ion was fragmented and the two most intense fragment ions (y4 and y5) were chosen for the method (see Table 4-1). The signal responses obtained from both SRMs of the signature peptide were summed in the final quantitation method to achieve lower detection limits.

Column trapping to allow online sample enrichment prior to separation on the analytical column was used. After initial digestion studies, the gradient conditions of the method were further modified by varying the gradient steepness to resolve a digestion by-product (GDSVVY) peak from the signature peptide peak. Evaluation of hOPN calibration standards, prepared in digestion buffer, showed that the calibration curve was linear over the range of 100 – 1000 pg/mL with a mean correlation coefficient of 0.9972. A weighting factor of 1/x was used. No internal standard was used during analysis on this system.

### 4.3.1.3 Digestion Optimization

Initial digestion studies showed that the time profile plot of the signature peptide concentration was biphasic indicating that the signature peptide obtained on digestion of hOPN was unstable (Figure 4-4). Such trends have been observed in other studies involving tryptic digestions (Agger, *et al.* 2010, Brownridge, *et al.* 2011a, Proc, *et al.* 2010, van den Broek, *et al.* 2013b). To

investigate the decrease in peptide concentration over time, the synthetic signature peptide was incubated with and without trypsin. A decrease in peptide concentration over time was seen which was not seen with controls that did not contain trypsin. The results indicated that the signature peptide was being degraded in the presence of trypsin. To determine possible degradation products, the signature peptide was incubated with trypsin and the digests were analyzed using LC-MS/MS in the precursor ion scan mode. The probable masses of cleaved products of the signature peptide were obtained using Protein Prospector software and the chromatograms were scanned for the presence of these cleaved products. The chromatograms showed a peak with mass of 693.4 Da corresponding to the cleaved product 'GDSVVY'. The authenticity of the peak was verified by conducting a product ion scan. As the cleavage took place on the carboxy terminal end of tyrosine, it indicated an enzymatic activity similar to chymotryptic digestion. Trypsin is known to undergo autolysis and may show a chymotrypsin-like activity(Tryspin gold mass spectrometry grade technical bulletin Part # TB309 Revised 03/13). It was speculated that cleavage of the signature peptide was a result of trypsin autolytic activity. A SRM method was developed to measure the cleaved product (see Table 4-1). In addition, the chromatographic gradient was modified to resolve the peaks of the signature peptide and cleaved product.
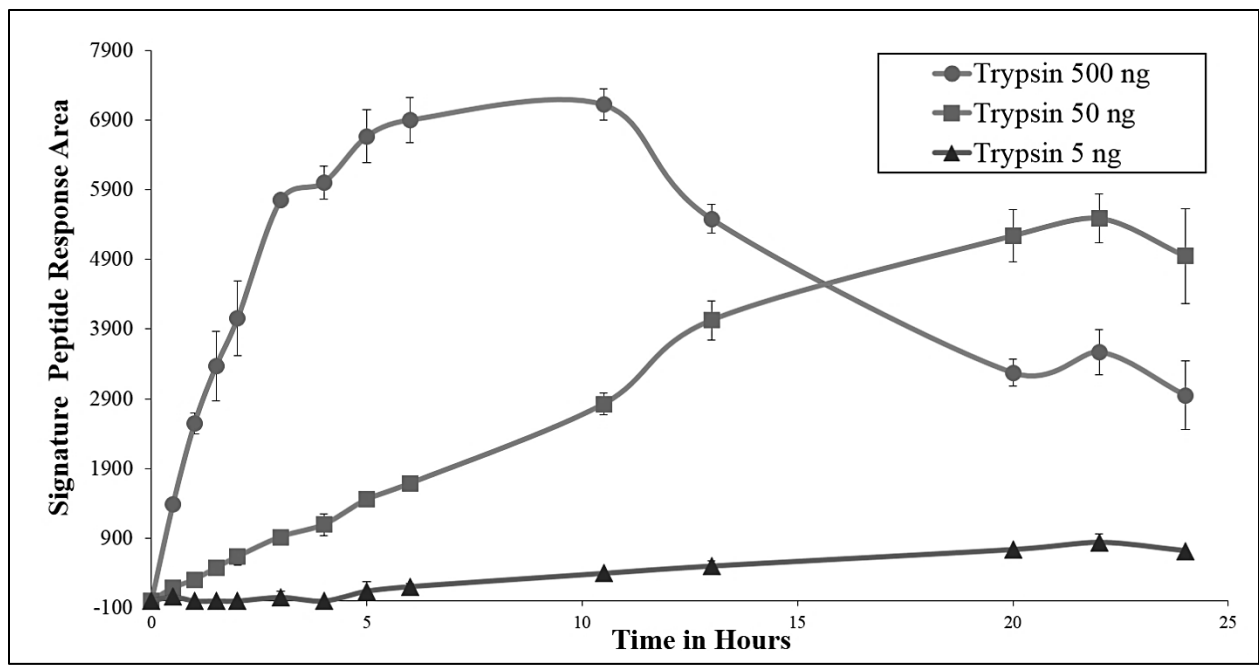
*Figure 4-4: Digestion profile of hOPN with varying amount of tryspin.*

Digestion studies with different amount of trypsin showed that the highest signature peptide yields were obtained between 5-10 hours using 500 ng of trypsin per sample (see Figure 4-4). The digestion efficiency was found to be around 35-40 %.

#### 4.3.1.4 Immunocapture method optimization

##### *4.3.1.4.1 Order of addition*

The signature peptide yields using the Strategy I, II and II are given in Figure 4-5. Adjusted signature peptide concentrations were obtained after subtracting the average blank plasma concentrations.

From Figure 4-5, it can be seen that the highest signature peptide yield was obtained when the samples were processed using Strategy I i.e. when immobilization of the antibody on a solid support is carried out prior to the immunocapture reaction. A probable reason could be that the immobilization of the "naked" capture antibody is more efficient compared to that of the preformed antigen-antibody complex due to steric hindrance. Also, no significant difference in signature peptide yield was seen between immunocapture incubation carried out for 4 hours and 20 hours for Strategy I. Hence, Strategy I with 4 hour immunocapture incubation was used for further studies. In the final method, the immobilization time was changed from 4 hours to 2 hours.
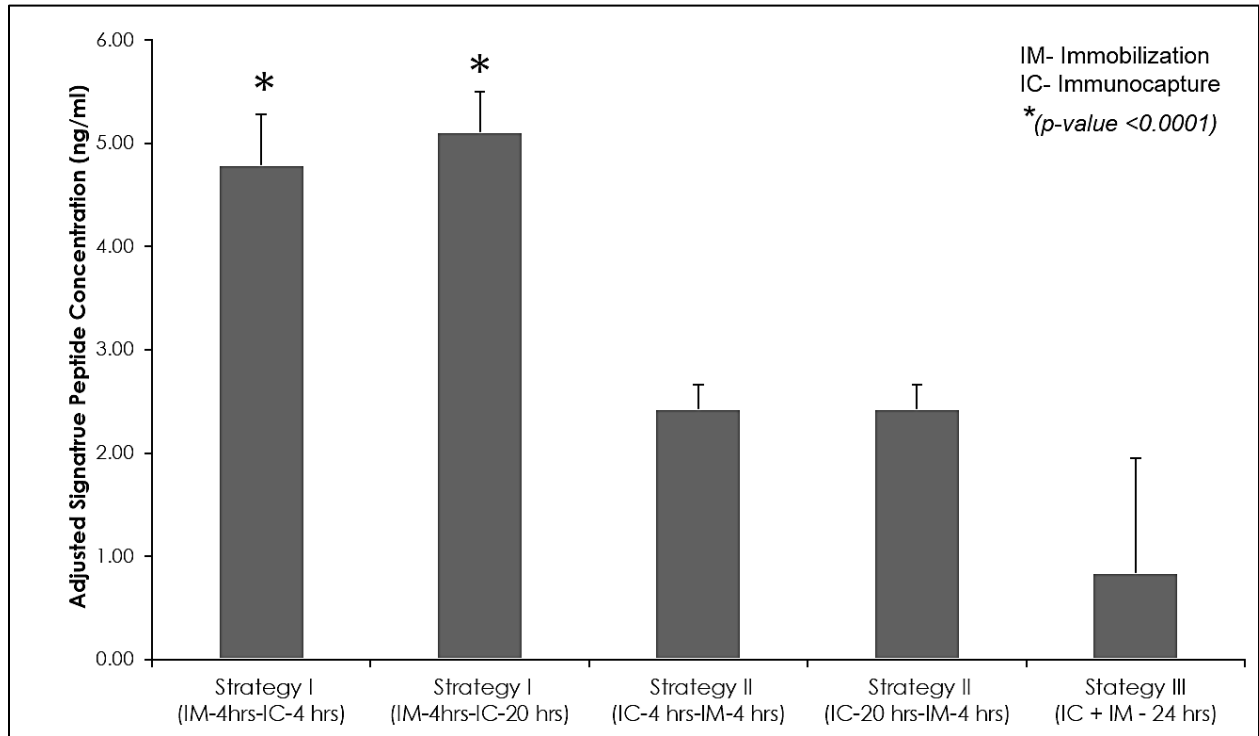
*Figure 4-5: Immunocapture optimization studies (Order of addition).*

### 4.3.1.4.2 Optimization of the amount of Antibody

The result of antibody amount optimization studies is given in Figure 4-6. A 'hook-effect' was seen due to excess coating of monoclonal antibody on the streptavidin coated plates. An assumption is that overcrowding of the immobilized antibodies results in steric hindrance, thus, negatively affecting the immuno-complex formation. When more than 0.5 µg of antibody was used per well, a decline in response was seen followed by response saturation with an increasing amount of antibody. The final method used 0.5 µg of antibody per well, since the highest response was obtained at this concentration.

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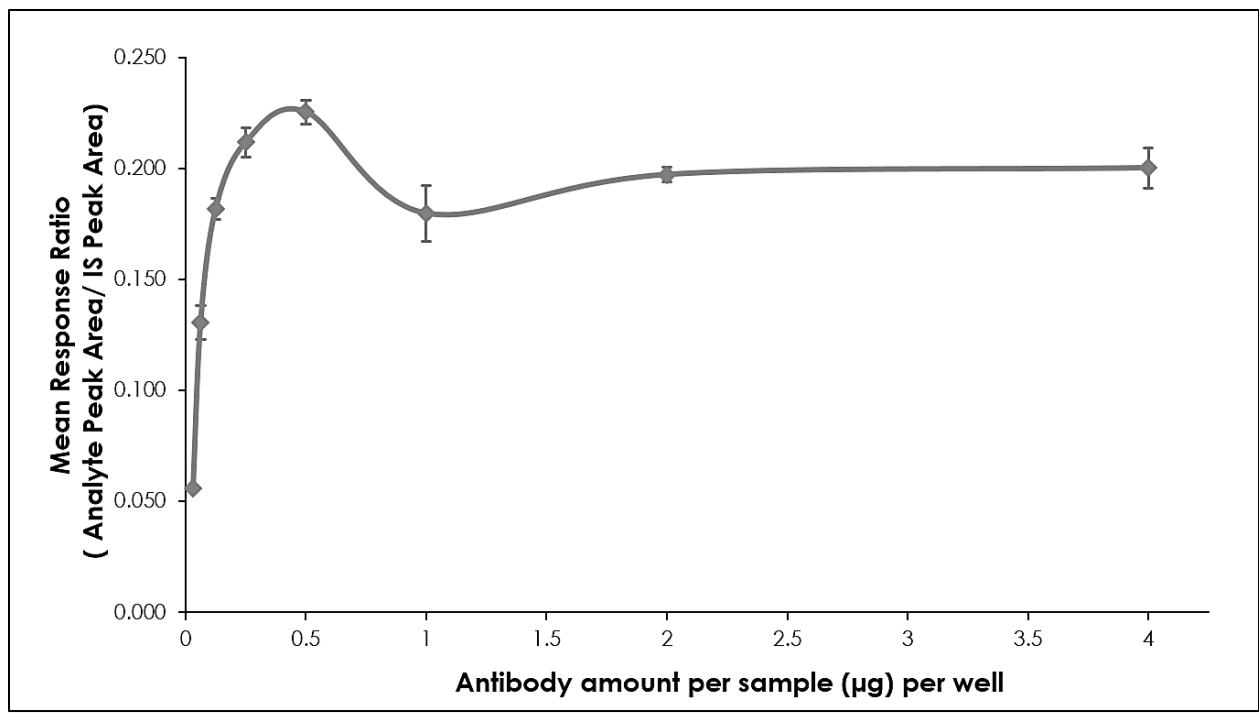
*Figure 4-6: Immunocapture optimization studies (Antibody amount).*

### 4.3.1.5   Evaluation of internal standards

A peptide 'GDSVVYGLR*' having the same sequence as the signature peptide and containing an arginine labeled with $^{13}C_6$ and $^{15}N_4$ was used as the SIL-IS peptide. Another peptide 'TYDGRGDSVV*YGLRSKSKKF' having the same sequence as the signature peptide along with additional amino acid chains flanking both terminal ends and containing a valine labeled with $^{13}C_5$ and $^{15}N$ was used as the extended SIL-IS peptide. The amino acid chains flanking the terminals were chosen from the hOPN sequence.  As hypothesized, comparison of the tryptic digestion time profiles showed that the extended SIL-IS peptide better mimicked hOPN digestion than the SIL-IS peptide (See Figure 4-7).

The extended SIL peptide and hOPN digestion profiles can be divided into three phases i.e. the 'formation phase', the 'transition phase' and the 'degradation phase'. The formation phase represent the incubation period between 0-5 hours which is dominant in the formation of signature peptide or SIL signature peptide from the digestion of hOPN and extended SIL peptide, respectively. The transition phase represent the incubation period between 5-10 hours where signature peptide formation and degradation processes occur at similar rates. The degradation phase represents the incubation period beyond 10 hours in which degradation of the signature peptide is the most dominant phenomenon. In the case of the SIL peptide, only degradation is occurring.
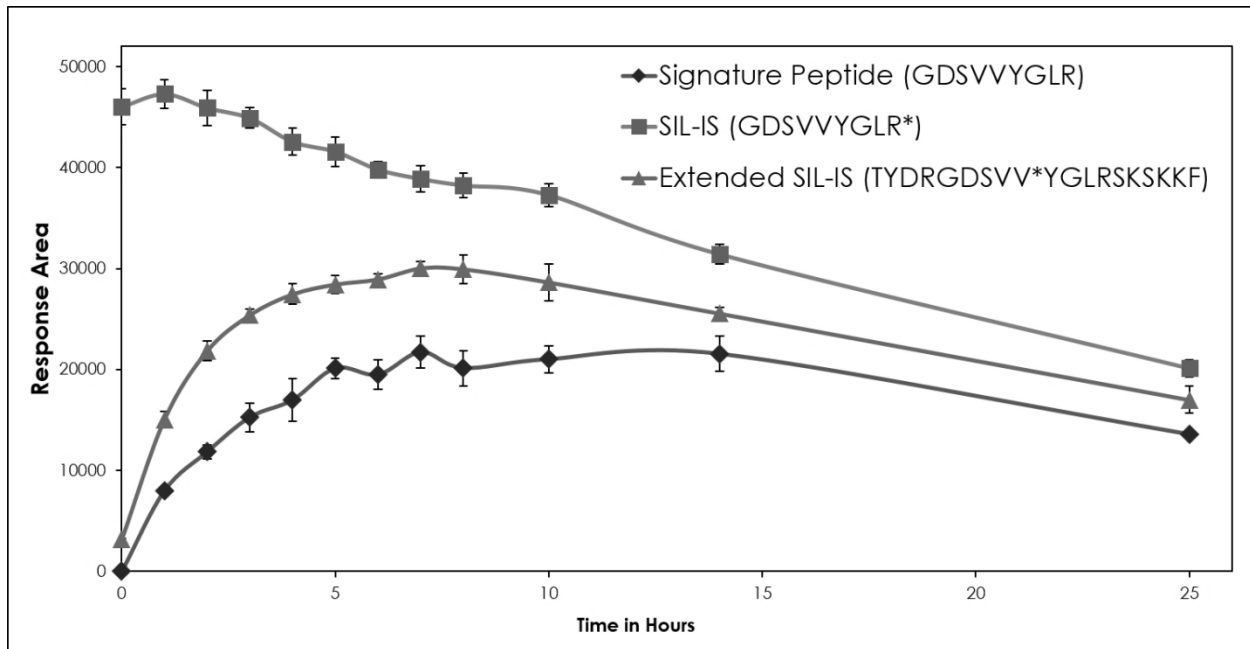
*Figure 4-7: Comparison of digestion profiles of hOPN and the two internal standards.*

Variability during trypsin digestion can occur due number of reasons including interfering substances in complex biological matrix, lot-to-lot variation in trypsin stock solution and inadequate removal of surfactants or chaotropic agents from prior sample processing steps. This variability can be tracked by adding an appropriate internal standard (Bronsema, *et al.* 2013, Brownridge, *et al.* 2011a, Proc, *et al.* 2010, van den Broek, *et al.* 2013a). In order to assess the ability of the two internal standards to account for digestion variability, digestion was carried out in replicate (n=8) at three different OPN concentration levels and three different amounts of trypsin (100ng, 500 ng and 900 ng). Digestion was stopped at three time intervals (2, 7 and 14 hours) representing the 'formation phase', 'the transition phase' and 'the degradation phase'. The average response of samples digested with 500 ng trypsin was used to calculate the variability which was expressed as the percent difference from the average. The term 'inherent digestion variability' was used to represent variability at 100 % trypsin activity i.e. using 500 ng of trypsin per sample, while 'forced digestion variability' was used to represent variability seen at 20 and 180 % trypsin activity.

