Applications of Mass Spectrometry in Clinical Chemistry and Biomedical Research

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List of abbreviations

3-NBA	3-nitrobenzyl alcohol
AC	alternating current
ADME	absorption, distribution, metabolism and elimination
AIDS	acquired immunodeficiency syndrome
AMS	accelerator mass spectrometry
APCI	atmospheric pressure chemical ionization
APPI	atmospheric pressure photoionization
AQUA	absolute protein quantification
ARV	anti retroviral drug
ARV	anti-retroviral
ATZ	amino acid anilinothialinone derivative
CI	chemical ionization
CID	collision-induced dissociation
CoA	coenzyme A
CRP	C-reactive protein
CV	coefficient of variation
DC	direct current
DTT	dithiothreitol
DIGE	differential gel electrophoresis
ECD	electron capture dissociation
EI	electron ionization
ESI	electrospray ionization
ETD	electron transfer dissociation
FAB	fast atom bombardment
FTMS	Fourier transform mass spectrometry
GCMS	gas chromatography coupled to mass spectrometry
HAART	highly active anti-retroviral therapy
hCT	human calcitonin
HIV	human immunodeficiency virus
HPLC	high performance liquid chromatography
ICAT	isotope coded affinity tagging
IgG	immunoglobulin G
IMMS	ion mobility mass spectrometry
ISTD	internal standard
ITRAQ	isotope tags for relative and absolute quantitation
LC	liquid chromatography
LCMS	liquid chromatography mass spectrometry
LLOQ	lower limit-of-quantitation
MALDI	matrix-assisted laser desorption ionization
MH^+	pseudo molecular ion
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
NNRTI	non-nucleoside reverse transcriptase inhibitors

PAGE	polyacrylamide gel electrophoresis
PI	protease inhibitor
PITC	phenylisothiocyanate
PK	pharmacokinetics
PKU	phenylketonuria
PMF	peptide mass fingerprinting
PTH	amino acid phenylthiohydantoin derivative
QC	quality control
RF	radio frequency
RIA	radioimmunoassay
sCT	salmon calcitonin
SDS	sodium dodecyl sulphate
SELDI	surface enhanced laser desorption ionization
SILAC	stable isotope labelling with amino acids in cell culture
SIM	selected ion monitoring
SIMS	secondary ion mass spectrometry
T3	triiodothyronine
T4	thyroxine
TDM	therapeutic drug monitoring
TFA	trifluoroacetic acid
TOF	time-of-flight
UV	ultra violet

English abstract

Clinical chemistry is a medical discipline whose aim is to diagnose and assess disease by analysis of biological specimens. Modern laboratories can perform several hundred different tests using many different methods developed over the last century. The classical, more traditional assays are typically labour-intensive, not multiplexed (only measure one analyte or disorder per assay), expensive, require a long turnaround time, and may not provide adequate sensitivity and specificity. Developments in mass spectrometry (MS) and related technologies over the last two decades have provided solutions for many if not all of these shortcomings. While MS based applications have not yet been widely implemented in clinical chemistry laboratories, current developments will encourage the replacement of traditional methods as well as the expansion of clinically diagnostic endpoints. Indeed, modern MS can be used to simultaneously analyze and quantitate multiple biomarkers in a single analysis. Currently, no other technique exists that can provide a comparable multiplexed analysis. In this thesis, current MS and related technologies were developed and applied to several important but distinct clinical chemistry applications. In particular, novel and useful methodologies were developed and implemented for the analysis and quantification of small molecule and peptide drugs (antiretroviral drugs and calcitonin), disease markers (C-reactive protein) and the biochemical state of an important drug target (human aromatase). The aim of this thesis is to describe, illustrate and discuss several novel examples of MS based clinical chemistry methods and applications.

The development and application of a high throughput liquid chromatography tandem mass spectrometry (LC-MS/MS) assay for the multiplexed therapeutic drug monitoring of eleven antiretroviral drugs in human plasma is described. The method was validated, tested against external quality control samples, and used for the therapeutic drug monitoring of more than 450 patient samples spanning nine months of analyses.

A novel capillary LC TOF-MS method for the determination of a peptide drug (salmon calcitonin), a thirty-two residue peptide hormone, in human plasma and urine was developed. The method requires only a few microliters of sample and is sufficiently robust to be implemented in a clinical chemistry setting. The assay provides unparalleled specificity, a useful alternative to immunoassays, and can be readily adapted to the multiplexed analysis of other peptide drugs.

A generally applicable procedure for the multiplexed quantitation of target proteins in complex mixtures is described. The method involves trypsinization in the presence of $^{13}C_6$-labelled analog peptide internal standards i.e. Isotope dilution method. As an example of its utility, the method was used to quantitate rat urinary C-reactive protein (CRP) in a model of drug induced nephrotoxicity.

Finally, the application of MS and related technologies for identifying protein posttranslational modifications is discussed. A model membrane protein, human estrogen synthase, was thoroughly analysed for the presence of modifications. Extensive sequence coverage (90.2%) was obtained, a novel amino acid substitution was identified and the enzyme was confirmed to harbour an N-linked glycosylation site.

Résumé français

La chimie clinique est une discipline médicale qui a pour but de diagnostiquer la présence et la progression d'une maladie par l'analyse d'échantillons biologiques. Les laboratoires modernes peuvent exécuter des centaines d'analyses en utilisant plusieurs méthodes développées au courrant des cent dernières années. Les essais classiques, et plus traditionnels, sont souvent laborieux, non multiplexe (mesurent seulement un analyte par essai), cher, exige un long temps de rotation et risque de ne pas fournir une spécificité adéquate. Pendant les deux dernières décennies, les développements dans le domaine de la spectrométrie de masse (MS) et les technologies rattachées ont fourni des solutions à plusieurs, pour ne pas dire tous, manques retrouvés dans les méthodes d'analyse traditionnelles.

Bien qu'actuellement la MS ne soient pas largement utilisee dans les laboratoires de chimie clinique, de nouveaux developpements dans ce domaine encouragerons le remplacement de méthodes traditionnelles ainsi que l'expansion du nombre de parametres de diagnostique clinique. En effet, la MS moderne peut etre utilisée pour simultanément analyser et quantifier de multiples marqueurs biologiques en une seule analyse. Actuellement il n'existe aucune autre technique qui procure une telle analyse multiplexe. Dans cette thèse, des technologies courantes de MS ainsi que des technologies connexes ont été développées et utilisées dans plusieurs applications importantes et distinctes en chimie clinique. En particulier, de nouvelles méthodologies utiles ont été développées et mises en application pour l'analyse et la quantification de petites molécules et peptides médicamenteux (médicaments antiretroviraux et calcitonine), des marqueurs de maladie (protéine

C-reactive), et l'état biochimique d'une cible de médicament importante (aromatase humaine). L'objectif de cette thèse est de décrire, illustrer, et discuter plusieurs nouveaux exemples d'applications basées sur la MS en chimie clinique.

Une méthode capillaire de chomatographie liquide-MS a été développé pour la détermination d'un peptidique synthetique (salcatonine), dans le plasma et l'urine humain. La méthode exige seulement quelques microlitres d'échantillon et est suffisamment précise pour être exécutée dans un cadre de chimie clinique. L'essai fournit une spécificité incomparable et est une alternative utile aux méthodes immunoenzymatiques.

Ensuite, une technique généralement appliquée dans la quantification multiplexée de protéines retrouvées dans des mélanges complexes est décrite. Cette méthode, qui utilise la dilution isotopique, implique la trypsinisation des protéines en présence de peptides analogues de standards internes qui sont étiquetés $^{13}C_6$. Pour démontrer son utilité, la méthode a été appliquée dans la quantification de la protéine réactive C (CRP) urinaire chez un modèle de rat souffrant de néphrotoxicité induite par médicaments.

Finalement, l'application de la MS et de technologies rattachées pour identifier les modifications post-translationnelles des protéines est discutées. L'œstrogène synthétase humain, une importante cible chemiotherapeutique, a été étudiée à fond pour la présence de modifications. Une couverture consciencieuses de la séquence (90.2 %) a été obtenue, avec laquelle une substitution originale d'acidaminé au N-terminus a été identifiée et l'enzyme a été confirmée de contenir un site de N-glycosylation.

Original contributions to knowledge

- Development and validation of a novel tandem LCMS method for simultaneous therapeutic drug monitoring of eleven HIV antiretroviral drugs in human plasma.

- Novel capillary LC TOF-MS approach for peptide drug quantification applied to salmon calcitonin in μL volumes of human urine and plasma.

- Generally applicable strategy for protein quantification applied to urinary C-reactive protein in a rat model of drug induced nephrotoxicity. Analysis involves μL sample volumes, ^{13}C-labelled tryptic internal standard peptides and capillary LC-TOF MS.