The inherent and forced digestion variability, across the three phases with the two internal standards, is shown in Figure 4-8 and 4-9, respectively. In these figures, the QC levels 01, 02, 03 represent the 80, 200 and 500 ng/ml of OPN concentrations, respectively. The whiskers of the box plots seen in these figures represent range and is a measure of the variability seen in the response. Brown Forsythe test of unequal variance was used to compare the standard deviations of the percent deviation from average in the individual groups. The statistical results of this test are available in supporting information (Table 4-3). The standard deviation of the percent deviation from average can be used as measure of precision i.e. lesser standard deviation values would represent higher precision and vice-versa.

From Figure 4-8, one can observe that the inherent digestion variability decreased with increase in digestion time when SIL-IS was used. For results analyzed with extended SIL-IS, the precision was least at 7 hours and highest at 14 hours. The high variability at 7 hours could be attributed to the transition phase wherein there is simultaneous occurrence of signature peptide formation and degradation. Highest precision was seen at 14 hours primarily because at this time interval only a single degradation process is dominant. It was seen that there was no significant difference between the responses obtained using the two internal standards (see Table 4-3). Thus we can conclude that the use of either internal standard does not have an impact on the inherent variability. Also, except for QC_02 samples at 2 hours, all the box plot in Figure 4-8 had their box plot whiskers within $\pm$ 20%. This indicates good precision using both internal standards.

From Figure 4-9, it can be observed that the forced digestion variability decreased with increasing digestion time when SIL-IS was used. However, when extended SIL-IS was used the variability at 7 hours was higher than 2 and 14 hours. The least variability was seen at 14 hours. This trend is similar to the inherent trend observed for inherent digestion variability. At each time interval, the variability decreased with increasing OPN concentration. This was more prominent for the responses analyzed using SIL-IS. Also, the variability in the responses analyzed using SIL-IS was significantly higher (see Table 4-3) in comparison to the response analyzed using extended SIL-IS, except for QC_03 samples at 7 and 14 hours. It can be seen that the data points analyzed using SIL-IS are more focused near the interquartile range instead of the median.
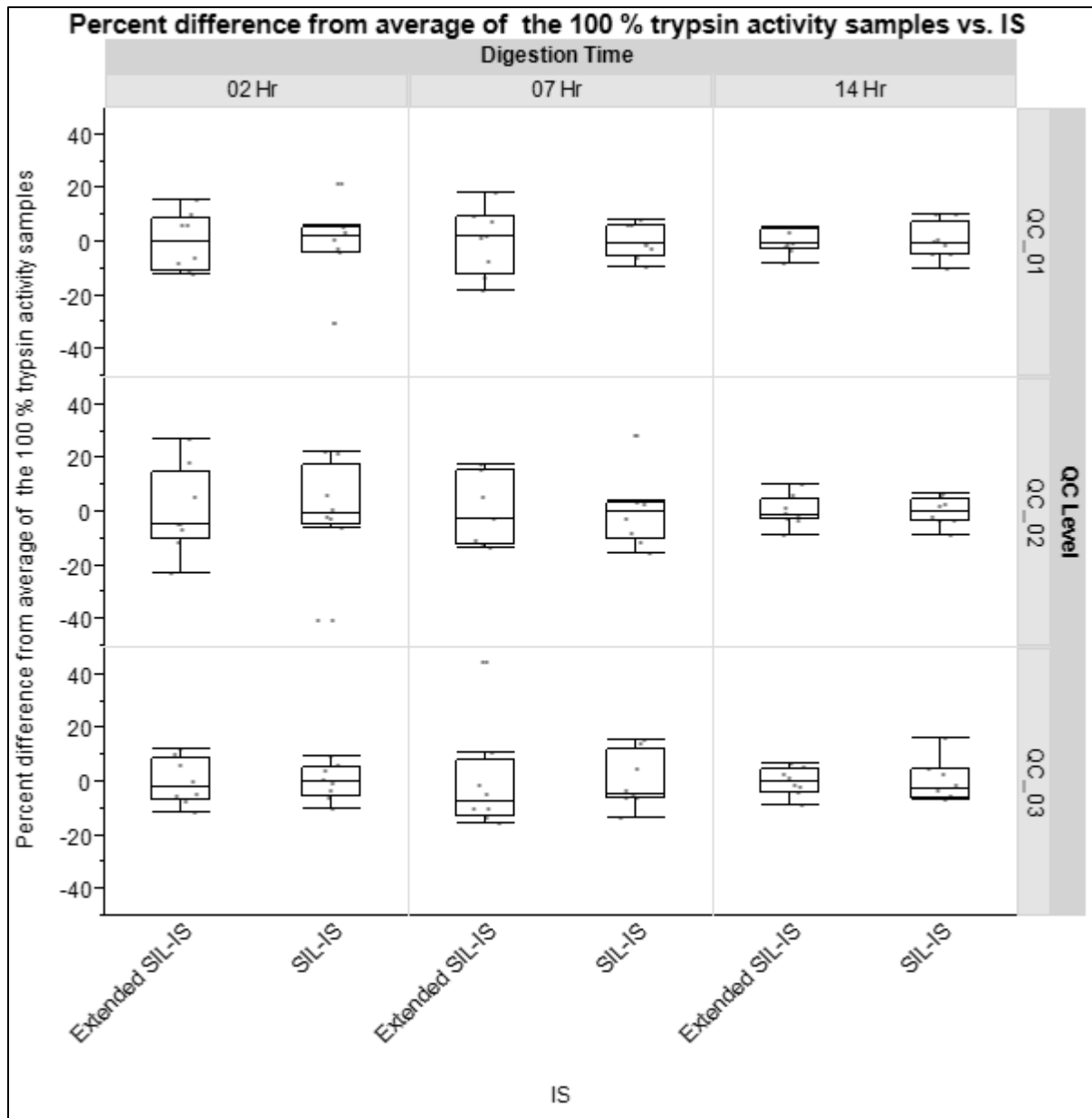
*Figure 4-8: Evaluation of internal standards ability to account for digestion variability*

*(inherent).*
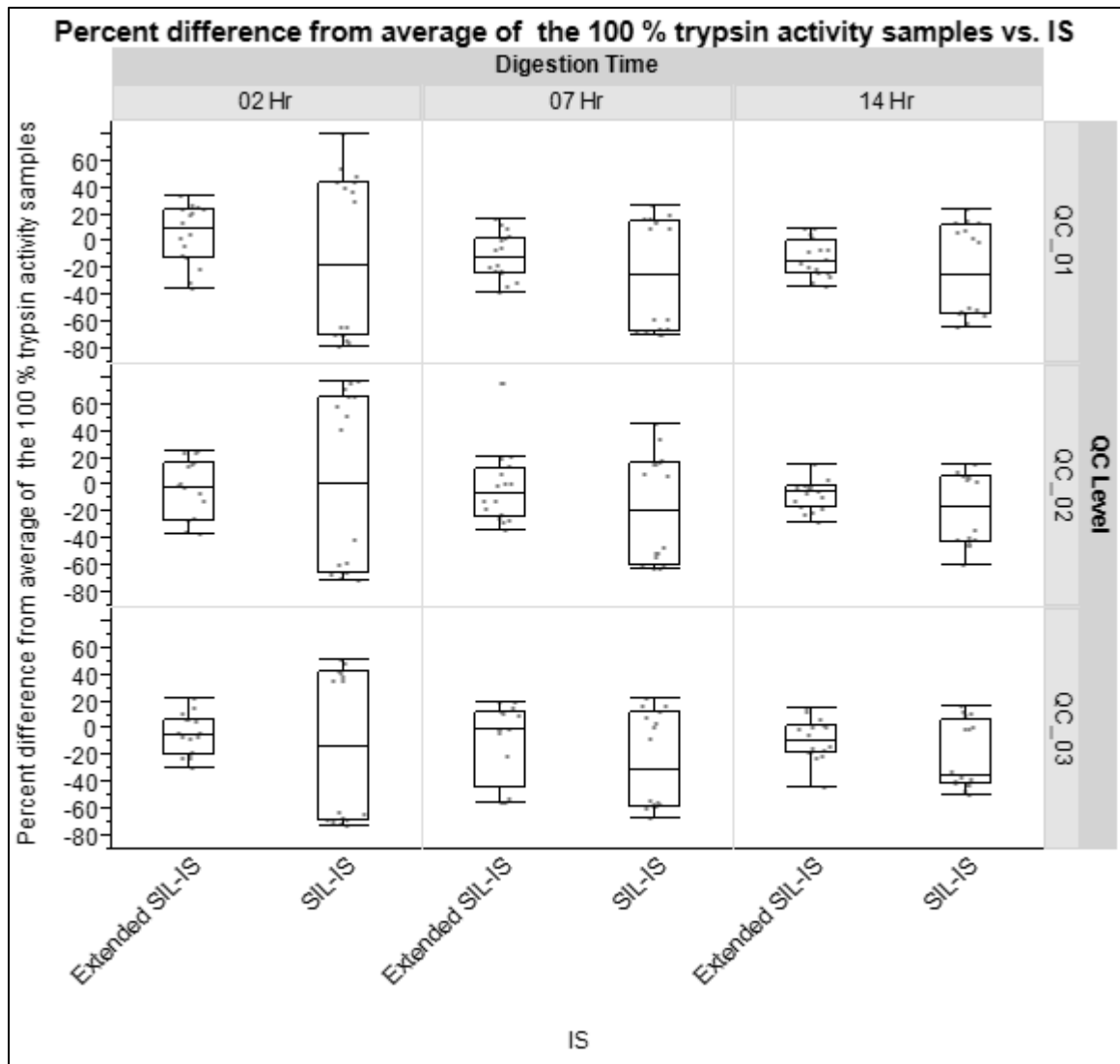
*Figure 4-9: Evaluation of internal standards ability to account for digestion variability*

*(forced).*

In contrast, the responses analyzed using extended SIL-IS are more focused around the median indicating that the trypsin activity variation had a smaller impact on the final response. Also, it can be seen that there is a significant difference in the precision at all-time points except QC-03 samples at 7 and 14 hours (see Table 4-3). Response obtained using extended SIL-IS showed higher precision in comparison to SIL-IS. Thus we can conclude that the extended SIL peptide would be a better internal standard when a digestion variability between samples is expected.

*Table 4-3: Results of digestion variability studies using different internals standards*

| Variability Type | Time (Hours) | QC Level | Extended SIL-IS (Std Dev) | SIL-IS (Std Dev) | Brown-Forsythe Test (F Ratio) | (p-value) |
|---|---|---|---|---|---|---|
| Inherent | 2 | 1 | 10.68 | 14.67 | 0.0040 | 0.9502 |
| Inherent | 2 | 2 | 16.29 | 19.62 | 0.0314 | 0.8618 |
| Inherent | 2 | 3 | 8.57 | 6.51 | 0.9208 | 0.3536 |
| Inherent | 7 | 1 | 12.31 | 6.34 | 2.4068 | 0.1431 |
| Inherent | 7 | 2 | 13.22 | 13.51 | 0.4010 | 0.5376 |
| Inherent | 7 | 3 | 19.86 | 10.52 | 0.4795 | 0.5 |
| Inherent | 14 | 1 | 4.65 | 7.12 | 0.8653 | 0.368 |
| Inherent | 14 | 2 | 5.81 | 5.22 | 0.0018 | 0.9665 |
| Inherent | 14 | 3 | 5.10 | 7.77 | 0.5267 | 0.48 |
| Forced | 2 | 1 | 21.91 | 61.61 | 87.3208 | <.0001* |
| Forced | 2 | 2 | 22.65 | 65.71 | 119.4768 | <.0001* |
| Forced | 2 | 3 | 14.81 | 56.98 | 324.2888 | <.0001* |
| Forced | 7 | 1 | 17.30 | 42.16 | 120.9018 | <.0001* |
| Forced | 7 | 2 | 27.30 | 40.62 | 12.0396 | 0.0016* |
| Forced | 7 | 3 | 28.96 | 35.46 | 4.5444 | 0.0413 |
| Forced | 14 | 1 | 14.16 | 34.50 | 65.2119 | <.0001* |
| Forced | 14 | 2 | 11.22 | 26.49 | 46.4727 | <.0001* |
| Forced | 14 | 3 | 15.06 | 25.24 | 3.6225 | 0.0666 |

\* *p-value* < Level of significance α =0.003

### 4.3.2 Method Transfer

The initial method used conventional flow HPLC coupled to a Sciex API 4000 Qtrap mass spectrometer. This method had a lower limit of quantification of 100 pg/ml for the signature peptide prepared in digestion buffer. The detectability limit of the signature peptide and the low overall sample processing efficiency of the method (17%) restricted the method range to a lower limit of 5 ng/ml of hOPN using 500 µL of sample. Biomarker investigations usually have limited sample availability. This was the primary reason for method transfer to the MFLC-MS/MS system. In addition, we wanted to evaluate the microflow LC system for regulated quantification of large molecules. The microflow LC system had a 1226 times higher signal-to-noise ratio as compared to the conventional flow LC system. This allowed us to lower the sample volumes to 20 uL while achieving similar detection limits. The conventional flow method used streptavidin coated magnetic beads for immunocapture which were necessary due to the high sample volume. The low sample volume of the new method allowed the immunocapture to be performed on more convenient high binding capacity streptavidin coated plates. In addition, the lower detection limits of the microflow LC system allowed for further sample dilution to avoid matrix interferences. The use of low flow rates also resulted in a 100-fold reduction in solvent consumption.

### 4.3.3 Method Validation Studies

A prime concern during method development was establishment of the method validation range. As discussed earlier, there is ambiguity in reported absolute concentrations of hOPN in biologically samples, primarily due to the differences between individual ELISA kits. For this reason, eights lots of healthy individual plasma lots were randomly chosen from the in-house inventory at PPD and screened to establish baseline plasma OPN levels. An average

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concentration of 49±17 ng/mL (mean + SD) was obtained from the screened samples. A 4-10 fold increase in OPN levels has been observed in diseased populations relative to healthy individuals from previous studies involving immunoassays(Rodrigues, *et al.* 2007). Hence, a concentration range of 25-600 ng/mL, which includes mean baseline concentration and a 10-fold increase in baseline concentrations, was chosen for the validation studies.

### 4.3.3.1 Selectivity and Matrix Effect

A common challenge in biomarker analysis is to select an appropriate matrix in which to prepare the standard curve. Ideally, this should be free of target analytes and identical to the sample matrix. To avoid matrix effects between individual lots, each sample was diluted 40-fold prior to immunocapture. This high dilution allowed use of the IC buffer as a surrogate matrix.

From Figure 4-10, it can be seen that the calibrations curves prepared using plasma standards and surrogate matrix standards are parallel. The plasma concentrations were not adjusted to account for baseline levels. The slope of the surrogate matrix i.e. IC buffer curve was 0.00073 (95% Lower confidence interval = 0.0067 and 95% Upper confidence interval = 0.00078) while the slope of the plasma curve was 0.00074 (95% Lower confidence interval = 0.0066 and 95% Upper confidence interval = 0.00081). The overall of 95% confidence interval of both slopes indicates that the slopes are not significantly different. Hence, we can conclude that any matrix difference that may exist would not preclude selective quantification provided the samples are diluted 40-fold prior to immunocapture.