- Biochemical MS analysis of human estrogen synthase resulting in 90.2% protein sequence coverage, identification of a novel amino acid substitution and confirmation of N-linked glycosylation.

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Preface and contribution of authors

With the rapid developments in MS and related technologies over the last two decades, a number of reports have appeared illustrating the potential for numerous clinical applications that can capitalise on the developing technology. In this thesis, several novel MS based methods related to clinical biochemistry are described. Chapter 1 provides an overview of traditional biochemical assays and applications in clinical chemistry. Chapter 2 presents an overview of current mass spectrometry. Chapters 3-6 present experimental work outlining novel applications of current MS technology as applied to quantitative analysis of antiretroviral drugs (Chapter 3), a peptide hormone (Chapter 4), a biomarker protein (Chapter 5), and biochemical characterization of an important chemotherapeutic target, human estrogen synthase (Chapter 6). Chapters 4, 5 have previously been presented as published material.

Chapter 4 is a published article: Aguiar, M., B. F. Gibbs, Masse R (2005). "Sensitive capillary chromatography mass spectrometric methods for the determination of salcatonin in human biological matrices." Journal of Chromatography B **818**(2): 301-308.

Mike Aguiar performed the bench work, sample workup, data analysis and drafted the manuscript. Bernard F. Gibbs conceived of the experiment, ordered necessary reagents and chemicals, operated and optimised the capillary LCMS system for data collection and edited the manuscript. Robert Masse arranged lab facilities and edited the manuscript.

Chapter 5 is a published article: Aguiar, M., R. Masse, B. F. Gibbs (2006). "Mass spectrometric quantitation of C-reactive protein using labelled tryptic peptides." Analytical Biochemistry **354**(2): 175-181.

Mike Aguiar performed the bench work, sample workup, data analysis and drafted the manuscript. Bernard F. Gibbs conceived of the experiment, ordered necessary reagents and chemicals, optimised and operated the capillary LCMS system for data collection and edited the manuscript. Robert Masse arranged lab facilities and edited the manuscript.

The introduction for chapter 6 has been adapted from a published review article: Aguiar, M., R. Masse, B. F. Gibbs (2005). "Regulation of cytochrome P450 by posttranslational modification." Drug Metabolism Reviews **37**(2): 379-404.

Mike Aguiar performed an extensive literature search and drafted the manuscript. Bernard F. Gibbs provided scientific feedback and edited the manuscript. Robert Masse edited the manuscript.

Chapter 7 provides a thesis summary and conclusions. Overall, this thesis is perhaps more notable for the approaches and analytical characteristics of the methods developed and applied rather than application to specific clinical analytes. The goal of the author was to illustrate the utility, versatility and analytical characteristics of current MS techniques within the context of the methodologies developed and described herein.

1.0 Introduction

Clinical chemistry is a sub discipline of pathology primarily concerned with assessing health and disease by analysis of biological tissues. The discipline dates back over a century to the introduction of simple chemical tests for the analysis of blood and urine specimens. Since then, many techniques have been developed to monitor enzymatic activities and measure components of biological samples using a variety of biophysical techniques including immunoassays, electrophoresis, chromatography, and various spectroscopic methods.

Current clinical chemistry laboratories are highly automated, with the ability to perform hundreds of different tests. These fall into four broad categories; general chemistry, endocrinology, immunology, pharmacology and toxicology. High throughput laboratories, found in hospitals and commercial clinical research organizations, perform hundreds or even thousands of analyses on a daily basis. Rigorous analytical standards are respected in order to ensure high-quality patient care.

In the last decade, mass spectrometric based techniques have blossomed to become an enabling technology in clinical chemistry and basic biomedical research. Modern day mass spectrometers are versatile workhorses that can perform multiplexed analyses quickly, with excellent sensitivity and specificity on virtually any type of analyte molecule. Clinical applications of the mass spectrometer include simultaneous analyses of metabolic intermediates for detecting dozens of inborn errors of metabolism (Chace, Kalas et al. 2003),

including disorders related to steroid and thyroid hormones (Soldin, Soukhova et al. 2005; Guo, Taylor et al. 2006), toxicological evaluation of patients and athletes (Allen, Azad et al. 2005), therapeutic drug monitoring to ensure compliance and optimization of drug dosages (Gu and Soldin 2007), and quantitation of biomarker peptides and proteins (Whiteaker, Zhao et al. 2007), all with unparalleled speed, specificity and sensitivity. The breadth of MS clinical applications reported in recent years will encourage the implementation of the developing technology in clinical chemistry laboratories throughout the world within the foreseeable future. Thus, future prospects for the application of MS based technologies in fundamental medical research and molecular medicine hold much promise.

1.1 Congenital Metabolic Diseases

Congenital metabolic diseases are a large group of genetic disorders of metabolism. The term "congenital metabolic diseases" is synonymous with "inborn errors of metabolism" and "inherited metabolic diseases". The notion of an "inborn error of metabolism" was originally proposed by British physician Archibald Garrod (1857-1936) who presented the "one gene, one enzyme" hypothesis (Garrod 1923). The hypothesis was derived from studies of familial alkaptonuria which Dr. Garrod used to establish a clear link between genetics and disease inheritance. Most congenital metabolic diseases are the result of mutations of individual genes required for the metabolism of various endogenous substrates. The inactive gene product causes a block in a particular metabolic pathway such

that toxic metabolites or intermediates accumulate and give rise to various physiological problems and abnormal physiological function. Alternatively, a defective gene may cause the lack of some essential metabolic species, again causing abnormal physiological function.

Historically, congenital metabolic diseases have been categorized as disorders of four general processes i) carbohydrate metabolism, ii) amino acid metabolism, iii) organic acid metabolism, or iv) fatty acid metabolism and storage. In the post genomic era, hundreds of new congenital metabolic diseases have been discovered with a corresponding proliferation in their categorization. Although individual metabolic disorders are rare, their combined contribution becomes significant (> 1/4000 births). The challenge for the clinical chemist is to screen for multiple congenital metabolic diseases simultaneously in a high throughput fashion with accuracy.

1.2 Clinical diagnosis of Congenital Metabolic Diseases

Given the hundreds of congenital metabolic diseases identified, there are many symptoms a patient may present that might indicate an inborn error of metabolism. In order to identify affected individuals in a timely fashion, avoiding irreversible morbidity, a number of diagnostic tests have been developed over the last several decades.

1.2.1 Guthrie bacterial inhibition test

Phenylketonuria (PKU) is one of the earliest inborn errors of metabolism identified. The disorder is rare (1 in 12,000 – 15,000 newborns) and is most often the result of a defect in the gene encoding phenylalanine hydroxylase, an enzyme required for the conversion of phenylalanine to tyrosine. Affected individuals accumulate abnormally high levels of phenylalanine in their blood and urine. If not detected and treated in time (within the first few weeks of life), the disorder can lead to irreversible brain damage and mental retardation. Other symptoms include irritability, restlessness, destructive behaviour and seizures.

Since the source of the disease is a high level of circulating phenylalanine, treatment is based on a phenylalanine-restricted diet. Prior to 1961, the disorder was typically diagnosed only after brain damage and accompanying symptoms became apparent. In order to detect the illness early enough to begin dietary intervention, before neurological damage, a method for the measurement of phenylalanine in newborn blood was devised by Dr. Robert Guthrie (Guthrie and Susi 1963). Briefly, a blood sample from a newborn collected several days after birth is spotted on filter paper, dried and applied to an agar plate inoculated with a strain of *Bacillus Subtilis* and a phenylalanine analog (β-2-thienylalanine) that prevents bacterial growth. If the dried blood spot contains sufficiently high phenylalanine levels to overcome the inhibitory affects of the analog (diagnostic for phenylketonuria), the bacteria grow a colony which is readily observed.

The Guthrie test was the first of its type of only a handful of newborn screening assays implemented in the years that followed. Similar assays included tests for congenital hypothyroidism, galactosemia and sickle cell anaemia.