No interfering peak was present at the retention time of an internal standard.   Spike recovery ranged from -13.1 to 19.2% in all the fortified samples after adjusting for the baseline levels. Representative chromatograms of the blank samples prepared in buffer and plasma are given in

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Figure 4-11 and 4-12, respectively. Representative chromatograms of LLOQ standard prepared in buffer and plasma are given in Figure 4-13 and 4-14, respectively.
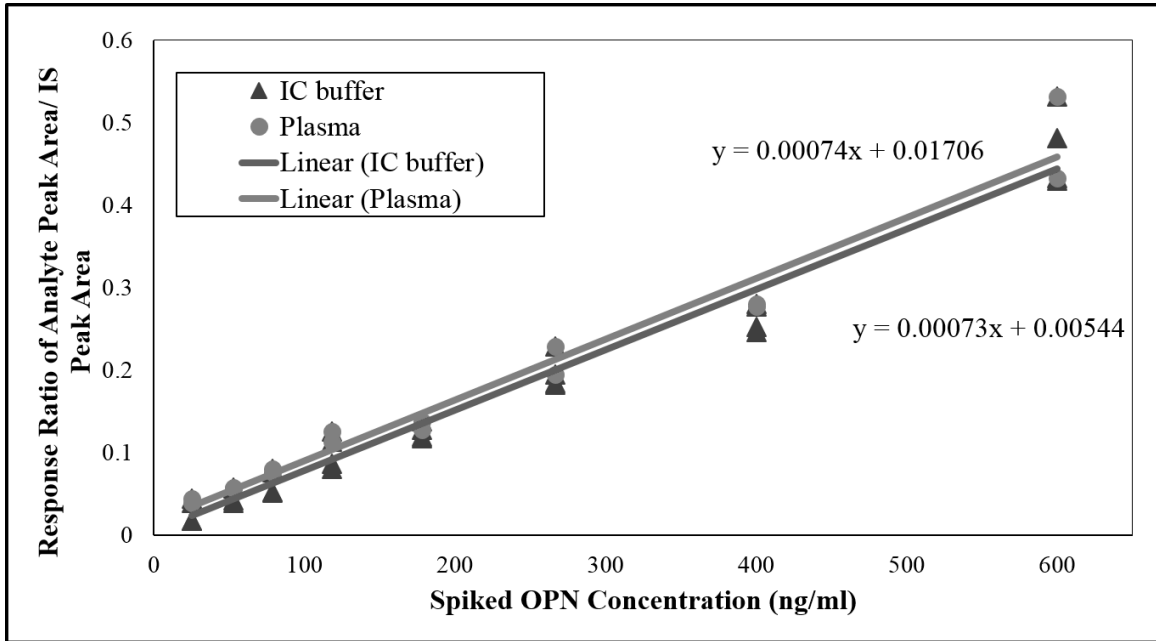


*Figure 4-10 : Parallelism experiment to demonstrate the suitability of the surrogate matrix i.e.*
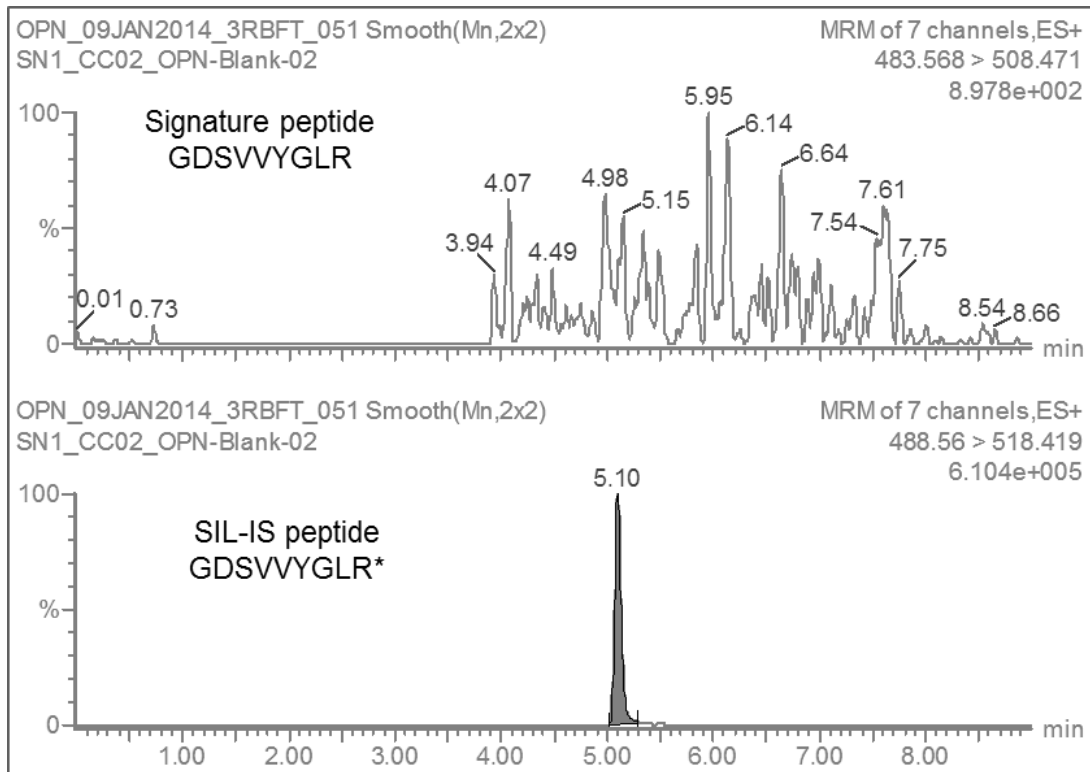
*IC buffer.*

*Figure 4-11: Representative chromatogram of blank (IC buffer) with SIL-IS.*
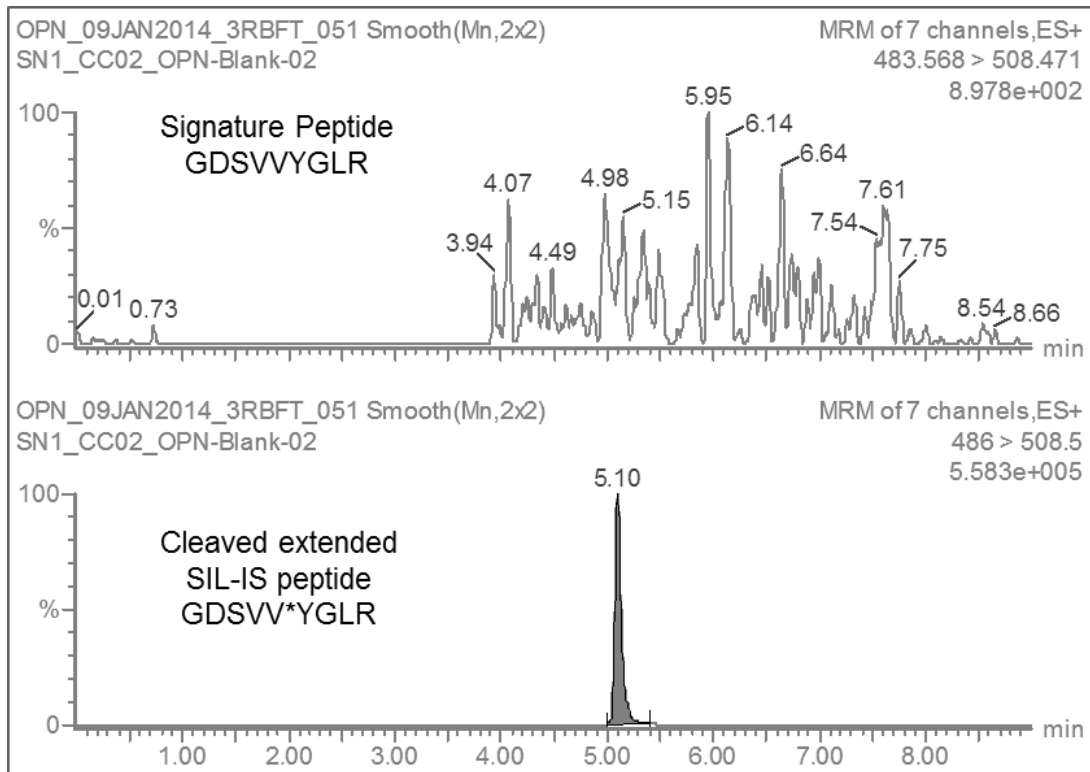
*Figure 4-12: Representative chromatogram of blank (IC buffer) with extended SIL-IS.*
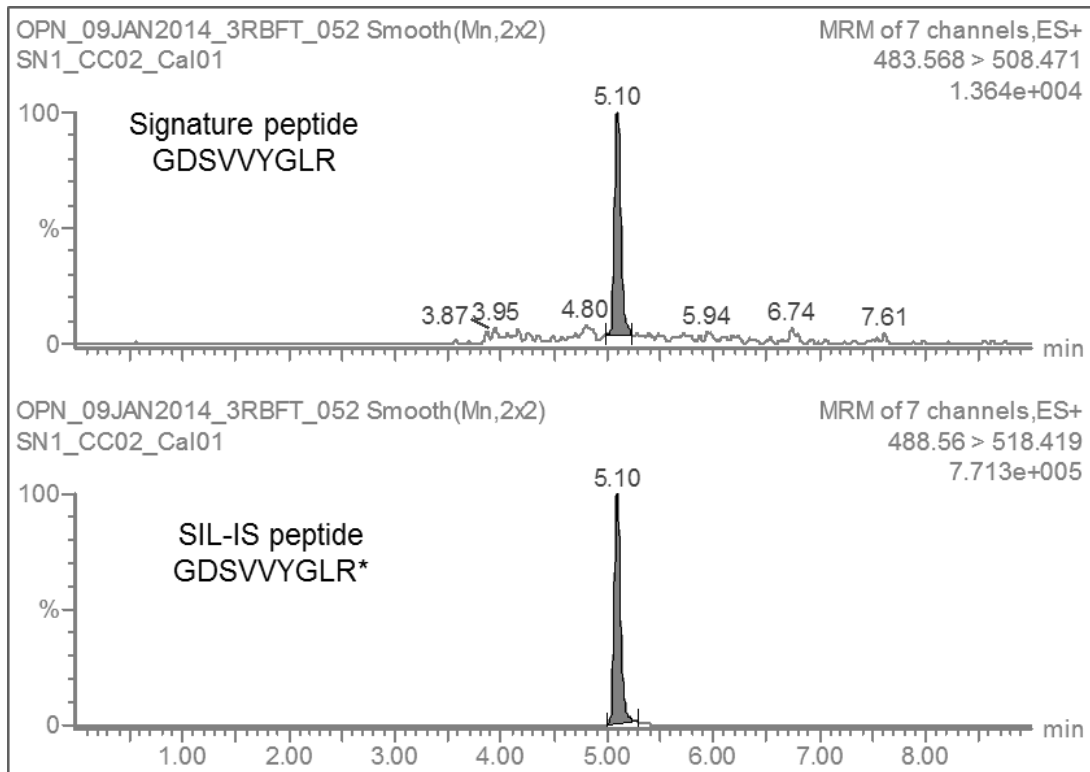
*Figure 4-13: Representative chromatogram of LLOQ standard (IC buffer).*
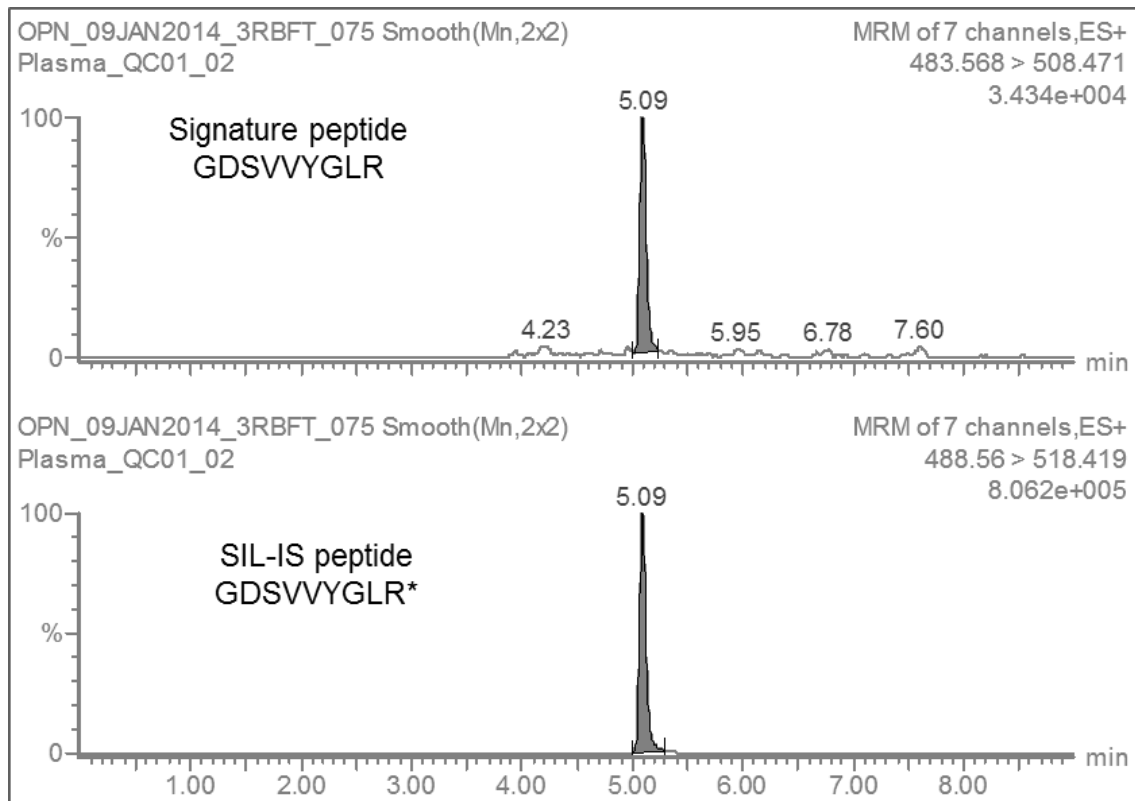
*Figure 4-14: Representative chromatogram of LLOQ QC (plasma).*

### 4.3.3.2 Linearity

The standard curves demonstrated a linear response over the concentration range of 25-600 ng/mL. The calibration curves yielded a mean (n=3) correlation coefficient of 0.9967. The percent deviation from nominal (%DFN) for the mean back-calculated values of the calibration standards were between -2.16% and 3.69%. Precision of the calibration standards measured as the percent relative standard deviation for the mean back-calculated values ranged from 2.98% to 9.59%.

### 4.3.3.3 Accuracy and Precision

The limit of quantification (LLOQ) was established at 25 ng/mL and demonstrated a precision of 10.7 % and 5.93 % in surrogate matrix and in plasma, respectively. The LLOQ demonstrated accuracy of -12.0 % and -1.21 % in surrogate matrix and in plasma, respectively. Intra-assay results of accuracy and precision for quality control samples using SIL-IS and extended SIL-IS are shown in Table 4-4 and 4-5. Inter-assay results of accuracy and precision for quality control samples using SIL-IS and extended SIL-IS are shown in Table 4-6 and 4-7. One LQC sample was excluded from statistically analysis citing sample processing error as demonstrated by low internal standard peak response.   Intra-assay and inter-assay precision with both internal standards was within 15%. No substantial advantage was seen in accuracy or precision with the use of extended peptide. Dilution integrity was established by analyzing 'over-the-curve' QC after a 2-fold and 4–fold dilution. The back calculated concentrations after analyzing DI QC samples post 2-fold and 4-fold dilutions yielded an accuracy of -11.83% and -7.60%, respectively and a precision of 6.97% and 5.04%, respectively.

#### *Table 4-4: Intra-day accuracy and precision (SIL-IS)*

| Matrix | IC Buffer | | | Plasma | | |
|---|---|---|---|---|---|---|
| | LQC | MQC | HQC | LQC | MQC | HQC |
| Nominal concentration (ng/ml) | 101.0 | 236.0 | 550.0 | 137.0 | 271.0 | 584.0 |
| Mean calculated concentration (ng/ml) | 99.3 | 230.0 | 568.7 | 127.8 | 253.8 | 540.3 |
| Standard deviation | 7.52 | 8.02 | 38.28 | 10.25 | 22.27 | 25.65 |
| Percent coefficient of variation | 7.57 | 3.49 | 6.73 | 8.02 | 8.77 | 4.75 |
| Percent deviation from nominal concentration | -1.67 | -2.54 | 3.39 | -6.70 | -6.33 | -7.49 |

## Table 4-5: Intra-day accuracy and precision (extended SIL-IS)

| Matrix | IC Buffer | | | Plasma | | |
|---|---|---|---|---|---|---|
| | LQC | MQC | HQC | LQC | MQC | HQC |
| Nominal concentration (ng/ml) | 101.0 | 236.0 | 550.0 | 137.0 | 271.0 | 584.0 |
| Mean calculated concentration (ng/ml) | 99.7 | 222.3 | 569.3 | 130.8 | 249.9 | 560.8 |
| Standard deviation | 9.03 | 8.43 | 35.90 | 10.88 | 16.16 | 25.22 |
| Percent coefficient of variation | 9.06 | 3.79 | 6.31 | 8.32 | 6.47 | 4.50 |
| Percent deviation from nominal concentration | -1.29 | -5.83 | 3.52 | -4.51 | -7.78 | -3.97 |

*Table 4-6: Inter-day accuracy and precision (SIL-IS)*

| Matrix | IC Buffer | | | Plasma | | |
|---|---|---|---|---|---|---|
| | LQC | MQC | HQC | LQC | MQC | HQC |
| Nominal concentration (ng/ml) | 101.0 | 236.0 | 550.0 | 137.0 | 271.0 | 584.0 |
| Mean calculated concentration (ng/ml) | 101.0 | 235.0 | 574.0 | 123.0 | 254.0 | 524.0 |
| Standard deviation | 6.78 | 12.8 | 33.9 | 22 | 23.2 | 42.9 |
| Percent coefficient of variation | 6.75 | 5.45 | 5.9 | 17.9 | 9.13 | 8.19 |
| Percent deviation from nominal concentration | -0.47 | -0.31 | 4.43 | -10.00 | -6.28 | -10.30 |

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*Table 4-7: Inter-day accuracy and precision (extended SIL-IS).*

| Matrix | IC Buffer | | | Plasma | | |
|---|---|---|---|---|---|---|
| | LQC | MQC | HQC | LQC | MQC | HQC |
| Nominal concentration (ng/ml) | 101.0 | 236.0 | 550.0 | 137.0 | 271.0 | 584.0 |
| Mean calculated concentration (ng/ml) | 103.0 | 234.0 | 580.0 | 130.2 | 254.0 | 542.0 |
| Standard deviation | 8.98 | 21.2 | 38 | 10.50 | 21.4 | 41.4 |
| Percent coefficient of variation | 8.72 | 9.06 | 6.56 | 8.06 | 8.43 | 7.65 |
| Percent deviation from nominal concentration | 1.92 | -0.57 | 5.43 | -4.94 | -6.38 | -7.25 |

#### 4.3.3.4 Stability Studies

The stock solution was stored at -80°C after reconstitution in phosphate buffered saline and it was stated to be stable for 3 months in those storage conditions as per supplier instructions. The plasma QC samples were found to be stable after 4-freeze thaw cycles. Short term stability was assessed for 18 days at -80°C and was found to be within specifications both matrices i.e. IC buffer and plasma. Samples thawed on the bench were found to be stable for 24 hours both matrices. Extracted samples from both matrices were found to be stable when stored at stored at 2-8°C for 9 days. The stability studies results are given in Table 4-8.