1.2.2 Profiling metabolic intermediates by Gas Chromatography Mass Spectrometry

Beginning in the late 1960's gas chromatography coupled to mass spectrometry (GCMS) had developed to the point where it could be used to identify a number of inborn errors of amino acid, fatty acid, and organic acid metabolism (Jellum, Stokke et al. 1971; Jellum, Stokke et al. 1972). Urine and serum samples as well as biopsies and other biological materials could be analyzed. The approach was also used for analysis of volatile fatty acids in urine (Gibbs, Itiaba et al. 1973). Non-volatile fatty acids could be derivatized to trimethylsilyl-esters to be made amenable to GCMS analysis (Mamer and Gibbs 1973). Other non-volatile analytes (organic acids, medium and long chain fatty acids, steroids, amino acids and peptides) also required a derivatization step in order to be made amenable to GC analysis. The use of GCMS for screening inborn errors of metabolism was a significant breakthrough as dozens of clinically diagnostic analytes extracted from biological specimens could be chromatographed and quantitated as individual species in a single GC separation. By the late 1970's GCMS had become a gold standard for identification of metabolic disorders from urine specimens (Goodman and Markey 1981; Niwa 1986). GCMS remains the principle method for clinically diagnosing organic acidemias (Duez, Kumps et al. 1996; Kimura,

Yamamoto et al. 1999; Kuhara 2001). Our understanding of organic acidemias and disorders of fatty acid oxidation has been mostly derived from GCMS studies of urine samples from affected patients. GCMS clearly demonstrated the potential for mass spectrometry (MS) based applications in the clinical laboratory. The principle shortcoming of GC is the requirement for volatile analytes. An ionization technique capable of ionizing the majority of clinically diagnostic analytes, which are not volatile, without derivatization and without thermal decomposition was needed. The development of matrix assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) would facilitate the ionization of non-volatile analytes (Chapter 2), opening the door to the direct analysis of a very wide array of clinically diagnostic analytes. ESI is especially convenient as it can be coupled directly to liquid chromatography, providing an additional dimension of specificity.

1.2.3 High throughput analysis of congenital metabolic disorders by tandem mass spectrometry

The essential breakthrough driving the application of MS based techniques in the clinical chemistry laboratory was the development and commercialization of tandem mass spectrometry (MS/MS) introduced in the early 1990's. Tandem MS based techniques have revolutionized newborn screening programs such that they now encompass more than thirty disorders of amino acid, organic acid and fatty acid metabolism (Table 1.1), all analyzed from a single dried blood spot sample. Typically, an analysis is completed within 5 minutes and costs less than two dollars to test for each disease (Sechi 2007). In practice, the tandem mass

Table 1.1 Disorders Detectable by Newborn Screening Using MS/MS
Source: Mayo Clinic, Rochester Minnesota

Disorder	Effectiveness of early treatment	Risk for acute crisis	Effectiveness of MS/MS
Disorders of amino acid metabolism			
phenylketonuria	+++	-	+
Other hyperphenylalaninemias	+	-	+
maple syrup urine disease	+++	+	+
Homocystinuria	+++	-	(+)
Tyrosinemia type I	+++	-	(+)
Tyrosinemia type II	+++	-	(+)
Citrullinemia	+++	+	+
Argininosuccinic aciduria	+++	+	+
Argininemia	+++	-	(+)
Disorders of organic acid metabolism			
Glutaric academia type I	+++	+	+
Propionic academia	+++	+	+
Methylmalonic acidemias	+++	+	+
Isovaleric academia	+++	+	+
3-Methylcrotonyl-CoA carboxylase deficiency	+++	+	+
3-methylglutaconyl-CoA hydratase deficiency	?	+/-	+/-
Multiple carboxylase deficiency	+++	+/-	+/-
2-methylbutyryl-CoA dehydrogenase deficiency	?	?	+
Disorders of fatty acid metabolism			
Carnitine transport defect	+++	+	+/-
carnitine palmitoyltransferase -1 deficiency (liver)	+	+	(+)
carnitine acylcarnitine translocase deficiency	+	+	+
carnitine palmitoyltransferase -2 deficiency			
neonatal onset	-	+	+
late onset	+	-	+

Disorder	Effectiveness of early treatment	Risk for acute crisis	Effectiveness of MS/MS
Disorders of fatty acid metabolism (continued)			
very long-chain acyl-CoA dehydrogenase deficiency	+	+	(+)
long-chain L-3-hydroxy acyl-CoA dehydrogenase deficiency	+	+	(+)
trifunctional protein deficiency	+	+	(+)
Medium-chain acyl-CoA dehydrogenase deficiency	+++	+	+
Medium-chain 3-ketoacyl-CoA thiolase deficiency	+	+	(+)
Medium/short chain L-3-hydroxy acyl-CoA dehydrogenase deficiency	+	+	(+)
short-chain acyl-CoA dehydrogenase deficiency	+	+	+
Functional SCAD deficiency	?	+	+
short-chain L-3-hydroxy acyl-CoA dehydrogenase deficiency (muscle)	+	+	-
short-chain L-3-hydroxy acyl-CoA dehydrogenase deficiency (fibroblasts)	+	+	+/-
short-chain L-3-hydroxy acyl-CoA dehydrogenase deficiency (liver)	+++	+	(+)
short-chain 3-ketoacyl-CoA thiolase (3-ketothiolase)deficiency	+++	+	(+)
electron transfer flavoprotein & electron transfer flavoprotein ubiquinone-oxidoreductase deficiency (glutaric acidemia type II)			
neonatal onset	-	+	+
late onset	+	+	(+)
Riboflavin responsive form(s) (glutaric acidemia type II)	+++	+	(+)

Disorder	Effectiveness of early treatment	Risk for acute crisis	Effectiveness of MS/MS
Disorders of fatty acid metabolism (continued)			
2,4-Dienoyl-CoA reductase deficiency	?	?	(+)
3-hydroxy 3-methylglutaryl - CoA synthase deficiency	+	+	-
3-hydroxy 3-methylglutaryl - CoA lyase deficiency	+	+	+

Effectiveness of treatment: +++, demonstrated; +, dietary and preventive measures, -, no effective treatment; §, liver transplantation; ?, insufficient information.
Risk for acute crisis: +, life-threatening metabolic crisis can occur at any age in untreated patients; -, symptoms usually do not present acutely; +/-, not all patients reported presented with acute symptoms; ?, number of patients reported is too small to allow reliable assessment.
Effectiveness of MS/MS: +, demonstrated in blood spots; (+), expected to be effective, but not yet conclusively demonstrated; +/-, questionable effectiveness; -, not effective.

spectrometer is capable of simultaneously monitoring many more analytes than those currently monitored in clinical applications. Indeed, current MS based proteomic investigations monitor the presence of hundreds or even thousands of proteins in a single LCMS run.

Tandem mass spectrometry involves successive stages of mass analysis separated by fragmentation of target analytes. Selection of an analyte precursor ion isolated for fragmentation, with subsequent mass analysis of fragment ions provides unequivocal specificity (Turecek, Scott et al. 2007).

For example, a mass analyzer is used to isolate ions of a specific m/z corresponding to a diagnostic acylcarnitine, steroid, peptide etc. from a mixture of

many ions entering the mass spectrometer. A typical mass analyzer can easily distinguish ions differing by a single m/z unit. The selected ion is channelled into a collision cell where the analyte is fragmented by gas phase chemistry through collision-induced dissociation (CID); that is, fragmentations resulting from gas phase collisions with a target gas (usually He, Ar, or N_2). A subsequent mass analysis is performed to scan or monitor specific and characteristic fragment ions produced. In particular, the tandem mass spectrometer can be set up to specifically monitor a series of precursor and product ion pairs for multiple reaction monitoring (MRM). The principle advantage being that the instrument focuses all analysis time (duty cycle) to monitoring discrete pairs of ions, which yields a gain in sensitivity of 1-2 orders of magnitude over an experiment where the instrument is operated in the full scan mode. The triple quadrupole operated in MRM mode is the instrument of choice for quantitative MS applications. Measured fragment ion intensities are proportional to analyte concentration, which can be estimated by comparison to an isotopically labelled internal standard signal or against a standard curve. The technique can be performed on a variety of instrument configurations (see section 2.4) but is typically run on a triple quadrupole which is relatively inexpensive and provides excellent precision and accuracy, ideal for implementation in a clinical chemistry laboratory.

1.2.4 Disorders of steroid synthesis and metabolism

Steroid hormones (glucocorticoid, mineralocorticoid, progestin, androgen and estrogen) are hydrophobic metabolites of cholesterol that mediate a multitude of

physiological processes. Cellular responses are signalled via members of the nuclear receptor superfamily, affecting transcription of target genes. Non-genomic affects mediated by alternative signal transduction pathways have also been described. Glucocorticoids such as cortisol are essential for survival and mediate metabolic, cardiovascular, immunologic and homeostatic functions in nearly every type of vertebrate tissue. Mineralocorticoids regulate blood pressure by affecting salt and water balance via kidney function. Sex steroids (androgens and estrogens) play a role in embryogenesis, reproductive function, development of secondary sexual characteristics, bone remodelling and maintenance, cognitive function, mood and behaviour, skin tone, and participate in directing the course of androgen and estrogen dependent malignancies such as prostate and breast cancer. Deregulated production or metabolism of steroids has been identified as the cause of a number of diseases. Diagnosis of steroid related diseases can be aided by determination of circulating steroid levels. A number of techniques have been employed to assay steroids in the clinical laboratory including immunoassays, GCMS and more recently LCMS. The principle challenge when assaying steroids is that circulating concentrations are very low (pM to fM) which necessitates an analytical system capable of routine quantitation with high sensitivity (Qu, Qu et al. 2007). Steroid immunoassays offer the greatest sensitivity but reagents are expensive and the technique lacks specificity, often overestimating concentrations due to cross reactivity towards related species. In addition, the immunoassays measure only one analyte per assay providing a low throughout. GC based assays for steroids offer adequate specificity and sensitivity but normally require lengthy

sample derivatization and may not be suitable for thermally labile steroids. LCMS offers enough sensitivity to measure physiological levels of steroids, unparalleled specificity, does not induce thermal degradation, and offers the ability to measure multiple steroids directly without lengthy derivatization. LCMS is analogous to GCMS in that compounds are resolved chromatographically before they are introduced to the mass spectrometer. LCMS differs from GCMS in that the mobile phase is liquid, usually a combination of water and organic solvents, instead of gas. While GC separations are normally performed at elevated temperatures which may lead to analyte decomposition, LC separations are typically performed at ambient temperature, avoiding thermal decomposition.

A key development in LCMS for the analysis of physiological levels of steroids was the introduction of atmospheric pressure photoionization (APPI) (Robb, Covey et al. 2000; Robb and Blades 2006). The technique is well suited for the efficient and specific ionization of non-polar analytes such as steroids. Liquid chromatography coupled to APPI and MS has been employed for the high throughout simultaneous quantitation of twelve steroids in a clinical laboratory setting. The method is useful for the diagnosis of several disorders of steroid metabolism including primary hyperaldosteronism, adrenal insufficiency, Cushing's syndrome, disorders of gonadal function and congenital adrenal hyperplasia (Guo, Chan et al. 2004; Holst, Soldin et al. 2004; Guo, Taylor et al. 2006; Holst, Soldin et al. 2007).

1.2.5 Disorders of thyroid hormone synthesis and metabolism

Thyroid hormones, thyroxine (T4) and triiodothyronine (T3) are iodinated derivatives of tyrosine produced in the follicular cells of the thyroid gland. Hormone biosynthesis involves iodination of thyroglobulin tyrosine residues. Iodotyrosines are then transferred and coupled to form T4 and T3. Proteolysis of thyroglobulin releases T4 and T3 for secretion into the blood stream. Circulating levels of T4 are on the order of 40-100 times greater than T3. Monodeiodination of T4 produces T3 locally by specific deiodinases present in target tissues. Both T4 and T3 are biologically active, but T3 is several fold more potent. The bulk of circulating T4 and T3 is protein bound in a biologically inactive state.

Thyroid hormone signalling is mediated via specific nuclear hormone receptors affecting gene transcription. Thyroid hormones are critically important regulators of systemic basal metabolic rate affecting protein, fat and carbohydrate metabolism. Disorders of thyroid hormone deficiency and excess have been characterized.

Patients affected by hypothyroidism typically present symptoms associated with a decreased metabolic rate such as poor muscle tone, chronic fatigue and intolerance to cold. Although uncommon, a lack of sufficient thyroid hormone during foetal development can result in cretinism. The principal causes of hypothyroidism are iodine insufficiency, particularly in areas where there is a low content of iodine in the soil (central Africa, central Asia, South American Andes and Indonesia). In developed countries, the primary cause is Hashimoto's thyroiditis, an autoimmune disorder where IgG type antibodies bind thyroglobulin

and form deposits in thyroid follicular cells. Iodine supplementation or thyroid hormone treatment is indicated for affected patients.

Symptoms of hyperthyroidism are just the opposite of hypothyroidism, with an accelerated basal metabolic rate and elevated body temperature. A prominent feature of hyperthyroidism is exophthalmos (bulging of the eyeball). The most common causes of hyperthyroidism are Graves' disease and toxic adenoma. Graves' disease is an autoimmune disorder whereby auto-antibodies bind to and chronically stimulate receptors on the thyroid gland. Chronic stimulation results in a hypertrophic thyroid visible as a goitre. The overactive thyroid produces excessive quantities of thyroid hormone without feedback inhibition, which in turn affects systemic metabolic homeostasis.

Adenoma of the thyroid can also lead to an overproduction of thyroid hormones. The disorders may be treated with radioactive iodine which normally concentrates in the thyroid, or ablative surgery to remove the neoplasm.

Clinical diagnosis of hypo and hyperthyroidism involves the determination of circulating thyroid hormone levels. Radioimmunoassays (RIA) have been used to measure thyroid hormone but these are subject to interference by iodothyronine-binding antibodies (Soukhova, Soldin et al. 2004). The auto antibodies may affect RIA results to yield erroneously high or low values which often are not consistent with clinical features. In addition, the immunoassays can lack specificity, with widely varying results depending on the particular immunoassay used. Alternative methods employing GCMS, HPLC, and LCMS have been reported (Burman, Bongiovanni et al. 1981; Thienpont, De Brabandere et al. 1994; De Brabandere,

Hou et al. 1998; Tai, Sniegoski et al. 2002; Soukhova, Soldin et al. 2004). The method of Soukhova el al., 2004 involves isotope dilution with a deuterium labelled L-thyroxin-d_2 internal standard for simultaneous determination of serum T3 and T4. Sample analysis is simple, specific, precise (%CV 3.5-9.0) and high throughput.

1.3 Pharmacokinetics and drug metabolism

Pharmacology is the study of substances that bring about physiological changes. Such substances may have medicinal properties and are therefore of vital importance to the practice of medicine. A critical aspect of developing a new medicine is to gain an understanding of the drug's absorption, distribution, metabolism and elimination (ADME). To achieve this, biological specimens (blood, urine, feces, etc…) are sampled from a study subject prior to and at defined intervals after drug administration. As the drug is absorbed, metabolized and cleared from the body, a pharmacokinetic profile can be obtained by quantitative analysis using a variety of techniques including immunoassays, and HPLC with a variety of detection modes including UV, fluorescence, amperometric, or MS. The choice of analytical method depends on several factors including the nature of the analyte(s), complexity of sample matrix, sensitivity and specificity required. Current pharmaceutical clinical testing (phases 1-4) is typically performed by reversed phase liquid chromatography with a triple quadrupole tandem mass spectrometer. The general applicability, sensitivity,

specificity and good analytical performance provided by the LCMS make it well suited for the analysis of numerous drugs in complex matrices (Blair and Tilve 2002; Ding, Yang et al. 2006). Typically, biological samples from animal or human subjects are mixed with an internal standard, extracted to remove particulates and bulk impurities and analyzed by LCMS. Analyte concentration is determined by back calculation against a suitable calibration curve. This type of instrumental setup has become the workhorse of the bioanalytical laboratory. More recently, there has been considerable interest in the use of Accelerator Mass Spectrometry (AMS; section 2.2.9) for a number of ultra-high sensitivity applications in early stage pharmaceutical development. In particular, AMS can be used as a tool for preclinical development (PK/ADME), phase 0 (microdosing) and phase 1 (metabolite profiling) studies. The technique provides quantitative sensitivity down to the attomol range, several orders of magnitude more sensitive than other forms of mass spectrometry. ADME information for a test substance can be gleaned from sub-therapeutic microgram doses (micro dose on the order of 1/100 of the dose required for therapeutic affect) of ^{14}C-labelled drug. Compounds that are difficult or extremely expensive to radio-label can be prepared in small quantities for use in phase 0 studies with AMS detection. The expectation is that AMS can be used to identify drug candidates that have poor ADME characteristics before committing considerable resources to further clinical testing, thus avoiding unnecessary animal testing and rendering pharmaceutical development more cost effective and efficient. At the moment, few facilities are equipped with the necessary instrumentation as the technique is

prohibitively expensive. The major pharmaceutical companies and select national research centers are equipped with AMS technology (Lappin, Kuhnz et al. 2006).

1.4 Therapeutic drug monitoring

Therapeutic drug monitoring (TDM) uses serum drug concentrations, pharmacokinetics, pharmacodynamics and clinical observations to optimize patient response to drug therapy. Most drugs do not require TDM as they can safely be taken over a wide range of dosage while still providing effective therapy. Furthermore, large variations in pharmacokinetic response can also be safely tolerated for most drugs. There are however a number of drugs, including the antiretroviral drugs used to treat HIV patients (Chapter 3), for which TDM is appropriate and may be necessary for optimal patient care. The goal is to maintain serum drug concentrations within an optimal therapeutic range. Concentrations below this range are ineffective while excessive concentrations result in toxicity. Where there is no clear endpoint to monitor patient response (e.g. Blood pressure for hypertensive patients, blood glucose for diabetic patients) drug serum concentrations are a useful surrogate endpoint. In addition to measuring serum drug concentration, every effort must be made to monitor additional clinical symptoms for a global assessment of patient status. TDM is especially useful to determine if a change in pharmacokinetics has occurred in response to an altered physiological state, a change in diet, or a change in pharmacokinetics induced by the introduction of an additional drug(s).