*Table 4-8: Results of Stability studies.*

| Study | Matrix | Level | N | % DFN | %CV |
|---|---|---|---|---|---|
| Freeze thaw Stability (4 cycles) | Plasma | LQC | 6 | 19.08 | 4.13 |
| | | HQC | 6 | 9.45 | 4.12 |
| Short Term Storage Stability (-80 °C) (18 days) | IC Buffer | LQC | 6 | 15.78 | 3.18 |
| | | HQC | 6 | 18.08 | 5.07 |
| | Plasma | LQC | 6 | 17.34 | 3.01 |
| | | HQC | 6 | 6.62 | 3.42 |
| Thawed Matrix Stability (24 Hours) | IC Buffer | LQC | 6 | 16.00 | 3.60 |
| | | HQC | 6 | 10.53 | 4.56 |
| | Plasma | LQC | 6 | 14.02 | 4.34 |
| | | HQC | 6 | 9.21 | 2.13 |
| Extracted Sample Stability (2-8 °C)      (9 Days) | IC Buffer | LQC | 6 | -0.49 | 3.60 |
| | | HQC | 6 | 5.84 | 2.55 |
| | Plasma | LQC | 5 | -2.38 | 2.54 |
| | | HQC | 6 | -9.78 | 6.09 |

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### 4.3.3.5 Evaluation of internal standards ability to account for digestion variability in the final method

Ideally an internal standard's ability to account for variability is measured in controlled settings. As seen earlier precision and accuracy studies showed that there was no significant difference in precision or accuracy when either internal standard peptide was used. However, from initial experiments, we had seen that the extended SIL-IS peptide should be a better internal standard to account for variability in trypsin activity. The impact of trypsin variability on precision was assessed from the slopes of plots of percent deviation from the average assayed concentration (at 100% trypsin activity) versus percent trypsin activity. A lower value for the slope would indicate that there is better precision and that the trypsin variability is lessened by the presence of the internal standard.

| IS | QC Level | Slope of fitted line |
|---|---|---|
| Extended SIL peptide | LQC | 0.031 |
| Extended SIL peptide | HQC | 0.136 |
| SIL peptide | LQC | 0.418 |
| SIL peptide | HQC | 0.567 |
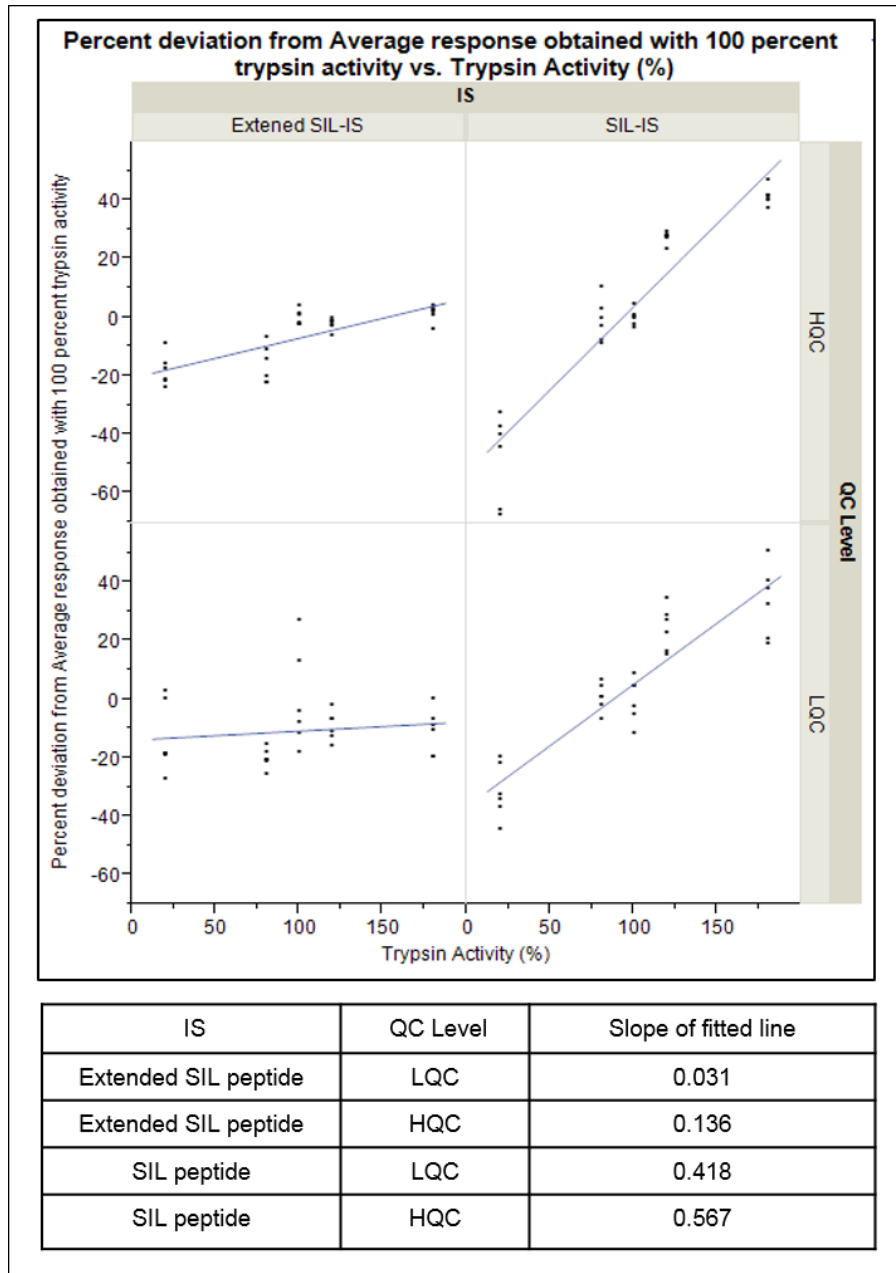
*Figure 4-15 : Plots of the percent deviation from average concentrations (100% trypsin activity) versus percent trypsin activity.*

From Figure 4-15, it can be seen that use of the SIL peptide as an internal standard resulted in slopes of 0.418 and 0.567, obtained for LQC and HQC s, respectively. Alternatively, when the extended SIL peptide was used as the internal standard, plots were obtained with slopes of 0.031 and 0.136 for LQC and HQC s, respectively. This suggests that during analysis of unknown samples, if a particular sample has diminished or enhanced trypsin activity, the impact of this on results would be less when using an extended SIL peptide as an internal standard.

### 4.3.4 Evaluation of plasma OPN levels in breast cancer patient and healthy individuals.

An average OPN concentration of $55.4\pm15.3$ ng/mL was determined in the plasma samples of healthy volunteers (see Figure 4-16). Plasma OPN concentrations in breast cancer patients were elevated and ranged from 85-637 ng/mL. In particular, 30% of the samples (n=10) showed more than 9-fold increase over the mean plasma concentrations in healthy volunteers. As OPN plays an important role in cancer progression (Rodrigues, *et al.* 2007), a probable speculation is that these plasma samples were from patients with advanced disease. Since these plasma samples were purchased from a commercial source, this could not be confirmed due to the lack of availability of additional disease information on these patients.

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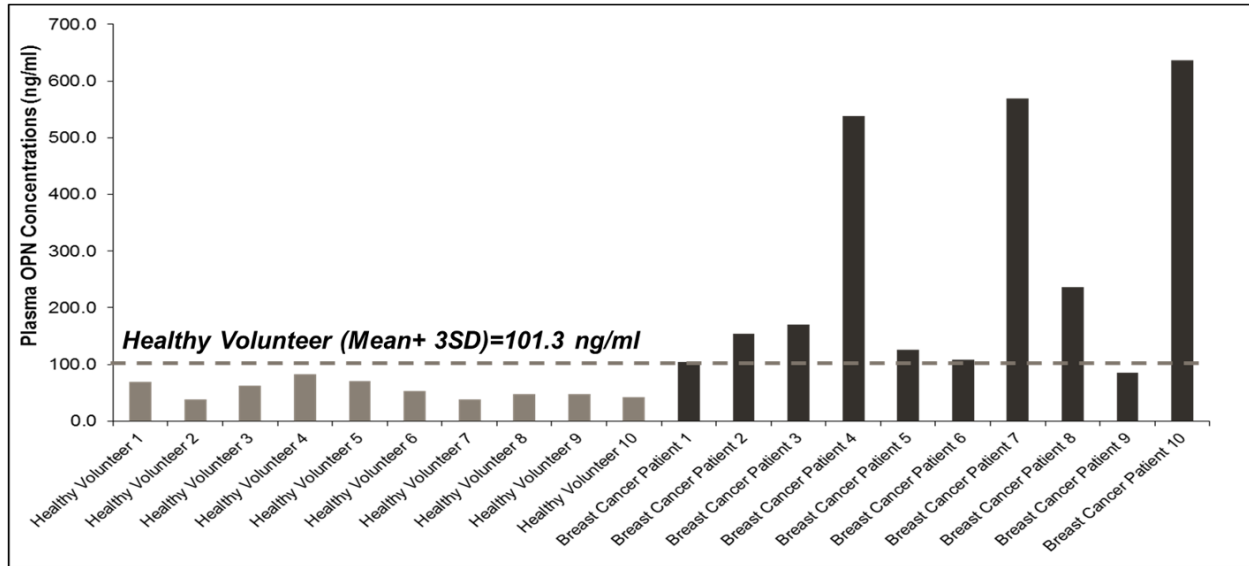
*Figure 4-16: Comparison of plasma OPN levels in healthy individuals and breast cancer patients.*

## 4.4   CONCLUSION

We developed and validated a method for quantification of human osteopontin from plasma using a capillary microflow LC-MS/MS system. The use of microflow LC-MS/MS platform resulted in 1226 times increase in signal-to-noise ratio, which in turn, allowed the use of lower sample volumes and reduced solvent consumption. This method demonstrated applicability of Waters iKey/MS™ separation device for regulated analysis of proteins.

The quantification was carried out by using a biologically relevant signature peptide GDSVVYGLR that was obtained after tryptic digestion of immunocaptured OPN. Validation was carried out using IC buffer as a surrogate matrix. A 40-fold dilution, prior to immunocapture, enabled avoidance of matrix effects. The method was accurate, precise and demonstrated good linearity over the range of 25-600 ng/mL. Stability studies demonstrated good bench-top, post preparative, freeze thaw and storage stability.

The selected signature peptide showed an additional chymotrypsin-like cleavage during digestion resulting in degradation of the peptide. In order to ensure that any variability that could arise from the two processes of peptide formation and degradation occurring simultaneously, internal standardization prior to digestion was incorporated in the method. A SIL peptide and an extended SIL peptide were evaluated as internal standards. Validation studies showed that under controlled conditions and long digestion time there was no significant difference in precision when either of the internal standards as used. However, when trypsin activity was forcibly varied, the extended SIL peptide was found to give better precision. This difference was more pronounced at when digestion was carried out at shorter time intervals.

The applicability of the method for measuring plasma OPN levels was demonstrated by analyzing samples obtained from breast cancer patients and healthy individuals. Breast cancer

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patient samples demonstrated elevated plasma OPN concentrations. The method range covers both healthy as well as diseased population plasma OPN concentrations. Human osteopontin can undergoes post-translational proteolytic cleavage which can interfere in its quantification in ligand binding assays. Considering its use as a biomarker, this method ensures that the captured hOPN is biologically active by using a biologically relevant signature peptide. Thus, this method is proposed as an alternative to ELISA and for measuring plasma OPN concentrations during oncological screening and monitoring.

# 5  DEVELOPMENT OF AN IN-VITRO CELL BASED SYSTEM FOR EVALUATION OF TOBACCO PRODUCTS FOR CARDIOVASCULAR RISK BY MEASURING THE SECRETED OSTEOPONTIN LEVELS IN TOBACCO EXTRACT EXPOSED ENDOTHELIAL CELLS

## 5.1  INTRODUCTION

Mass spectrometry based methods show great promise as an alternative to immunoassays for quantification of protein biomarkers (Makawita, *et al.* 2010, Ramanathan, *et al.* 2011, Rauh 2012). Mass spectrometry provides a universal platform for selective analysis of biomarker proteins. In particular, a mass spectrometer has the ability to differentiate between a biomarker protein and its post-transnationally modified form (Makawita, *et al.* 2010, Wang, *et al.* 2009). In this chapter, our objective was to develop a mass spectrometry based method to simultaneously measure secreted human osteopontin and its matrix metalloproteinase-3 (MMP-3) cleaved N-terminal fragment and to use this method to develop an in-vitro cell-based system to evaluate tobacco products for cardiovascular risk.

The tobacco plant has been smoked, chewed, or inhaled for many centuries (Willis, *et al.* 2012). Smoked tobacco, mainly cigarettes, has been the predominant form of tobacco use. In the US, smokeless tobacco (ST) has gained tremendous popularity in last few decades (Delnevo, *et al.* 2014). This popularity can be attributed to a number of factors, including an unsupported perception of safety; apparent health advantages of ST use over smoked tobacco; increased smoke-free laws across the country; the ease of use in a concealed manner; and a substitute product for smoking cessation (Piano, *et al.* 2010).

Many forms of ST products exist worldwide such as snus, snuff, chewing tobacco, gutka, pan and mishri. In the United States, the predominant forms of ST products are chewing tobacco and snuff (Delnevo, *et al.* 2014, Willis, *et al.* 2012).Chewing tobacco is available as loose leaves, plugs (bricks), or twists of rope and is placed between the cheek and lower lip, typically toward the back of the mouth (Willis, *et al.* 2012). It is either chewed or held in place. Snuff is finely cut or powdered tobacco. It is packaged moist or dry; most American snuff is moist (also referred to as snus). It is available loose, in dissolvable lozenges or strips, or in small pouches similar to tea bags. A pinch or pouch of moist snuff is placed between the cheek and gums or behind the upper or lower lip (Willis, *et al.* 2012).

Cigarette smoking has been associated with a number of harmful effects including an increased risk for cancer, cardiovascular diseases, chronic obstructive pulmonary disease and asthma . Cigarette smoking is known to promote atherosclerosis mainly through pathological processes such as endothelial dysfunction, inflammation and thrombosis (Unverdorben, *et al.* 2009, Winkelmann, *et al.* 2009). Presently, there is not a full understanding of the mechanisms underlying the susceptibility of tobacco users to cardiovascular disease which is due in part to a lack of knowledge about all the constituents present in tobacco products (Bishop, *et al.* 2012).

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Also, there is a lack of standardized *in vitro* testing systems for tobacco products (Johnson, *et al.* 2009).

A recent study showed that non-cytotoxic concentrations of cigarette smoke particulate matter increased osteopontin levels in cultured human endothelial cells (Bishop, *et al.* 2012). This effect was reduced in the presence of ascorbate, thus these researchers associated smoking particulate matter exposure to increased oxidative stress. In addition, a particulate matter concentration dependent rise in matrix MMP-3 was observed in these experiments. As osteopontin is known to be cleaved by MMP-3 to form a bioactive fragment (Agnihotri, *et al.* 2001), thus, a potential role of the osteopontin fragment is implicated. Also, the study showed that serum osteopontin levels were significantly lowered as compared to those measured prior to smoking cessation in smokers who quit smoking for 5 days, thus showing an agreement with the conclusion of the cell exposure studies (Bishop, *et al.* 2012).