While a number of methods have been developed to monitor serum drug concentration (Immunoassay, GC, LC), the advantages inherent in LCMS make this technology an attractive approach to be used in TDM. Chapter 3 outlines a method used for the simultaneous determination of eleven antiretroviral drugs in serum in a high throughput fashion. No other analytical technique can provide a comparable multiplexed analysis.

1.5 Proteomics

A reductionistic approach using purified components has allowed researchers to appreciate the principle machinery at work in a cell. While such an approach has been essential to arrive at our current understanding of the "nuts and bolts", the challenge for current cell and molecular biologists is to gain an understanding from a systems biology standpoint; to understand how all the parts work together. There have been several important scientific advances that place the scientific community in a position to begin addressing the systems biology questions. Genome sequencing efforts, including that of the human genome, have laid the foundation to be used in transcriptome and proteome studies. Microarray technology allows scientists to probe gene transcription from the complement of genes in an entire genome in one experiment. The cell biologist can now monitor genomic transcription in response to specific stimuli and as a function of different cell states. Comparative microarray studies can be performed in a semi-quantitative fashion. Thus, we are beginning to obtain an understanding of groups

of genes that are simultaneously up-regulated or down-regulated at different stages of the cell cycle and in response to different stimuli (Mann 1999). These groups of genes provide insight about cellular systems involving multiple genes that work together in a given cellular response system. The trouble with microarray studies is that they only provide an indication of which components might be participating. In order to understand systems biology from a functional standpoint, the cell biologist must turn to the gene products that carry out cellular functions; the proteins (Mann 1999).

Proteomics is the systematic analysis of all proteins in a cell or tissue. Over the last two decades, developments in modern mass spectrometry techniques have made it possible to perform large scale studies of protein identification, characterization, and quantitation (Domon and Aebersold 2006). Genomic sequences are used to relate peptide and protein sequences to specific genes. Prior to the use of mass spectrometry for determining primary structure (amino acid sequence), chemical methods using N-terminal sequencing (Edman sequencing) were employed. N-terminal sequencing involves sequential rounds of i) N-terminal derivatization with phenylisothiocyanate (PITC) ii) cleavage of the derivatized residue with trifluoroacetic acid (TFA) to yield the anilinothialinone derivative (ATZ-amino acid) iii) solvent extraction and conversion to a phenylthiohydantoin derivative (PTH-amino acid) iv) and reversed phase chromatographic separation for amino acid identification by retention time. A typical Edman sequencer can sequence one or two amino acids per hour and requires nanomolar quantities of analyte. In contrast, the tandem mass

spectrometer can sequence a typical tryptic peptide (10-30 residues) in one second with femtomol sensitivity.

There are two principle approaches that are being explored in proteomics. The "top down" approach aims to analyze intact proteins by MS while the "bottom up" approach analyses peptide fragments derived from hydrolysed proteins. The "bottom up" approach is much more common and involves enzymatic digestion using a hydrolytic agent such as trypsin or pepsin; a variety of other proteolytic enzymes (chapter 6) or chemical cleavage agents can also be used. Once proteins have been digested, the resulting peptides are readily applied to a variety of chromatographic separations and are detected with better sensitivity than intact proteins. Also, peptides of less than 3-4 kDa are readily fragmented by CID for peptide sequencing whereas larger peptides or proteins are not readily fragmented by CID. The most widely used instrument for peptide mass analysis and sequencing is the ion trap (section 2.2.4, 2.2.5), although other types of mass spectrometers have also been used.

The "top down" approach is used to measure the mass of large peptide and intact proteins and can be used to sequence these using electron capture dissociation (ECD) and more recently electron transfer dissociation (ETD) (Domon and Aebersold 2006). ECD and ETD are alternate fragmentation techniques to CID that are based on electron transfer reactions rather than gas phase collisions. Peptides and proteins can be efficiently fragmented along the peptide backbone (with minimal cleavage of amino acid side chains) for sequence analysis. Currently, ECD is only commercially available on FTMS instruments; which

typically cost $ 1,000,000 US or more, require highly trained users and constant attention to maintain (eg. the superconducting magnet must remain cooled with liquid Helium/Nitrogen). Within the last 2 years ETD has been integrated into commercially available ion trap and Orbitrap instruments; it is expected that ETD will find widespread use for top-down protein analysis as well as a complimentary bottom-up peptide fragmentation technique.

1.5.1 Protein and peptide fractionation

Most biological samples including serum, cells, tissues etc.. are a complex mixture of proteins, peptides and low molecular weight molecules. The various components can vary by 10 orders of magnitude in concentration (Frohlich and Arnold 2006). If a complex mixture is introduced into a mass spectrometer, the major components will generally be observed to the exclusion of others present at lesser concentration. In the case of a proteolytic digest, each protein typically gives rise to dozens of peptides which generates even greater complexity. Thus, a complex sample will generally necessitate some type of sample fractionation in order to study the vast majority of components which are present in moderate to low abundance. Fractionated samples also produce simplified mass spectra that are more readily interpreted.

A number of methods have been employed in order to fractionate a sample prior to mass analysis. Proteins in a complex mixture can be resolved by polyacrylamide gel electrophoresis (PAGE), ether in one or two dimensions. Two-dimensional gel electrophoresis is a powerful technique for resolving proteins and even protein isoforms. Gel spots are generally the result of a single

protein species. Protein bands/spots (containing one or a few proteins) can then be digested in-situ for subsequent mass analysis of peptides. Peptide masses resulting from the digestion of a pure protein spot can be determined by MS which are then used for identification using peptide mass fingerprinting (PMF). If this information does not allow unequivocal identification of the protein, individual peptides can be subjected to tandem mass spectrometry for peptide sequencing based protein identification.

An alternative approach is to avoid gel electrophoresis altogether. A protein mixture can be digested directly with subsequent fractionation of the complex peptide mixture by two or more dimensions of liquid chromatography prior to MS analysis. Such an approach is known as "shotgun proteomics". Because of the multitude of peptides generated from digestion of a complex protein sample, the approach necessitates the use of a tandem mass spectrometer for peptide sequencing based protein identification. Alternatively, a combination of gel separation followed by LCMS with tandem MS can also be employed when necessary.

Other, more focused, methods for protein or peptide enrichment prior to MS analysis include affinity isolation of glycoproteins, phosphoproteins/peptides, or immunological capture (Boyle, Hess et al. 2006).

1.5.2 Protein identification

There are two main ways MS is used to identify proteins; PMF and peptide sequencing using tandem MS. PMF uses the masses of peptides observed from protein hydrolysis (with defined cleavage specificity) to search against a database

of peptide masses arising from the in silico digestion of known proteins. If a protein in the reference list gives rise to a significant number of predicted masses that match experimental values, there is some evidence that this protein was present in the original sample. PMF works best for purified proteins or individual gel spots present in a 2D-Gel. The confidence of PMF-based protein identifications can be greatly enhanced by using accurate measurement of monoisotopic peptide masses with instruments that provide low ppm to sub ppm mass accuracy (Chapter 2). Tight constraints on peptide masses can discriminate between peptides that are isobaric but do not have just the right elemental composition. PMF protein identification software is available as part of instrument provided packages and freely accessible software available over the internet (Sequest, Mascot, OMSSA and X!Tandem).

Tandem MS is a more sophisticated and powerful method for protein identification. Peptides sampled into the MS are subject to CID to generate a set of information-rich product-ion fragments. The fragmentation process primarily gives rise to cleavage products that break along the peptide backbone in a predictable fashion. An in-silico database of theoretical fragmentation spectra derived from peptides that have the correct m/z and digestion specificity constraints is then used for peptide (and protein) identification. Since a product ion spectrum can be obtained in 1 second, a single LC tandem MS run of a complex peptide mixture can measure thousands of peptide masses and their corresponding product-ion spectra. The most popular algorithms for searching tandem MS data against genomic databases are Sequest and Mascot. Digestion of

posttranslationally modified proteins will yield modified peptides (phosphorylation, methylation, acetylation, etc.) that can also be identified and mapped using tandem MS (Patricia Hernandez 2006).

When a genomic database is not available, de novo peptide sequencing can be performed using specialized tandem MS interpretation software; PEAKS, LuteFisk and Sherenga.

Sequence tag based searching is a less automated alternative for tandem MS interpretation. The user inputs information about enzyme specificity, a peptide mass observed and a partial peptide sequence derived from a series of several prominent product ions observed. This information is then searched against genomic databases to assign peptides consistent with the entered information. Sequence tags can be searched using appropriate software; SPIDER and GutenTAG.

1.5.3 Protein quantitation and Clinical biochemistry

The ability to monitoring changes in protein abundance as a function of cell state or response to stimulus will provide a deeper understanding of biological systems (Mann 1999). For example, a comparison of cells grown under different conditions, cancerous vs. normal, different stages of the cell cycle, stimulated vs. non-stimulated etc. can inform about fundamental biological processes. One method for monitoring relative protein quantities is to use differential gel electrophoresis (DIGE). The technique involves labelling samples with dyes or fluorescent reagents prior to 2D-PAGE. Equivalent but differentially labelled proteins migrate to the same position in a gel. Absorption or fluorescence

differences in the labelled proteins can then be used to monitor relative protein abundances. Differentially expressed proteins can subsequently be interrogated by MS for protein identification. However, DIGE is restricted to those proteins that can be applied to 2-D gel electrophoresis. Very large, very small or membrane bound (hydrophobic) proteins may not be amenable to 2-D gel electrophoresis. As an alternative, the MS based techniques used for protein identification described above can be adapted for protein quantitation purposes. Several MS based strategies have been described for the large scale quantitation of proteins and for a more targeted hypothesis-driven quantitation of select proteins or posttranslationally modified proteins (Bantscheff et al., 2007). Indeed, once proteins and posttranslational modifications have been identified, the targeted MS based approaches are especially useful for protein quantitation or quantitation of protein modifications (e.g. phosphorylation, ubiquitination) (Gerber, Rush et al. 2003; Kirkpatrick, Denison et al. 2005). Typically, stable (non-radioactive) enriched isotopes of carbon (^{13}C) nitrogen (^{15}N) or oxygen (^{18}O) are incorporated into or used to label proteins in one sample while a comparison sample uses naturally abundant isotopes (^{12}C, ^{14}N and ^{16}O). The two samples are mixed and applied to gel electrophoresis or digested without prior gel separation. Isotopic variants of peptides derived from the different samples are easily distinguished by their m/z but are otherwise chemically identical in terms of chromatographic and MS behaviour. Heavy and light isotopic MS signal intensities correspond to

Table 1.2 Clinically diagnostic proteins
Source: Mayo Clinic, Rochester Minnesota

Diagnostic protein (Disease)	**Tissue**	**Analysis method**
Acetylcholinesterase (Organo-Phosphate Poisoning)	Amniotic fluid, Erythrocytes	Spectrophotometric-thiocholine production
α-Fucosidase (Fucosidosis)	Fibroblast, Erythrocytes	Fluorometric
α-Galactosidase (Glycogen Storage Disease, Type II)	Serum, Fibroblasts, Leukocytes	Fluorometric
α-Glucosidase	Fibroblasts	Fluorometric
Aldolase	Serum	Ultraviolet, Kinetic
α -L-Iduronidase (Hurler Disease)	Fibroblasts Leukocytes	Fluorometric
Alkaline phosphatase	Serum	Chemical inhibition and differential inactivation
α -Mannosidase (Lysosomal Storage Disorders)	Fibroblasts, Leukocytes	Fluorometric
Aminolevulinic Acid Dehydratase and Porphobilinogen Deaminase (Acute Intermittent Porphyria)	Erythrocytes	Porphyrin quantitation by Fluorometry
Amylase	Body fluids	Substrate kinetic, Exclusion Column Chromatography
α-N-Acetylglucosaminidase (Sanfilippo Syndrome Type B)	Serum, Fibroblasts	Colorimetric
Angiotensin Converting Enzyme (Sarcoidosis)	Serum	Spectrophotometry
Arylsulfatase A (Metachromatic Leukodystrophy)	Fibroblasts, Urine, Leukocytes	Colorimetric
Arylsulfatase B (Maroteaux-Lamy Syndrome)	Fibroblasts	Colorimetric
β-Galactosidase (Mucopolysaccharidosis IVb)	Fibroblasts, Leukocytes	Fluorometric
β-Glucosidase (Gauchers Disease)	Fibroblasts, Leukocytes	Fluorometric

Diagnostic protein (Disease)	Tissue	Analysis method
β-Glucuronidase (Sly Syndrome)	Cerebrospinal fluid, Fibroblasts	Fluorometric
Biotinidase	Blood	Colorimetric
Carbohydrate Deficient Transferrin (Carbohydrate Deficient Glycoprotein Syndrome)	Serum	Affinity chromatography/MS
Ceruloplasmin, (Wilson's Disease)	Serum	Colorimetric, Kinetic
Creatine Kinase (CK) Isoenzyme Electrophoresis	Serum	Electrophoresis, densitometry
Cystathionine β-Synthase	Fibroblasts	Enzymatic reaction with radiolabel marker
Familial Amyloidosis	Blood	LC-MS
Galactokinase (Galactosemia)	Blood	Radioisotopic
Galactose-1-Phosphate Uridyltransferase (Galactosemia)	Blood	Ultraviolet, Kinetic
Galactosylceramide Beta-Galactosidase (Krabbe Disease)	Fibroblast Leukocytes	Radioisotopic
Hexosaminidase (Tay-Sachs Disease)	Amniotic Fluid Serum Fibroblasts Leukocytes	Fluorometric
Iduronate Sulfatase (Hunter syndrome)	Fibroblasts	Enzymatic, Radio labelled
Lactate Dehydrogenase Isoenzymes	Serum	Electrophoresis densitometry
Aspartate Aminotransferase	Serum	Electrophoresis
Macroamylase	Serum	Colorimetric rate reaction, Exclusion column chromatography
N-Acetylgalactosamine-6-Sulfate Sulfatase (Galactosamine-6-Sulfatase Deficiency)	Fibroblasts	Enzymatic/Radio labelled/Anion exchange chromatography
5'Nucleotidase	Serum	Enzyme kinetic

Diagnostic protein (Disease)	Tissue	Analysis method
Pancreatic Amylase	Serum, Body fluid	Monoclonal antibody
Sphingomyelinase (Sphingomyelinase Deficiency)	Fibroblasts	Colorimetric
Thiopurine Methyltransferase (Myelotoxicity)	Erythrocytes	Enzymatic end point/LC-MS/MS
Uroporphyrinogen Decarboxylase (Erythrohepatic uroporphyria)	Erythrocytes	HPLC
Uroporphyrinogen III Synthase (Gunthers Disease)	Erythrocytes	HPLC
Albumin (Calcipylaxis, nephrotic syndrome)	Plasma, Urine	bromocresol green method/ Colorimetry

relative peptide (and protein) abundance. The most popular methods for isotope labelling are stable isotope labelling with amino acids in cell culture (SILAC), trypsin-catalyzed ^{18}O-labeling, isotope coded affinity tagging (ICAT) which is also useful for affinity isolation of tagged peptides prior to MS analysis, and isotope tags for relative and absolute quantitation (ITRAQ) (Masaru Miyagi 2007). An alternative strategy is to use isotope dilution with exact amounts of labelled internal standards for absolute protein quantitation (AQUA) (Gerber, Rush et al. 2003). The AQUA strategy is an attractive approach for implementation in a clinical setting as equipment already in routine use for small molecule quantitation (i.e. LC-triple quadrupole tandem MS) can be readily adapted for quantitation of target peptides using MRM in a high throughput fashion (Hortin 2007). This avoids investment in more specialized instrumentation typically used in proteomic investigation. In terms of clinical applications, a number of diagnostic enzymes and proteins are normally monitored to indicate disease status

(Table 1.2). While clinically relevant enzymes can be monitored indirectly by following enzyme kinetics, non-enzymatic proteins (e.g. Albumin) must be detected directly, typically using immunological methodologies which are low throughput and require expensive reagents (Becher, Pruvost et al. 2006). Enzyme kinetic assays are also low throughput, only measuring the activity of one enzyme per assay. In contrast, LCMS is ideal for multiplexed analysis and solvents/reagents are inexpensive (water, acetonitrile, methanol, TFA). Labelled internal standard peptides are easily synthesized and can be ordered from a number of commercial sources.

2.0 Overview of Mass Spectrometry

Mass spectrometry is an analytical technique used to resolve and measure gas phase ions of different mass to charge (m/z) ratios. The technique is amenable to analysis of many compound classes ranging from small gaseous molecules to macromolecules with virtually no limit in molecular weight. The basic mass spectrometer is composed of four parts a) ion source for producing gaseous sample ions, b) mass analyzer (or mass analyzers in the case of tandem mass spectrometry) capable of resolving ion species c) ion detector and d) computer system for operating the instrument and storing/displaying data (mass spectra). An elaboration of the basic instrument is the tandem mass spectrometer which is useful for obtaining characteristic product ion spectra from selected precursor ions. The tandem mass spectrometer can also be operated in several other scanning

modes including precursor ion scanning and neutral loss scanning. The inherent characteristics of the tandem mass spectrometer make it well suited for clinical chemistry laboratory analyses. Mass spectrometry is used for many applications including a) compound identification b) determining chemical composition (using isotope intensities) c) chemical structure determination using fragmentation spectra d) quantitative analysis and e) studying various physical, chemical or biological properties of analytes or analyte complexes.