Currently, there is no standard ELISA for measuring OPN, which has been the major limitation for clinical development of OPN as a biomarker in cancer. In fact, studies have shown that OPN blood concentrations measured with different ELISAs gave different absolute values ranging from nanogram per milliliter to milligram per milliliter (Anborgh, *et al.* 2009, Blum, *et al.* 2012, Plumer, *et al.* 2008). In addition, western blot is the only way to measure MMP-3 cleaved osteopontin fragments. Western blot methods are semi-quantitative and have issues with antibody cross-reactivity. Hence, there is a need for development of a quantitative method that can simultaneously measure osteopontin and osteopontin fragments (thrombin and/or MMP cleaved products) from a biological matrix. One of the objectives of this chapter was to develop a method to simultaneously measure secreted human osteopontin and its MMP-3 cleaved N-terminal fragment from supernatants of endothelial cells.

Considering osteopontin as a biomarker of endothelial dysfunction (Scatena, *et al.* 2007, Shimada, *et al.* 2005, Waller, *et al.* 2010), we hypothesized that a cell based system as described by Bishop et al, could be used to assess the cardiovascular risk. A specific objective of this chapter, was to develop a cell based in vitro system for evaluation of tobacco products for cardiovascular risk toxicity studies related to tobacco regulation. An immortalized human umbilical vascular endothelial cell line i.e. EA.hy926, was used to avoid variability arising due to use of primary cells. The study objective was to measure and correlate levels of secreted osteopontin and the MMP-3 cleaved N-terminal osteopontin fragment from the cell based system upon tobacco product exposure. A significantly high level of osteopontin in comparison to non-exposed controls would indicate endothelial dysfunction.

In our preliminary investigations, digestion studies of human osteopontin showed an interfering peak in the chromatogram of the MMP-3 cleaved N-terminal fragment of human osteopontin. Thus, we concluded the LC-MS/MS method, using signature peptides obtained from the biologically relevant zone i.e. 'RGDSVVYGLR', would not be able to distinguish between full human osteopontin and MMP-3 cleaved N-terminal fragment of human osteopontin. Additionally, EA.hy926 cells were exposed to cigarette smoke extract and its supernatant was evaluated for secreted osteopontin levels. It was observed that the secreted osteopontin concentration in supernatant of cell culture studies were below detection limits. Since the preliminary results did not indicate that this research objective could be addressed with the proposed system, this study was terminated.

## 5.2 MATERIALS AND METHODS

### 5.2.1 Reference Materials

Recombinant hOPN was purchased from R&D Systems (Minneapolis, MN, USA). Signature peptides having the amino acid sequence 'GDSVVYGLR' and 'GDSVVYG' for hOPN and the MMP-3 cleaved N-terminal hOPN fragment, respectively, were synthesized by Elim Biopharmaceuticals (Hayward, CA, USA). A stable isotope labeled (SIL) peptide having the sequence 'GDSVVYGLR*', containing an arginine residue labeled with $^{13}C$ and $^{15}N_4$ was synthesized by Thermo Scientific (Rockford, IL, USA) and used as the SIL-IS. The labeled arginine residue resulted in a mass shift of 10 amu from the mass of the OPN-SP. In this chapter, we will refer to the full OPN signature peptide (GDSVVYGLR) and to the MMP-3 cleaved N-terminal hOPN fragment signature peptide (GDSVVYG) as 'OPN-SP' and 'OPN-SP-1', respectively.

### 5.2.2 Reagents and Chemicals

Cigarette smoke concentrate was purchased from Murthy Pharmaceuticals (Lexington, KY, USA). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), trypsin EDTA solution (1x), Dulbecco's phosphate buffered saline (D-PBS) and EA.hy926 cells were purchased from American Type Culture Collection (ATCC). Protease inhibitor cocktail tablets were obtained from Roche (Indianapolis, IN, USA). HPLC grade water with 0.1% formic acid and acetonitrile with 0.1% formic acid were purchased from Honeywell Burdick & Jackson (Muskegon, MI, USA). High grade water was obtained using a Milli-Q integral water purification system (Billerica, MA, USA). Formic acid, methanol, Trizma hydrochloride (1M), bovine serum albumin and tween 20 were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Glacial acetic acid was obtained from Macron Fine Chemicals (Center Valley, PA, USA).

Sodium chloride solution (5 M) and EDTA solution (0.5M) were obtained from Ambion (Foster City CA, USA). Trypsin Gold was obtained from Promega (Madison, WI, USA). Biotinylated antibodies specific to hOPN (MAB193P) were obtained as a gift from Maine Biotechnology Services (Portland, Maine, USA). Immuno-capture (IC) buffer and IC wash buffer were prepared in-house and were comprised of 0.1/ 5.0/ 3.0 /0.2 /91.7 /0.1(v//v/v/v/w) Tween 20/ Trizma HCl (1M)/ NaCl (5M)/EDTA (0.5M)/water/ bovine serum albumin and 5.0/3.0/0.2/91.8/0.1 (v/v/v/v) Trizma HCl (1M)/ NaCl (5M)/ EDTA (0.5M)/ water, respectively. Digestion solution, containing of 500 ng/100 µL of trypsin gold,  0.05 pg/100 µL of SIL-IS peptide and 0.68 pg/100 µL of extended SIL-IS peptide in 50 mM ammonium bicarbonate buffer, was freshly prepared prior to digestion for the validation studies.

### *5.2.3*  **LC-MS/MS method**

The LC-MS/MS system consisted of a Waters NanoAcquity UPLC system coupled to at Waters TQ-S mass spectrometer fitted with an ion key/MS™ separation device. The separation device consisted of a compact cartridge with an in-built capillary column having an internal diameter of 150 µm and packed with 1.7 µm particles. HPLC grade water with 0.1% formic acid and acetonitrile with 0.1% formic acid were used as mobile phase A1 and B1, respectively. The LC system was operated in the trap mode using a single pump system. A Symmetry C18 Guard Column, 300 µm x 50 mm from Waters (Milford, USA) was used as the trap column. Trapping was carried out for 2 minutes at a flow rate of 20 µL/min using 99.5% mobile phase A1. Analytical separation was performed on a Waters BEH 130 C18 iKey™ Separation Device (100 mm x 150 µm I.D., 1.7 µm). Gradient conditions were as follows: 0.0–0.5 min, isocratic 5% B1; 0.5 –3.0 min, linear from 5% to 20% B; 3.0 –5 min, linear from 20% to 30% B; 5.0 –7.0 min, linear from 30% to 95% B; 7.0 –8.4 min, isocratic 95% B; 8.4 –8.5 min, linear from 95% to

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5%; 8.4 –8.5 min, isocratic 5% B. The total running time was 9 min and the injection volume was 20 µL in the full loop mode. The initial flow rate from 0.0 to 0.5 min was kept at 0.5 µL/min to avoid high back pressure during switch over from trapping to analysis mode. From 0.5 to 8.5 minutes, the chromatographic separation was carried out at a flow rate of 2.5 µL/min. The flow rate was changed to 1.0 µL/min after 8.5 min. The cone voltage and collision energy were optimized for each compound using automatic tuning (Intellistart system) in the TQ-S. The parameters for each compound are listed in Table5-1. The capillary voltage was 3.6 kV, the source temperature was 100 °C, the source offset was 60 V, and the collision gas was argon. Dwell times for all transitions were 0.044s.

### 5.2.4   Sample Preparation

Biotinylated anti-hOPN monoclonal antibodies (1 mg/ well) were immobilized on high capacity streptavidin coated 96 well plates (Thermo scientific, USA). Samples were diluted 4-fold with IC buffer and 100 µL of the diluted sample were added to each well. Immunocapture was carried out at room temperature for 4 hours using a constant vortex of 450 rpm. After 4 hours, the plates were washed 3 times with immunocapture wash buffer. Digestion was carried out by adding 100 µL of digestion solution to each well and incubating the plates at 37°C in a water bath for 14 hours. Digestion was terminated by adding 2 µL formic acid to each well. The digested samples were transferred a Protein LoBind 96 well plate and analyzed using LC-MS/MS.

### 5.2.5   Digestion Studies

Mass spectrometric parameters for OPN-SP1 were determined using a Waters Intellistart system. The retention time for this peptide was determined using the chromatographic method developed in chapter 4. Human osteopontin stock solution was spiked in 50 mM ammonium bicarbonate solution to obtain OPN standard solution having concentrations of  39.1, 78.1, 156.3, 312.5.625,

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1250 and 2500 ng/mL.    A 500 uL aliquot of the OPN standard solution was incubated with 200

ng of MMP-3 in a 2 ml Lobind Eppendorf centrifuge tube for 3 hours at 37 °C. The reaction is

terminated by heating at 95 °C for 15 minutes. These samples were then digested with 500 ng of

trypsin at for 14 hours at 37 °C along with controls that were not treated with MMP-3. The

digestion was terminated by adding 10 uL formic acid. The samples were then transferred to an

autosampler plate and analysed by LC-MS/MS.

### 5.2.6    Cigarette Smoke Extract Exposure studies

### 5.2.6.1    Method modifications

Human osteopontin standard solutions (100 and 500 ng/mL) were prepared by spiking

recombinant hOPN in IC buffer or DMEM media, with and without complete protease inhibitor,

from cell culture flasks used for sub-culturing the EA.hy926 cells. The standards were analyzed

for the amount of human osteopontin using GDSVVYGLR as the signature peptide using the

LC-MS/MS method. A modified method that was developed in Chapter 4 was used.   The

modification included a 4-fold dilution of the samples prior to immunocapture instead of   a 40-

fold dilution. The method was evaluated for capture differences between the DMEM media (with

and without complete protease inhibitor) and IC buffer.

### 5.2.6.2    Exposure studies

Immortalized human umbilical vein endothelial cells (EA.hy926 cells) were cultured in DMEM

and fetal bovine serum (10%). The plates were incubated in at 37°C in a 5% CO2 humidified

atmosphere. Cells were seeded at $1 \times 10^5$ cells/well to allow approximately 80% confluence at the

time of media harvest, after all exposure studies. Cigarette smoke extract (CSE) containing

40mg/ml particulate matter and 1.7 mg nicotine was purchased from a commercial vendor and

diluted with DMEM + 10% FBS.

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Cells were treated for 24hr either with cigarette smoke extract or nicotine or with 0.5% DMSO as a diluent control. In all exposure studies, the concentrations used of particulate matter was 20, 40, 60 and 100 µg/ml, and concentration used of nicotine was 0.85, 1.7, 2.6 and 4.25 µg/ml in the exposure studies. After 24 hours, the media from the plates was aliquoted into a 1.5 ml Lobind Eppendorf centrifuge tube containing complete EDTA-free protease inhibitor cocktail. The samples were processed as per sample preparation procedure and analyzed using LC-MS/MS along with hOPN standards (8-500 ng/ml).

## 5.3   RESULTS AND DISCUSSION

Mass spectrometric parameters obtained during tuning of the OPN-SP1 are given in Table 5-1. The b5 fragment ion (m/z 458.31) had the highest intensity and its mass transition was included in method developed in Chapter 4. Chromatographic evaluation of the synthetic signature peptide using the LC-MS/MS method developed in Chapter 4 showed a peak at the retention time of 4.6 mins.

*Table 5-1: Mass spectrometric Parameters of GDSVVYG*

| Compound | | Parent m/z | Cone Voltage | Daughters | Collision Energy |
|---|---|---|---|---|---|
| GDSVVYG | 1 | 696.67 | 50 | 458.31 | 18 |
| | 2 | 696.67 | 50 | 72.04 | 50 |
| | 3 | 696.67 | 50 | 136.03 | 50 |
| | 4 | 696.67 | 50 | 341.22 | 28 |

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### 5.3.1 Digestion Studies

OPN digestion study results with MMP-3 and trypsin are given Table 5-2. It can be seen that OPN digestion with MMP-3 and trypsin resulted in the formation of GDSVVY. A linear increase in the peak area of the GDSVVYG peptide was seen in the digestion carried out with MMP3 and Trypsin. Digest fragments increased with an increasing amount of OPN as seen Figure 5-1. However, when OPN was digested only with trypsin, small amount of GDSVVYG peptide were obtained as see in Table 5-2. This unexpected GDSVVYG peptide formation during trypsin digestion would interfere in the simultaneous quantitative estimation of full hOPN and MMP-3 cleaved N-terminal fragment of hOPN.

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*Table 5-2: Evaluation of the GDSVVYG fragment from OPN digest samples*

| OPN Concentration (ng/ml) | Average OPN-SP2 (GDSVVYG) Peak Area (cps) | |
|---|---|---|
| | Trypsin Digestion (n=2) | MMP-3 + Trypsin Digestion (n=3) |
| 39.1 | 850 | 2858 |
| 78.1 | 2299 | 3465 |
| 156.3 | 1335 | 2506 |
| 312.5 | 1312 | 5345 |
| 625.0 | 1087 | 8133 |
| 1250.0 | 2918 | 9494 |
| 2500.0 | 3092 | 16051 |

132
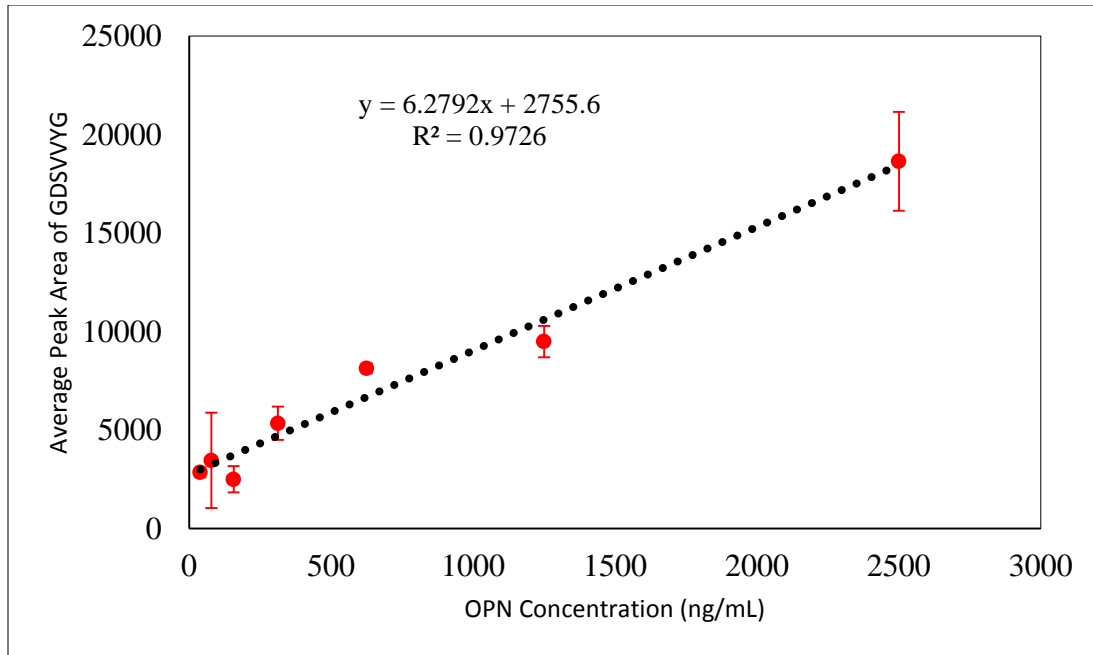
*Figure 5-1: GDSVVYG formation upon digestion of hOPN with MMP-3 and trypsin*

### 5.3.2   Cigarette smoke extract exposure studies

The method developed in chapter 4 was modified for the immunocapture step to accommodate changes in the sample matrix i.e. DMEM cell media and to increase the amount immunocaptured. The method developed in chapter 4 was modified for the immunocapture step wherein a 4-fold dilution of the sample was carried out prior to immunocapture instead of a 40-fold dilution. This modification in the method enabled higher amount of immunocaptured protein. From Figure 5-2, it can be observed that there was no significant difference in the immunocaptured hOPN amount between the hOPN standards prepared in IC buffer and DMEM buffer. This modified procedure was used for analyzing samples obtained in the cigarette extract exposure studies in EA.hy926 cells.

The study conducted by Bishop et al, used HUVECs to demonstrate secretion of hOPN upon exposure to cigarette smoke particulate matter.  HUVECs are primary cells and have an average life span of 10 serial passages and can be kept in culture for maximum of 5 months (BouÃ¯s, et al. 2001). We wanted a more rugged immortalized cell based system that could be routinely used in different laboratories ensuring good reproducibility. In order to improve reproducibility and life span in culture, we chose an immortalized HUVEC cell line i.e. EA.hy926 cells for this study.  EA.hy926 are obtained from a fusion of HUVEC and lung carcinoma cell A549 and it is the most widely used immortalized HUVECs (BouÃ¯s, *et al.* 2001).

In the study conducted by Bishop et al, HUVECs were exposed for 24 hours to cigarette smoke particulate matter and this resulted in a dose dependent increase in the concentration of secreted osteopontin in the media (Bishop*, et al.* 2012). In our study, the cigarette smoke extract was obtained from a commercial source. The extract was certified to contain 40 mg/ml of particulate matter and 1.7 mg/ml of nicotine. The cigarette smoke extract was obtained by smoking

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University of Kentucky's 3R4F Standard Research Cigarettes on an FTC Smoke Machine. Nicotine exposure in human-derived osteoblast-like cells, aortic vascular smooth muscle cells and human pancreatic ductal adenocarcinoma cells have shown an increase in osteopontin levels (Chipitsyna*, et al.* 2009, Walker*, et al.* 2001, Wang*, et al.* 2012b). We wanted to investigate whether or not nicotine alone had an impact on the osteopontin levels in our study. Hence, in our investigation, cells were treated with nicotine controls at concentrations similar to those seen in the cigarette smoke extract.