2.1 Ionization Sources

2.1.1 Electron and Chemical ionization for GCMS

Initial applications of the mass spectrometry involved the use of GCMS for monitoring metabolic intermediates. GC is ideally suited for coupling to mass spectrometry since analytes elute from the column already in the gas phase and are conveniently directed into the MS. Typical GCMS ionization sources include electron ionization (EI) and chemical ionization (CI). Electron ionization (EI) is the oldest and most well established of the MS ionization sources. It is suitable for ionizing volatile, thermally stable neutral organic analytes of low molecular weight (< 700 Da). In EI, the sample (or effluent from GC column) is directed into the ionization source where the analytes encounter a beam of electrons emitted from a metallic filament (typically 70 eV) to which a voltage is applied. The interaction of sample molecules with the electron beam results in the formation of ionized radical species which are amenable to MS analysis. The radical species are generally unstable and fragment readily, producing mass

spectra characteristic of the parent molecule (McLafferty 1980). The appearance of diagnostic fragments can be useful for rationalizing the structure of an unknown species. As a complimentary alternative to EI, CI produces ionized analyte species by charge exchange reactions occurring in the source region with reagent gasses (Munson and Field 1966). CI reagent gasses (methane, ammonia) are present in a large excess relative to analyte species. Reagent gasses are ionized by interaction with the EI electron beam producing charged reagent gasses that react with sample analytes by acid/base reactions. Protons or other charged species are transferred from the reagent gas to the analyte or visa versa. Charge transfer is dependent on the relative proton affinities of the analyte and reagent gasses. CI is a "soft" ionization process as compared to EI and produces predominantly ionized analyte species such as pseudo-molecular ions $(M+H)^+$ or $(M-H)^-$ which tend to fragment less than the radical species formed by EI. Typically, GCMS instruments are equipped with quadrupole mass analyzers (section 2.2.3) electron multiplier detectors.

2.1.2 Fast atom bombardment and secondary ion mass spectrometry

Although the GCMS revolutionized the analyses of clinically diagnostic volatile analytes and other small organic molecules made amenable to GCMS analysis through derivatization, more powerful methods for the analysis of polar and charged non-volatile analytes without the need for lengthy derivatization were sought. Two MS related developments introduced at roughly the same time did much to greatly expand the scope of amenable analytes with simplified sample handling procedures. The introduction of fast atom bombardment (FAB) was an

important step in the development of ionization sources for mass spectrometry (Barber, Bordoli et al. 1981). The technique is suitable for the ionization of polar or charged non-volatile and/or thermally labile molecules such as low molecular weight drugs, steroids, amino acids, acylcarnitines, peptides and proteins without derivatization.

The physicochemical processes that produce FAB ion species are complex and different from EI or CI. FAB works by bombarding a metal target coated with a liquid matrix in which the sample has been dissolved with a high energy (4-10 keV) beam of neutral atoms (typically Argon or Xenon). Most of the energy is absorbed by the matrix, from which analyte ions are ejected by a poorly understood process. A key element of FAB is the choice of a suitable matrix. Typical matrices include glycerol and 3-nitrobenzyl alcohol (3-NBA). FAB is also a "soft" ionization technique that produces primarily pseudo molecular ions (positive and negative) which are similar to those produced by CI, together with fragment ions at lower mass. Secondary ion mass spectrometry (SIMS) is a related technique used for enhanced sensitivity of moderate to high molecular weight analytes (>5000 amu). SIMS uses a beam of high energy cesium ions rather than the Argon or Xenon atom beam used in FAB.

Commercial tandem mass spectrometers were introduced roughly at the same time as FAB, and the two techniques were coupled for the multiplexed analysis of a variety of clinically diagnostic analytes including steroids, fatty acids, organic acids, acylcarnitines, drugs and peptides (Chace, DiPerna et al. 1999). The specificity and ability to perform high throughput multiplexed analyses afforded

by the tandem mass spectrometer were key factors driving the implementation of mass spectrometry based techniques in the clinical chemistry laboratory.

2.1.3 Matrix assisted laser desorption ionization

Matrix assisted laser desorption ionization (MALDI) is a "soft" ionization technique well suited for the ionization of biomolecules (peptides, proteins, nucleotides). An acidified liquid sample is mixed with an energy absorbing material (sinapinnic acid, alpha-cyano-4-hydroxycinnamic acid or 2,5-dihydroxybenzic acid), the mixture is spotted on an inert metal surface and allowed to dry to form co-matrix crystals. The dried sample spot is then irradiated with a laser (typically a nitrogen laser) which vaporizes a portion of the crystallized material. The laser energy is primarily absorbed by the MALDI matrix to protect analyte molecules from thermal decomposition. Sample vaporization results in the gas phase production of charged analyte species, typically singly protonated adducts, by processes which are poorly understood. MALDI is commonly coupled to time-of-flight (TOF) mass analyzers which resolve species in the time domain. The MALDI laser pulse serves as a convenient reference time point for the TOF analyzer (Karas and Hillenkamp 1988).

2.1.4 Ionization sources compatible with liquid chromatography

2.1.4.1 Electrospray Ionization

Electrospray ionization (ESI) (Whitehouse, Dreyer et al. 1985) has largely replaced FAB and has become the ionization source of choice for many MS applications. ESI is amenable to the largest variety of analyte species including

drugs and biomolecules and can afford sensitivities in the attomol range under optimal conditions. In ESI a liquid solution containing the analyte of interest flows through a capillary to which a voltage of 1-5 kV is applied. As the liquid emerges from the capillary, it forms a fine plume of charged solvent droplets. The solvent in the charged droplets evaporates until charged analytes are ejected into the gas phase as a result of Coulombic repulsion. ESI operates in the nL to μL /min flow rate range but can accommodate higher flow rates using a variety of nebulization assisted techniques with or without a heated drying gas. In particular, ESI is well suited to be coupled directly to a variety of liquid chromatography techniques (Whitehouse, Dreyer et al. 1985).

2.1.4.2 Atmospheric pressure chemical ionization

Atmospheric pressure chemical ionization (APCI) is a complimentary ionization technique for certain polar analytes that do not ionize well by ESI; typically compounds that do not readily ionize in solution. APCI involves high temperature (480 °C) nebulization of HPLC effluent flowing in the 50 μL – 2 mL /min range. Solvent vapours are then directed towards a corona discharge needle which ionizes the vapours to form reagent gasses. APCI works in an analogous fashion to CI, in that analytes are ionized by a complex series of reactions with charged reagent gasses formed in the source region.

2.1.4.3 Atmospheric pressure photoionization

Another ionization source for analytes in the condensed phase is atmospheric pressure photoionization (APPI). HPLC effluent is mixed with a photoactive reagent (toluene), nebulized with a probe similar to that used for APCI and

exposed to laser light which reacts with the toluene to form reagent gasses for the charge exchanged ionization of polar and non-polar analytes. (Robb, Covey et al. 2000; Robb and Blades 2006). The technique is well suited for compounds such as steroids to the exclusion of ionizing many ionic or highly polar species. Consequently, spectra produced by APPI are largely devoid of chemical background, which is desirable in high sensitivity analytical applications.

2.2 Mass analyzers

Mass analyzers separate the ions according to their mass-to-charge ratio. All mass spectrometers (with the exception of the ion mobility mass spectrometer which operates under a partial vacuum, section 2.2.8) are based on dynamics of charged particles in electric and magnetic fields in vacuum where the following two laws apply:

(1) $\mathbf{F = z(E + v \times B)}$ **(Lorentz force law)**

(2) $\mathbf{F = ma}$ **(Newton's second law of motion)**

where F is the force applied to the ion, m is the mass of the ion, a is the acceleration, z is the ionic charge, E is the electric field, and v x B is the vector cross product of the ion velocity and the magnetic field.

Equating the above expressions for the force applied to the ion yields:

(3) $\mathbf{(m/z)a = E + v \times B}$

Expression 3 is the classic equation describing the motion of charged particles in a vacuum under a given set of electric and magnetic fields. When applied with the knowledge of a particle's initial conditions, expression 3 accurately describes the

particle's motion in space and time. An interesting observation to be made here is that any two particles with the same physical quantity m/z will behave the same way in the mass spectrometer (under vacuum). Although the field is referred to as mass spectrometry, the quantity which is actually measured is the ratio of mass to charge rather than mass. Graphical representation of MS data is normally presented with the dimensionless m/z ratio along the x axis and signal intensity along the y axis.