The media from the exposure study was evaluated using the modified immunocapture method described earlier along with calibration standards which were prepared by spiking recombinant hOPN in IC buffer. In the study conducted by Bishop et Al., the study showed an average concentration of 14 ng/ml in the DMSO controls.  Expecting similar hOPN concentrations, hOPN calibration standards having a concentration range of 8-500 ng/ml were used during the analysis of the exposure study samples.  The osteopontin levels were found to be below detection limits in all the samples in the exposure study including the DMSO controls. The study was repeated again with the same particulate matter concentrations for 48 hours. However, the osteopontin levels in all samples were still found to be below the detection limit. A possible reason could be that EA.hy926 cells secreted osteopontin at concentrations much lower than those seen in the HUVEC exposure studies.  Variability in the detected hOPN concentrations between different commercially available immunoassay kits has been reported. Thus, another reason could be that the absolute hOPN levels in the HUVEC study may be inaccurate i.e. overestimated.

As the exposure studies did not yield measurable osteopontin levels in the cell media, it would be very challenging to use this cell line for development of the in-vitro cell based system for routine

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screening of tobacco products. Since the preliminary results did not indicate that this research objective could be addressed with the proposed system, this study was terminated.
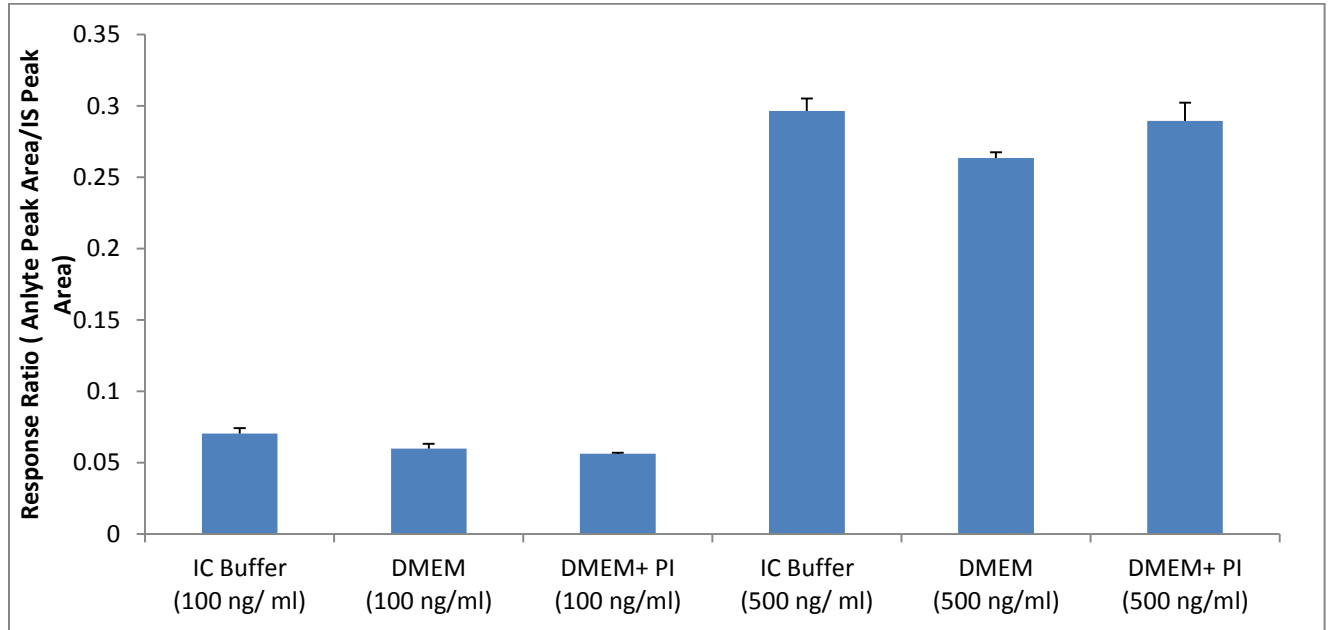


*Figure 5-2: Evaluation of matrix on hOPN immunocapture*

## 5.4 CONCLUSION

In 2009, the US Congress passed the Family Smoking Prevention and Tobacco Control Act mandating regulation of the tobacco industry by the Food and Drug Administration (FDA) through the FDA Center for Tobacco Products (CTP) (Prevention 2009). Since then there has been an increase in the need for in-vitro tools for regulating tobacco products (Hecht 2012, Leischow, *et al.* 2012). Based on the findings of Bishop et al., we proposed an in-vitro cell based system to evaluate tobacco products for cardiovascular risk. The primary objective of this system was to simultaneously measure secreted osteopontin and the MMP-3 cleaved N-terminal osteopontin fragment using LC-MS/MS from media of cells exposed to tobacco product extracts. However, our preliminary studies indicated that our proposed LC-MS/MS strategy would not be able to simultaneously measure full hOPN and its MMP-3 cleaved fragment due to an unexpected interference seen at the retention time of signature peptide for the MMP-3 cleaved fragment of hOPN.  In addition, our initial tobacco extract exposure studies in an immortalized endothelial cell line did not yield detectable osteopontin levels. Since the preliminary results did not indicate that this research objective could be addressed with the proposed system, this study was terminated.

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## 6    AN EXTENDED STABLE ISOTOPE LABELED SIGNATURE PEPTIDE INTERNAL STANDARD FOR TRACKING IMMUNOCAPTURE OF HUMAN OSTEOPONTIN FOR LC-MS/MS QUANTIFICATION

*Drawn from manuscript submitted to Biomedical Chromatography., August 2014*

### 6.1    INTRODUCTION

Internal standardization is an important aspect of the method development process for mass spectrometry based quantification of proteins. Various internal standardization strategies for mass spectrometry based protein quantification have been proposed previously and reported in several review articles (Bronsema*, et al.* 2012, Brun*, et al.* 2007, Pailleux*, et al.* 2012, van den Broek*, et al.* 2013a). Ideally internal standards should compensate for all variations encountered during sample processing as well as instrumental response fluctuations.  Sample processing steps usually involve a protein purification step and an enzymatic digestion step. The most commonly used internal standard for protein quantification is a stable isotope labeled (SIL) form of the signature peptide, commonly referred to as the SIL-IS peptide. A SIL-IS peptide contains amino

acids labeled with the stable isotopes of $^{13}C$ or $^{15}N$ thus it is physiochemically identical to the signature peptide but can be easily differentiated on the mass analyzer due to the mass difference imparted by the presence of the isotope. The major advantage of using a SIL-peptide is that these can be synthetized at relatively low cost.  A SIL-peptide IS is usually added before enzymatic digestion and can efficiently compensate for extraction recovery, peptide instability and LC-MS/MS variability. However, they do not account for proteolytic digestion variability or any variation in the protein purification processes (Brun, et al. 2009, Li, et al. 2012).

 A cost effective internal standardization variability in digestion efficiency is achieved by using extended SIL-IS peptides. An extended SIL-peptide, i.e. a SIL-IS peptide having cleavable groups flanking either side of side of a SIL-peptide,  is added prior to digestion  and these have shown to compensate for variability in digestion efficiency in addition to extraction recovery, peptide instability and LC-MS/MS instrumental  variability. (Barnidge, et al. 2004, Neubert, et al. 2013, Ocana, et al. 2010).

In order to compensate for variability arising during protein purification, a SIL-IS protein may be required. SIL-proteins can be obtained by incorporating stable isotope labeled amino acids into the target protein (Ong 2002, Picard, et al. 2012). This can be achieved using metabolic labeling by incubating cells in a medium containing stable isotope labeled amino acids popularly known as Stable Isotope Labeling by Amino Acids in Cell Culture (SILAC) (Ong 2002). Alternatively, SIL-proteins can also be made by in vitro protein synthesis in a cell free system or can be chemically synthesized if it a small protein (Jian, et al. 2013).  SIL-protein standards may be ideal internal standards for protein quantifications as they can compensate for immunoaffinity isolation variability, enzymatic digestion variability, extraction recovery, peptide instability and

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LC-MS/MS variability. However, a major drawback of SIL proteins is a lack of commercial availability and high cost.

Human osteopontin (hOPN) is a secreted biomarker protein which is present in various biological fluids including blood, milk and urine (Lund, *et al.* 2009). It is implicated in a variety of disease states, including cardiovascular disorders, cancer, and several chronic inflammatory diseases (Lund, *et al.* 2009, Rodrigues, *et al.* 2007, Waller, *et al.* 2010). In our previous work, we developed and validated an immunoaffinity coupled liquid chromatography tandem mass spectrometry (LC-MS/MS) method to quantify human osteopontin from plasma using a biologically relevant signature peptide GDSVVYGLR (Faria, *et al.*). We also demonstrated the advantages of using an extended stable isotope labeled (SIL) signature peptide as an internal standard (IS) to compensate for digestion variability. In that method however, the internal standardization did not compensate for immunocapture variability as internal standard was added post immunocapture.

We hypothesized that an extended SIL-IS peptide could be used to compensate for immunocapture variability during protein quantification provided that immunocapture is carried out with an antibody that binds to a common epitope present on both the protein and SIL-IS peptide. This hypothesis is based on the assumption that any change in immunocapture efficiency should impact analyte protein and IS peptide binding proportionally. In a proof of concept investigation, we demonstrated the ability of an extended SIL-IS peptide to compensate for immunocapture variability using a hOPN specific antibody (MAB222P) that has an epitope in the signature peptide region (DSVVYG) of the protein. A schematic of the quantification strategy is shown in Figure 6-1.

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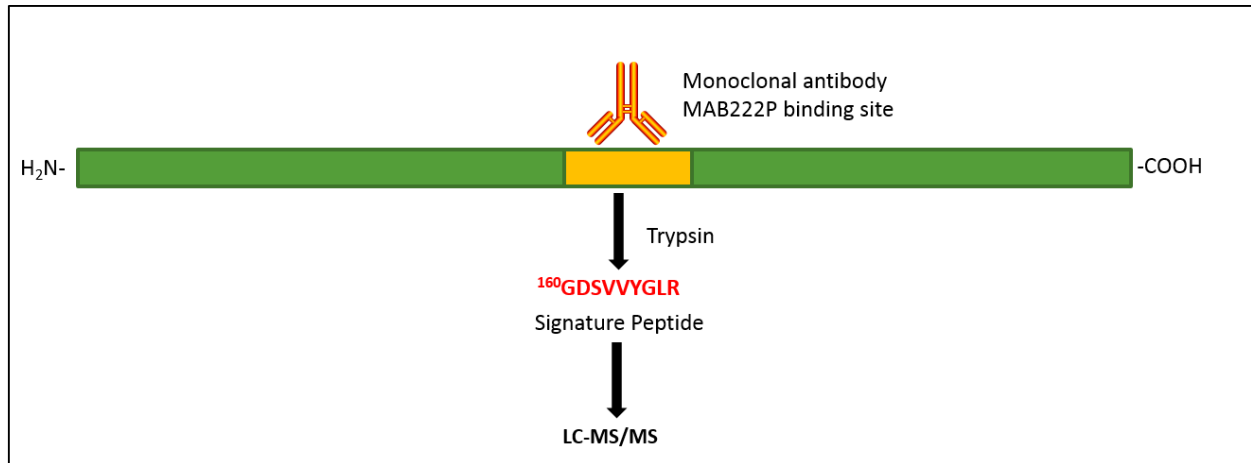
*Figure 6-1: Strategy for human osteopontin qunatification using an antibody selective for the*

*signature peptide*

## 6.2 EXPERIMENTAL

### 6.2.1 Reagents and Chemicals

Recombinant hOPN (rhOPN) was purchased from R&D Systems (Minneapolis, MN, USA). The OPN signature peptide (OPN-SP), with an amino acid sequence 'GDSVVYGLR', was synthesized by Elim Biopharmaceuticals (Hayward, CA, USA). An extended SIL peptide (TYDGRGDSVV*YGLRSKSKKF) and a SIL peptide (GDSVVYGLR*), obtained from Thermo Scientific (Rockford, IL, USA), were used as the IS peptide I and IS peptide II, respectively. The IS peptide I upon cleavage by trypsin yielded GDSVV*YGLR. The labeled amino acids V* and R* had additional mass of 6 and 10 Da, respectively. HPLC grade water with 0.1% formic acid and acetonitrile with 0.1% formic acid were purchased from Honeywell Burdick & Jackson (Muskegon, MI, USA). High grade water was obtained using a Milli-Q integral water purification system (Billerica, MA, USA). Bovine serum albumin, Dulbecco's phosphate buffered saline, formic acid, methanol, Trizma hydrochloride (1M) and tween 20 were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Glacial acetic acid was obtained from Macron Fine Chemicals (Center Valley, PA, USA). Sodium chloride solution (5M) and EDTA solution (0.5M) were obtained from Ambion (Foster City CA, USA). Trypsin Gold was obtained from Promega (Madison, WI, USA). Biotinylated antibodies to hOPN (MAB222P) were obtained as a gift from Maine Biotechnology Services (Portland, Maine, USA). The product information for MAB222P antibodies state that they have affinity towards the 'DSVVYG' amino acid sequence of hOPN. Immuno-capture (IC) buffer was prepared in-house and was comprised of 0.1/5.0/3.0/0.2/91.7/0.1 (v//v/v/v/w) Tween 20/ Trizma HCl (1M)/ NaCl (5M)/EDTA (0.5M)/water/ bovine serum albumin. IC wash buffer was prepared in-house and was comprised of 5.0/3.0/0.2/91.8/0.1 (v/v/v/v) Trizma HCl (1M)/ NaCl (5M)/ EDTA (0.5M)/

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water, respectively. Digestion solution, containing of 500 ng/100 μL of trypsin gold, 0.05 pg/100 μL of SIL-IS peptide II in 50 mM ammonium bicarbonate buffer, was freshly prepared prior to digestion for validation studies.

### 6.2.2   Instrumentation

The MFLC-MS/MS system consisted of a Waters NanoAcquity UPLC coupled to at Waters TQ-S mass spectrometer fitted with a prototype ion key/MS™ separation device. The separation device consisted of a compact cartridge with an in-built capillary column having an internal diameter of 150 μm and packed with 1.7 μm particles. HPLC grade water with 0.1% formic acid and acetonitrile with 0.1% formic acid were used as mobile phase A1 and B1, respectively. The LC system was operated in the trap mode using a single pump system. A Symmetry C18 Guard Column, 300 μm x 50 mm from Waters (Milford, USA) was used as the trap column. Trapping was carried out for 2 minutes at a flow rate of 20 μL/min using 99.5% mobile phase A1. Analytical separation was performed on a Waters BEH 130 C18 iKey™ Separation Device (100 mm x 150 μm I.D., 1.7 μm). Gradient conditions were as follows: 0.0–0.5 min, isocratic 5% B1; 0.5 –3.0 min, linear from 5% to 20% B; 3.0 –5 min, linear from 20% to 30% B; 5.0 –7.0 min, linear from 30% to 95% B; 7.0 –8.4 min, isocratic 95% B; 8.4 –8.5 min, linear from 95% to 5%; 8.4 –8.5 min, isocratic 5% B. The total running time was 9 min and the injection volume was 20 μL in the full loop mode. Initial flow rate from 0.0 to 0.5 min was kept at 0.5 μL/min to avoid high back pressure during switch over from trapping to analysis mode. From 0.5 to 8.5 minutes, the chromatographic separation was carried out at a flow rate of 2.5 μL/min. The flow rate was changed to 1.0 μL/min after 8.5 min. The cone voltage and collision energy were optimized for each compound using automatic tuning (Intellistart system) in the TQ-S.

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*Table 6-1 : Sumamry of LC-MS/MS Paramters*

| Peptide Sequence | Retention time (mins) | Selected Reaction Monitioring (SRM) Transitions (Parent ion → Fragment ion) | Cone Voltage (V) | Collision Energy (V) |
|---|---|---|---|---|
| GDSVVYGLR | 5.0 | m/z 483.6-508.5 | 15 | 16 |
| GDSVV*YGLR | 5.0 | m/z 486.0-508.5 | 19 | 14 |
| GDSVVYGLR* | 5.0 | m/z 488.6-518.4 | 19 | 14 |
| TYDGRGDSV*VYGLRSKSKKF | 4.6 | m/z 568.6-669.4 | 37 | 16 |

*Stable Isotope labeled amino acid

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The parameters for each compound are listed in Table 6-1. The capillary voltage was 3.6 kV, the source temperature was 100 °C, the source offset was 60 V, and the collision gas was argon. Dwell times for all transitions were 0.044 s.

Table 6-1. Sumamry of LC-MS/MS Paramters

### 6.2.3 Preparation of Standard Solution

Recombinant hOPN standard was reconstituted in dulbecco's phosphate buffered saline to prepare a stock solution of 100 µg/ml. Osteopontin calibration standard solutions of varying concentrations ranging from 25-1000 ng/ml were obtained by spiking hOPN stock solution in IC buffer.

### 6.2.4 Sample Preparation Procedure

Commercially available monoclonal antibodies 'MAB 222P', which were specific to hOPN and which had affinities for a specific site on the signature peptide ('DSVVYG'), were used for immunoaffinity isolation. Biotinylated anti-hOPN monoclonal antibodies were immobilized on high capacity streptavidin coated 96 well plates (Thermo scientific, USA). 100 µL of the osteopontin calibration standard solution was added to each well along with a 50 µL aliqout of IS peptide I solution. Immunocapture was carried out at room temperature for 4 hours using a constant vortex of 450 rpm. After 12 hours, the plates were washed 3 times with immunocapture wash buffer. Digestion was carried out by adding 100 µL of digestion solution containing IS peptide II to each well and incubating the plates at 37°C in a water bath for 14 hours. Digestion was terminated by adding 2 µL formic acid to each well. The digested samples were transferred to a Protein LoBind 96 well plate and analyzed using LC-MS/MS.

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### 6.2.5 Immunocapture variability evaluation

Biotinylated anti-hOPN monoclonal antibodies (750 ng per well) were immobilized on high capacity streptavidin coated 96 well plates and used for analysis varying hOPN concentration (25-600 ng/ml) to evaluate linearity of the method.

Immunocapture variability was forcibly induced by varying the antibody amount i.e. 150, 240, 400, 650, 1050, 1710, 2770 and 4500 ng per well, thus resulting in varying immunocapture efficiency. A hOPN standard solution of 250 ng/ml was used for all samples in this study. The samples were processed as per sample preparation procedure and analysed using LC-MS/MS. The results were expressed as the percent difference from the average response ratio (analyte/IS peptide I and analyte/IS peptide II) obtained in samples analyzed with the nominal samples.

### 6.3 RESULTS AND DISCUSSION

Each sample was processed with internal standard addition prior and post immunocapture using two different IS peptide. Our earlier investigations in chapter 4 showed that under controlled digestion conditions, the precision is the same when either internal standard is used. This investigation was conducted under controlled digestion conditions, so we assume that there was no bias in the digestion variability when either IS peptide is used.

The immunocapture efficiency of the antibody used in this study was 300 fold less than the antibody used in our previous method. Due to this low absolute recovery, this method was used only as a proof-of-concept study and was not developed into a fully validated method.
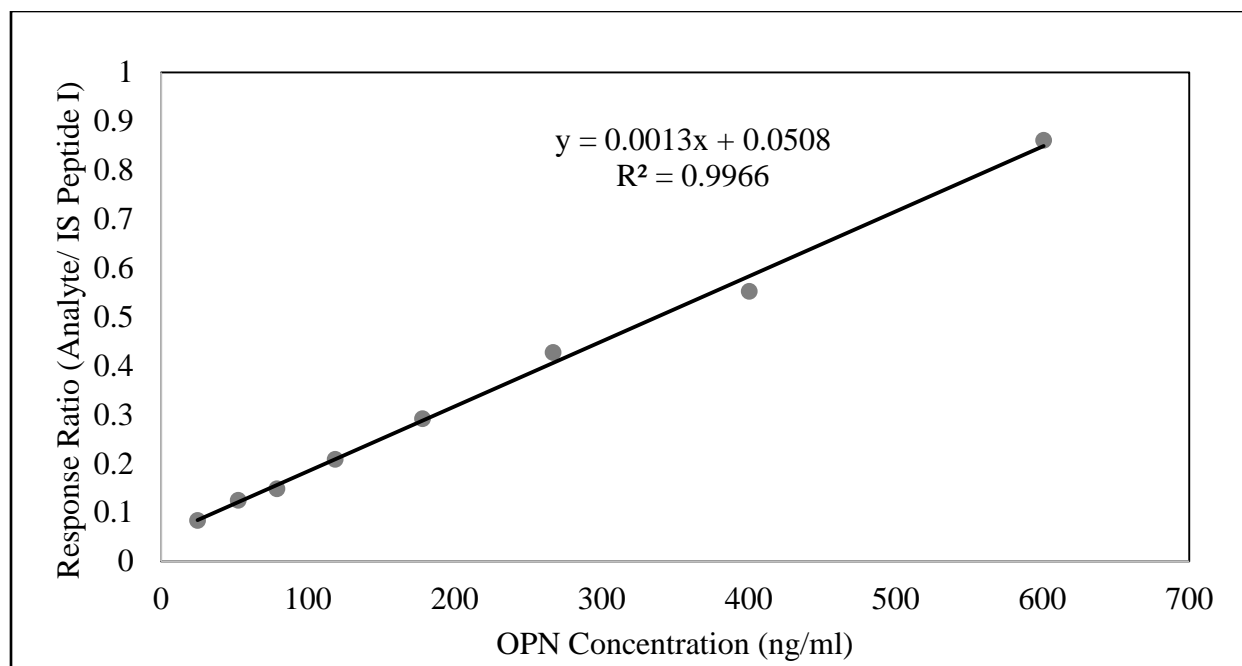
*Figure 6-2: Linearity using modified method*

The method developed in Chapter 4 was modified, wherein the immunocapture was carried out with a different antibody whose epitope site' DSVVYG" was within the selected signature peptide (GDSVVYGLR). The modified method yielded a linear response with varying concentration of hOPN as seen from Figure 6-2. This indicates that the system is not saturated for this hOPN range (25-600 ng/ml) using wells coated with 750 ng of antibody.

A logarithmic increase in analyte peak area was seen with increasing amount of antibody per well (see Figure 6-3). A deviation from linearity was observed at 1050 ng in the antibody amount versus analyte area curve for that OPN concentration, indicating saturation of the immunocapture capacity. Hence, samples analyzed with 1050 ng antibody per well were used as nominal samples.

A logarithmic increase in IS peptide I (IS added prior to immunocapture) peak area was seen with an increasing amount of antibody per well (see Figure 6-4). On the other hand, a flat relationship was seen in the IS peptide I (IS added after immunocapture) peak area was seen with an increasing amount of antibody per well (see Figure 6-5).

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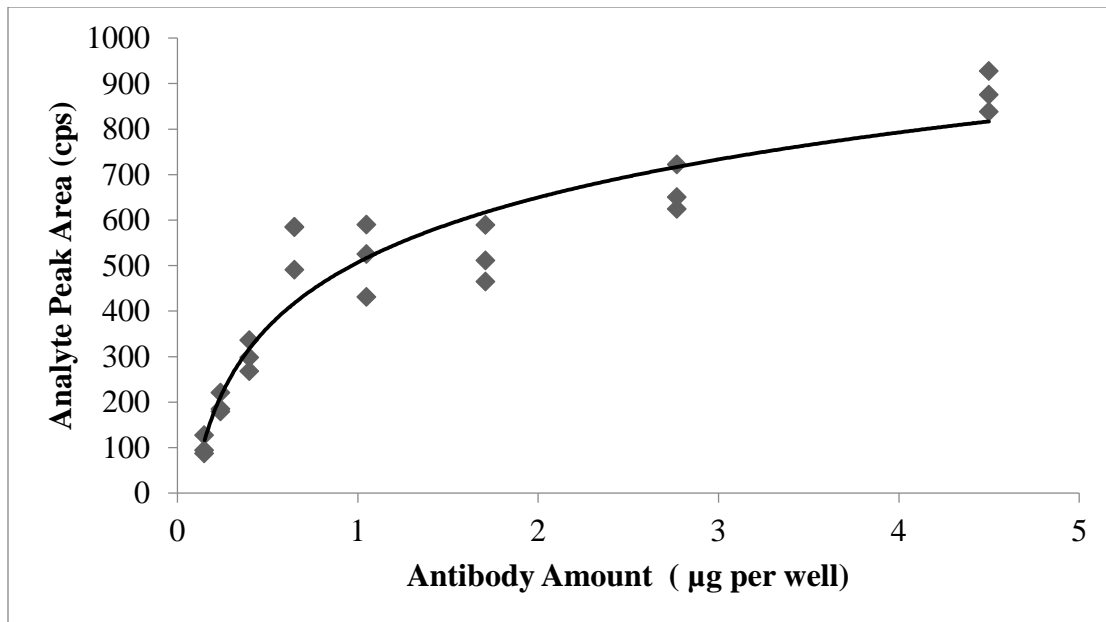
*Figure 6-3: Plot of Analyte peak area versusamount of immunocapture antibody.*

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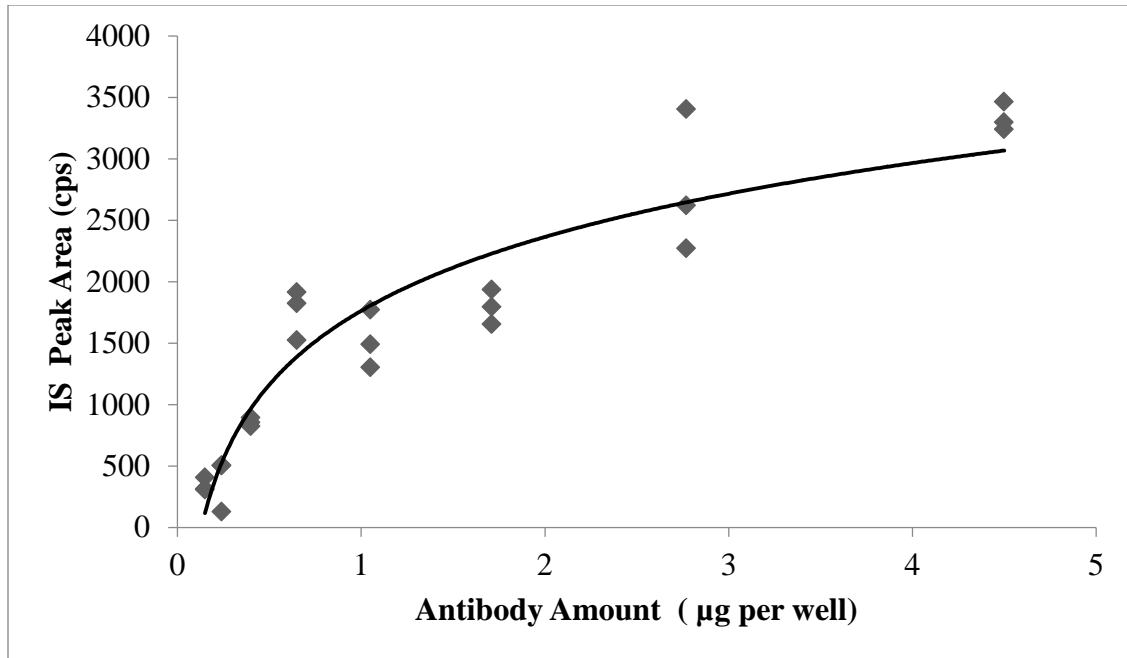
*Figure 6-4: Internal standard peptide I (added prior to immunocapture) peak area with a*

*varying amount of immunocapture antibody.*
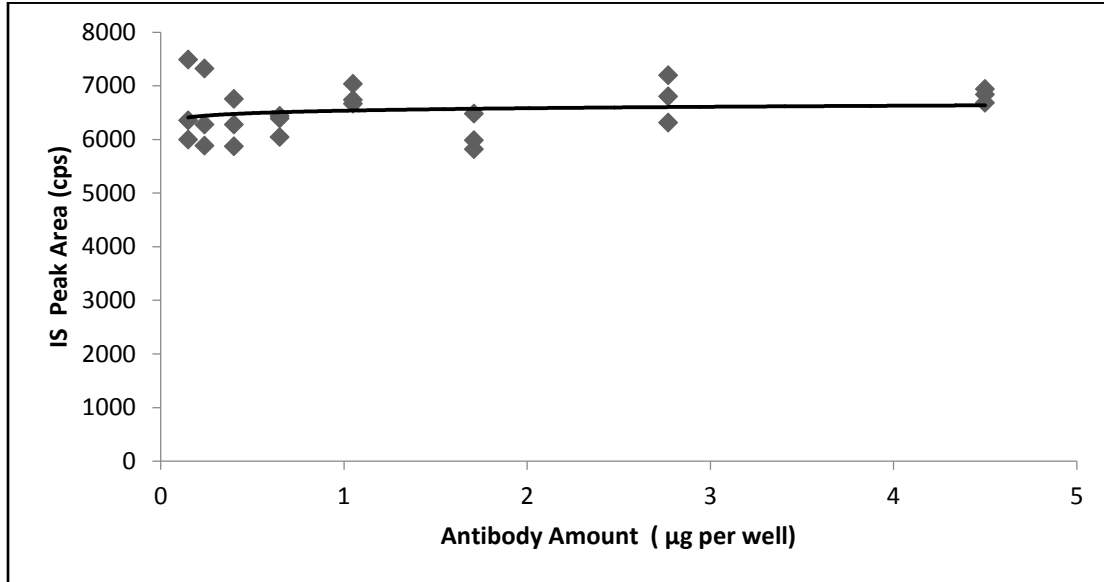
*Figure 6-5: Internal standard peptide II (added after immunocapture) peak area with a*

*varying amount of immunocapture antibody*
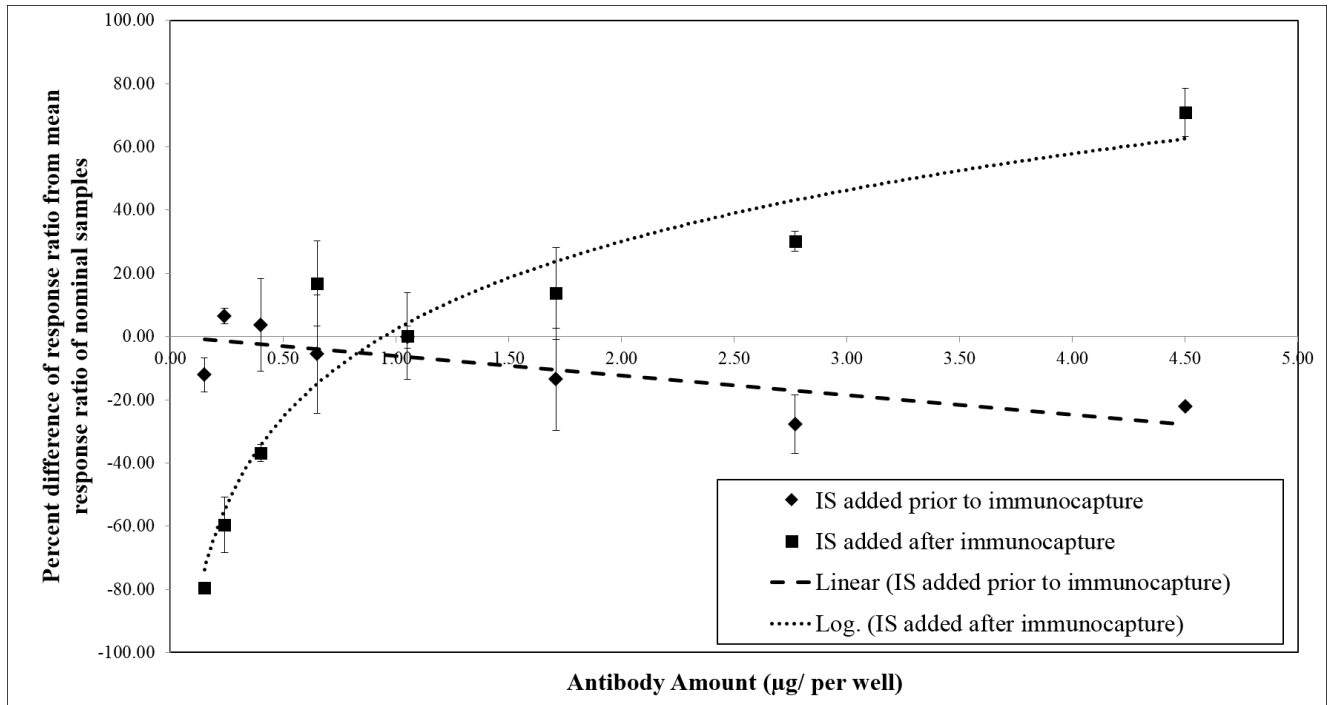
*Figure 6-6: Evaluation of internal standard peptide addition in the immunocapture variability*

*study*

The comparison of impact of IS addition was before and after immunocapture was done by evaluating trend of the percent difference from the average response ratio (analyte/IS peptide I and analyte/IS peptide II) obtained in samples analyzed with the nominal samples. From Figure 6-6, it can be observed that the addition of an extended SIL-peptide IS before immunocapture compensated for immunocapture. This is shown as the flat trend (variability ranging from -37.5% to 20.3%), as compared to the logarithmically increasing trend (variability ranging from -80.9 % to 77.0 %) when IS was added post immunocapture.

## 6.4 CONCLUSIONS

This study demonstrates that an extended SIL-IS peptide can be used as an internal standard to compensate for immunocapture variability during quantification of hOPN by LC-MS/MS. Extended peptides may be considered as potential internal standards to account for sample processing variability (immunocapture and digestion) during protein quantification using immunoaffinity coupled LC-MS/MS. Use of extended SIL-peptides provides a cost effective internal standardization strategy as an alternate to the use of SIL-proteins. This internal standardization strategy could be used for quantification of low abundance biomarker proteins which require immunocapture purification, but do not have commercially available SIL-IS proteins.

# CHAPTER 7

## 7    OVERALL CONCLUSIONS

Biomarker quantification plays an important role in clinical disease diagnosis, patient stratification, therapy selection and therapeutic monitoring (Fleming, et al. 2012, Goodsaid, et al. 2007, Pletcher, et al. 2011). In addition, biomarkers are increasingly being used by the pharmaceutical industry as surrogate end point markers during drug development to have shorter clinical trials (Kola, et al. 2004, Lee, et al. 2009). Proteins are the functional biomolecules in biologically processes. Proteins found in biological fluids such as blood, urine, saliva and milk are preferred as biomarker molecules, primarily due to the ease of sampling (Dhingra, *et al.* 2005, Rogers, *et al.* 2008). Protein biomarker application can involve measurement of the amount of protein or of the protein activity. Currently, protein biomarker quantification is dominated by immunoassays. Over the last few decades, immunoassays platforms have improved from the use of radioimmunoassay to enzyme linked immunosorbent assays (ELISA) (Hoofnagle, *et al.* 2009). Immunoassays provided a high sensitivity, high throughput and cost effectiveness platform to quantify biomarkers. However, immunoassays suffer from selectivity issues leading to method inaccuracies. Method inaccuracy arising from lack of selectivity can

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lead to biased results, in turn affecting clinical decisions (Hoofnagle, *et al.* 2009). This led us and others to evaluate alternative methods for quantification of biomarker protein molecules. This dissertation focused on developing and validating mass spectrometry based methods for quantification of protein biomarkers.

Liquid chromatography coupled to mass spectrometry has been the small molecule gold standard of quantitative analysis for quantification of small molecules for many years now. Over the last decade, mass spectrometry is increasingly being evaluated as an alternative to immunoassays for protein biomarker applications (Makawita, *et al.* 2010, Ramanathan, *et al.* 2011, Rauh 2012). In the first chapter, strategies for quantitative analysis of protein biomarker molecules using liquid chromatography tandem mass spectrometry were discussed. Currently, the most widely used protein quantification strategy using LC-MS/MS involves the formation of unique peptides upon enzymatic digestion of the analyte protein (Berna, *et al.* 2009, Keshishian, *et al.* 2009, Makawita, *et al.* 2010, Rauh 2012, Wang, *et al.* 2009). These unique peptides, also known as signature peptides, are then analyzed as surrogate analytes using LC-MS/MS. Method development begins with *in-silico* digestion studies of the analyte protein, to identify potential signature peptides and their mass spectrometric transitions. Enzymatic digestions are then carried out preferably using recombinant analyte protein and the digests are screened for the presence of the signature peptides identified during *in-silico* studies. In the final optimization studies, the signature peptide that is most selective and has the highest signal intensity is chosen and used as the surrogate analyte for the protein.

If the biomarker proteins are quantified from complex biological matrices, it is essential that there is a purification step to isolate the analyte protein or the signature peptide from unwanted matrix components (Berna, *et al.* 2009, Keshishian, *et al.* 2009, Makawita, *et al.* 2010, Rauh

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2012, Wang, *et al.* 2009). Biomarker protein concentrations and their physicochemical properties are main considerations while choosing an appropriate purification strategy. An immuno-based purification is usually required if the biomarker protein is found in low nanogram levels. Sometimes a 'double clean-up' involving a protein purification step followed by a peptide purification step is used to achieve cleaner samples and lower detection limits. (Ahn, *et al.* 2009, Kushnir, *et al.* 2013, Neubert, *et al.* 2013).

Internal standard (IS) selection is an essential method development step for mass spectrometry based quantitative analysis (Bronsema, *et al.* 2012, Brun, *et al.* 2007, Pailleux, *et al.* 2012, van den Broek, *et al.* 2013a). Though stable isotope labeled SIL-proteins are considered ideal internal standards during protein quantification by LC-MS/MS, SIL-peptides are used for internal standardization as they are more readily available and have lower cost. However, SIL-peptides lack the ability to compensate for variability arising from sample processing steps such as protein purification and enzymatic digestion (Bronsema, *et al.* 2012, Brun, *et al.* 2007, Pailleux, *et al.* 2012, van den Broek, *et al.* 2013a). Extended SIL-peptides, i.e. SIL-peptides having additional amino acid residues flanking on either side, can be used as alternatives to SIL-peptides to account for digestion variability (Bronsema, *et al.* 2012, Brun, *et al.* 2007, Pailleux, *et al.* 2012, van den Broek, *et al.* 2013a). In chapter 4, we have demonstrated the ability of an extended SIL-IS peptide to account for digestion variability. In addition, we have demonstrated that an extended SIL-IS peptide can compensate for immunocapture variability when the epitope lies within the region of the signature peptide.

A 'fit-for-purpose' method validation is traditionally used for biomarker assays i.e. the method validation should be evaluated based on its intended purpose. (Bower, *et al.* 2014, Lee 2009, Lee, *et al.* 2006, Stevenson, *et al.* 2013). Selectivity of the mass spectrometry based biomarker

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assay is ensured by choosing a unique signature peptide and appropriate mass transitions. Additionally, selectivity can be incorporated in the method through selective isolations such as protein and peptide immunocapture. Accuracy and precision of the method within an analytical batch and between batches is evaluated during validation. As biomarkers are endogenous molecules, calibration curve standards and quality control samples are prepared by spiking protein reference standards into pooled matrix samples which have low endogenous levels of the biomarker (Lee, *et al.* 2009). Alternatively, a surrogate matrix such as analyte stripped matrix or buffer matrix can be used. A parallelism experiment in which comparison of the sensitivity in the sample matrix and the surrogate matrix can be used to demonstrate that there are no matrix differences between the sample matrix and surrogate matrix (Lee, *et al.* 2009). In addition, storage stability, in-process stability and post preparative stability is also established during validation. Method validation ensures that the developed biomarker assay is selective, accurate, precise and reproducible. This builds greater confidence in the biomarker based decisions in clinical settings.

In chapter 3, a LC-MS/MS method was developed and validated for measurement of the activity of the biomarker protein thymidine kinase 1 (TK1) in serum. TK1 is an enzyme involved in DNA synthesis whose activity in serum is indicative of tumor proliferation and the severity of blood malignancies (Topolcan, *et al.* 2008). TK1 activity was measured by monitoring the conversion of 3'-deoxy-3'-fluorothymidine (FLT), a specific exogenous substrate for TK1, to 3'-deoxy-3'-fluorothymidine monophosphate (FLT-MP) (Faria, *et al.* 2012). Protein precipitation and on-line SPE was used for analyte isolation prior to LC-MS/MS analysis. The amount of FLT-MP generated was quantified using LC-MS/MS. The method was linear over the range of 0.5-500 ng/mL for FLT and 2.5-2000 ng/mL for FLT-MP with a mean correlation coefficient of

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0.9964 and 0.9935 for FLT and FLT-MP, respectively. The lower limit of quantification was 0.5 ng/mL for FLT and 2.5 ng/ml for FLT-MP. Intra-assay accuracy and inter-assay accuracy was within ±12 % for both FLT and FLT-MP. Intra-assay precision was 2.8% to 7.7% for FLT and 3.3% to 5.8% for FLT-MP. Inter-assay precision was 4.6% to 14.9% for FLT and 4.9% to 14.6% for FLT-MP. Typically, an activity assay is more sensitive to formation of product in comparison the consumption of the substrate; hence serum TK1 activity was assessed by the formation of FLT-MP. Serum TK1 activity was measured in serum from hepatocellular carcinoma (HCC) patients and age-matched controls under standardized conditions. A sub-population of the HCC patient samples showed an almost 20-fold enhanced TK1 activity compared to the controls. This method provided a robust alternative to radiometric and immunochemical assays for rapid and selective determination of serum TK1 activity during oncological screening and monitoring.

In chapter 4, a microflow LC-MS/MS method was developed and validated for measurement of the biomarker protein human osteopontin from plasma using a biologically relevant signature peptide. Human osteopontin (hOPN) is a secreted cell signaling protein which is implicated in the pathogenesis of a variety of disease states, including cardiovascular disorders, cancer, and several chronic inflammatory diseases (Lund, *et al.* 2009, Rodrigues, *et al.* 2007, Waller, *et al.* 2010). The primary integrin binding site on hOPN is [159]RGDSVVYGLR. This region is also a site known to undergo proteolytic cleavage and is devoid of any post-translational phosphorylation. The signature peptide' (GDSVVYGLR) was chosen from this biologically active region of hOPN. The use of a biologically relevant signature peptide ensures that the captured hOPN is biologically active. Immunocapture, using hOPN specific antibodies, was used to isolate hOPN from the plasma matrix prior to tryptic digestion. Online sample enrichment was performed using column trapping prior to chromatographic separation. Initial

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method development was carried out using a conventional flow liquid chromatographic (LC) system coupled to an AB Sciex API 4000 Qtrap. The method was transferred to a capillary microflow Waters ionkey/MS system in order to achieve the detection limits needed for normal endogenous concentrations of osteopontin. Immunocapture buffer was used as a surrogate matrix for validation studies. Samples were diluted prior to analysis to eliminate matrix effects. The method was validated over a range of 25-600 ng/mL. The performance of the method was found to be compliant with the USFDA validation guidance (Center for Drug Evaluation and Research (U.S.)*, et al.* 2001).

During method development, the signature peptide was observed to undergo an unexpected chymotryptic-like cleavage leading to its degradation. An important objective of this chapter was to evaluate the ability of the stable isotope labeled (SIL) peptide GDSVVYGLR* and an extended SIL peptide TYDGRGDSVV*YGLRSKSKKF' as internal standards (IS) to account for variability and instability of the signature peptide during digestion. Inherent digestion variability was not significantly different with either peptide IS. However, when the trypsin activity was varied, the extended SIL peptide was found to be better internal standard to account for digestion variability. In the digestion variability studies, the use of extended SIL peptide as internal standard limited the total variability within ±30%. Alternatively, when SIL peptide was used as internal standard the variability ranged from -67.4% to 50.6 %.

The applicability of the method for measuring plasma OPN levels was demonstrated by analysis of samples obtained from breast cancer patients and healthy individuals. The method range covered both healthy and diseased population plasma OPN concentrations. The plasma OPN concentrations in healthy individuals ranged from 38-85 ng/mL with a mean concentration of 55.4±15.3 ng/mL. A 1.5-12 fold increase in OPN concentrations, ranging from 85-637 ng/mL,

was seen in breast cancer patient samples. Human osteopontin undergoes post-translational proteolytic cleavage which can interfere in its quantification in ligand binding assays. The use of a biologically relevant signature peptide ensures that the captured hOPN is biologically active. Thus, this method is proposed as an alternative to ELISA and for measuring plasma OPN concentrations for biomarker applications.

In chapter 5, prelimary studies were conducted to develop a cell based system to evaluate tobacco products for cardiovascular risk based on the LC-MS/MS measurement of secreted osteopontin and MMP-3 cleaved osteopontin fragments. Our first objective was to develop a LC-MS/MS to quantify full hOPN and the MMP-3 cleaved hOPN fragments from cell media. From our in-silico studies, we predicted that tryptic digestion of hOPN and its MMP-3 cleaved N-terminal hOPN fragment would yield two different signature peptides i.e. 'GDSVVYGLR' and 'GDSVVYG', respectively. However, in our preliminary LC-MS/MS evaluation of full hOPN tryptic digests, we saw that tryptic digestion resulted in the production of only a small amounts of the 'GDSVVYG' peptide, thus negating our ability to quantify the MMP-3 N-terminal hOPN cleaved fragments using LC-MS/MS. Our second objective was to develop an in vitro method for evaluating the cardiovascular risk potential of tobacco products by measuring secreted hOPN levels from tobacco extract exposed endothelial cells. EA.hy926 vascular endothelial cells were exposed to cigarette smoke extract and its supernatant was evaluated for secreted osteopontin levels along with nicotine and solvent controls, using a modified LC-MS/MS method developed in chapter 4. However, it was observed that the secreted osteopontin concentrations in the supernatant of cell culture studies were below detection limits. Since the preliminary results did not indicate that this research objective could be addressed with the proposed system, this study was terminated.

In chapter 6, in a proof-of-concept study, we evaluated the ability of an SIL-peptide IS peptide to compensate for immunocapture variability during quantification of human osteopontin (hOPN) by immunoaffinity coupled LC-MS/MS. For this study, we modified the LC-MS/MS method to quantify hOPN with a hOPN specific antibody that had an epitope present on the SIL-IS peptide. Immunocapture variability was induced by varying the antibody amount per well from 150-4500 ng and analysis was carried out with internal standards added before and after the immunocapture step. The immunocapture variability ranged from -80.9 % to 77.0 % when the IS was added after immunocapture and from -37.5% to 20.3% when the IS was added before immunocapture. The lower variability demonstrates the ability of the SIL-IS peptide to compensate for variation during immunocapture. The immunocapture efficiency was significantly lower than that of the original method. The low immunocapture efficiency will limit the detectability of the method for its biomarker application. Hence, this method was not validated.

With increase in the biomarker applications in drug development and clinical settings, there is a need for improving analytical technology for biomarker quantification. LC-MS/MS shows great promise as an analytical tool for quantification of protein biomarkers (Makawita, *et al.* 2010, Ramanathan, *et al.* 2011, Rauh 2012). We developed and validated a method to precisely and accurately measure the activity of serum thymidine kinase 1 using LC-MS/MS. In addition, we developed and validated a method to precisely and accurately measure human osteopontin from plasmas using LC-MS/MS. The method focused on using a signature peptide from the bioactive region of the human osteopontin, thus, enabling better reliability of this method for biomarker applications. In addition, our investigations were focused on internal standard correction for

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protein quantification by LC-MS/MS, and enhanced our present understanding of internal standardization for protein quantification. We were able to demonstrate that extended SIL-IS peptides can effectively compensate for immunocapture and digestion variability during human osteopontin quantification from plasma by LC-MS/MS. These methods provide an alternative to immunoassay quantification for evaluation of protein biomarkers in clinical settings. In the future, technological developments in mass spectrometric instrumentation and sample processing to achieve lower detection limits coupled with better workflow to achieve more rugged methods will result in greater acceptance of mass spectrometry based quantification methods by regulatory authorities and the clinical sciences community.

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**VITA**

Morse Faria was born on January 07, 1982 in Goa, India. He received his Bachelor's degree in Pharmaceutical Sciences from the Goa University, Mumbai, India in 2003. He received a Master's degree in Pharmacy from Mumbai University, India in 2007. After his master's degree, he worked for 2 years as Research Associate at Wockhardt Ltd., Mumbai, India and for another 2 years as a Lecturer in Pharmacy at VES College of Pharmacy, Mumbai, India. He joined the Ph.D. program in Pharmaceutics at Virginia Commonwealth University, Richmond, VA in 2010. Morse has authored 3 accepted manuscripts to date and these have been published in journals such as Journal of Chromatography B, Indian drugs and, Journal of Applied Pharmaceutical Sciences. In addition, Morse has also co-authored six invited podium presentations and several posters related to his graduate research. During his graduate studies at VCU, he completed a 1 year Pre-doctoral fellowship at PPD, Richmond, VA, USA. In addition, he has competed a Clinical and Translational Research Course 2013 at NIH, Bethesda, USA.

Morse was recipient of the VCU Leadership and Service Award and Who's Who among Students in American Universities and Colleges in 2012. He was also the recipient of the AAPS-APQ Graduate Student Symposium Award in 2013 and AAPS-APQ Graduate Student Research Award 2014. Apart from this, he has also been awarded the AAPS Travelship award in 2012-2014. Morse was awarded the John Wood Excellence in Pharmaceutics Award 2014 by the VCU Department of Pharmaceutics. He was also awarded the Altria Fellowship 2013 and

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Charles T. Rector & Thomas W. Rorrer, Jr. Dean's Award 2014 by VCU School of Pharmacy. He was inducted in the Alpha Epsilon Lambda (AEL) Honor Society Membership in 2013 and Rho Chi Rho Chi Honor Society, Lambda Chapter in 2014.

Morse has served as the Vice President and Advisory Committee member in Tiranga, Indian Natiaonals at VCU from 2011-2013. He was the founder member of Tiranga's community engagement program – Reaching Out Dil Se (RODS). Morse has also served as an appointed Graduate Student Representative in the executive committee of the AAPS – Analysis and Pharmaceutical Quality Section (APQ) from 2012-2014. He has also served as the Treasurer and Secretary in the VCU-Pharmaceutics Graduate Student Association as well as the AAPS Student Chapter at VCU from 2011-2013. He was nominated as the Graduate Student Ambasssor of VCU School of Pharmacy for the year 2012-13.

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