Many types of mass analyzers have been developed and commercialized over the last few decades. Some use static, dynamic, magnetic or electric fields, but all operate according to expression 3. Every mass analyzer has its strengths and weaknesses. The following section describes the more common mass analyzers which have been developed and commercialized, most of which have made their introduction into major clinical chemistry laboratories.

2.2.1 Sector mass spectrometer

The sector mass spectrometer is among the earliest designs implemented for mass spectrometric analysis. The instrument uses an electric and/or magnetic field to direct the path and/or velocity of charged particles (ions) (Thomas W. Burgoyne 1996). The instrument changes the direction of ions transiting the mass analyzer. Ion trajectories are bent such that the ion path depends on the m/z ratio. The curvature of the trajectory of ions with a smaller ratio of m/z is more pronounced as compared to ions of larger m/z ratio. By manipulating the strength of the electric and/or magnetic fields, ions of specific m/z can be directed through a curved path towards the detector which measures ion abundances. The sector

instrument can be operated with fixed settings of electric and/or magnetic field strength to select a narrow range of m/z, or it can be operated to scan through a range of m/z in order to identify ionic species.

2.2.2 Time-of-flight

Perhaps the easiest mass spectrometer to understand is the Time-of-flight (TOF) analyzer. It uses an electric field to accelerate a population of ions through the same potential, and then measures the time they take to reach the detector. The kinetic energy imparted to every ion is the same. Thus, ion velocities will depend only on the quantity m/z. Lighter ions will travel through a field free vacuum region to reach the detector first. Ion drift times are calibrated to equate the time-of-flight with m/z.

2.2.3 Quadrupole

The quadrupole mass analyzer is among the most popular used in mass spectrometry. Quadrupole instruments are easy to use, cover a good mass range (typically 10-3000 m/z), provide good linearity for quantitative work, sufficient resolution to resolves isotopic clusters of singly charged species (typically R = 1000), mass accuracy of 0.1-0.2 m/z, and good quality mass spectra. Most importantly, quadrupole instruments are relatively inexpensive.

The quadrupole uses oscillating electrical fields applied to two pairs of metal rods (Frohlich and Arnold 2006). At any instant, one pair of rods is held at a positive electric potential while the other pair is held at a negative potential. Voltages applied to the rods are alternated at a radio frequency (RF). Simultaneously, an appropriate direct current (DC) potential is also applied to the rods. The

combination of RF and DC applied determine the resolution of mass filtering. The amplitude of RF and DC voltages will determine which ions (of a particular m/z) will have a stabilized oscillatory trajectory to emerge from the quadrupole and arrive at the detector.

2.2.4 Quadrupole ion trap

The quadrupole ion trap (3 dimensional trap) was initially applied to GCMS and subsequently adapted to LCMS. The trap stores ions between a ring electrode and two end cap electrodes. Ions are stabilized in the trap by applying an RF voltage to the ring electrode. For optimal resolution and sensitivity, ions are focused at the center of the trap region where electric fields approach ideality. In practice, this is achieved by collisional cooling with an inert damping gas (helium) to stabilize ions. Mass resolution achievable with an ion trap is a function of scan range and scan speed. Scanning a range of several hundred m/z in less than 1 second will provide resolution comparable to a quadrupole. However, much higher resolution (R = 5000) can be obtained by scanning a reduced mass range at low speed. The ion trap can also be used for tandem MS or even MS^n experiments, all in one trap. (March 2000). For comparison, tandem mass spectrometry with a quadrupole instrument requires one quadrupole for each dimension of mass analysis and collision cell; hence the name triple-quadrupole for tandem quadrupole MS.

2.2.5 Linear quadrupole ion trap

A linear quadrupole ion trap uses four parallel rods, similar to that described above for the quadrupole, to define the region where ions can be trapped. The linear form of the trap can be used as a selective mass filter (like a quadrupole), or

as an ion trap by enclosing ions in a field for storage along and between the axis of the electrodes. The linear ion trap is also capable of performing MS^n experiments in a manner similar to the quadrupole ion trap. The main advantages of the linear trap (relative to the quadrupole ion trap) are increased ion storage capacity, yielding higher sensitivity, and faster scanning. The quadrupole and linear ion traps are excellent instruments for use in proteome analysis of complex peptides mixtures when used in conjunction with appropriate LC separation.

2.2.6 Fourier transform mass spectrometry

Fourier transform mass spectrometry (FTMS) offers the greatest mass resolution (can routinely exceed 1,000,000) and best mass accuracy (less than 1 ppm) of any type of mass spectrometry. The unparalleled mass accuracy is often useful for unequivocal determination of molecular composition while the very high resolution is useful for distinguishing compounds with similar but distinct m/z present in a complex mixture. The high resolution is especially useful for analysis of large macromolecules such as proteins that typically adopt a series of multiply charged states when produced from ESI. The isotopic distribution of a multiply charged protein species is readily resolved by FTMS, allowing unambiguous charge state assignment and estimation of exact molecular weight of the intact protein, which is extremely useful.

The FTMS traps ions in a magnetic field causing them to travel in a circular path between two plates. As the ions travel in a circular motion, they produce a cyclical image current as they pass near detectors placed at fixed positions. The frequency of an ion's cycling, which is a function of its mass to charge ratio, is

deconvoluted by Fourier transform of the signal. (Comisarow and Marshall 1974; Alan G. Marshall 1998).

2.2.7 Orbitrap

The Orbitrap is the most recently invented mass spectrometer, commercially available since 2005. The instrument affords mass resolution in excess of 100,000, mass accuracy in the 2-5 ppm range, a m/z range exceeding 5000 and a dynamic range of at least $1x10^3$ (Qizhi Hu 2005). Thus, the Orbitrap performance approaches that of an FTMS but without the need for superconducting magnets and is considerably less costly to purchase. Instrumental design consists of a central spindle electrode and a coaxial outer barrel-like electrode defining an elongated chamber where ions are radially trapped and allowed to oscillate longitudinally along the length of the electrode. An ion with a given m/z is measured by monitoring the frequency of harmonic ion oscillations along the axis of the electric field. Ion frequency measurement is a non-destructive process that uses time-domain image current transients which are converted to mass spectra using fast Fourier transforms. Ion detection in the Orbitrap is similar to that employed in FTMS which measures an AC image current in a circuit between electrodes that sense ions as they travel past the detecting electrodes (Melvin A. Park 1994).

2.2.8 Ion Mobility Mass Spectrometry

Ion mobility (IM) mass spectrometry (IMMS) is a gas phase separation technique for ions based on their volume (collisional cross-section). The technique is well suited for the structural study of macromolecules such as peptides and proteins.

Prior to entering the IM region, ions experience an electrical potential gradient and are then allowed to drift through an inert buffer gas (usually helium) held at some pressure. Drift time is a function of the collisional cross section relative to the ions charge state. The time frame for IMMS is longer than the duty cycle associated with other forms of MS such that the course of an IM separation can be sampled at numerous time points by a subsequent stage of MS (typically TOF MS). Hence, IMMS provides a dimension of ion separation somewhat analogous to chromatography (Verbeck, Ruotolo et al. 2002). The time frame for an IMS separation is shorter than a typical LC or GC separation. Thus, IM can be integrated into a triply hyphenated technique such as LC/IM/MS.

2.2.9 Accelerator Mass Spectrometry

AMS is a technique used for quantitation of elemental (atomic) isotopes (eg, ^{12}C, ^{13}C, ^{14}C) rather than molecules. The technique provides attomol sensitivity, with unequivocal selectivity towards specific isotopes. Several nuclear physics techniques are used to distinguish a given "rare" radioisotope from other elements and molecules of the same m/z quantity. The technique involves the production of negatively charged elemental or ion molecules by bombardment of a solid sample with a Cs+ ion beam. Electrons are then stripped from the atomic anions to form positively charged nuclei. The cationic nuclei are then accelerated with MeV force for highly selective detection of the atomic isotope of interest using a multi-anode gas-ionization detector. Atomic cations of different isotopes are counted and quantitated as individual particles. The ability of AMS to form and isolate nucleic cationic isotopes derived from an organic compound (eg. Microdosing test

drug) allows the technique to be used for the quantitative analysis of trace amounts of analyte (Herbert Budzikiewicz 2006).

2.3 Ion detectors

A critical component of the mass spectrometer is the ion detector. Typically, the number of ions arriving at the detector is quite small, which necessitates signal amplification. The most common type of detector is the electron multiplier which senses an impinging ion and releases a cascade of electrons to produce an amplified electronic signal. Faraday cups have also been used as detectors in mass spectrometry. Electron multiplier and Faraday cup detectors have largely been replaced by the microchannel plate detector, which is an array of miniature electron multipliers aligned in parallel to each other (F. Dubois 1999).

The FTMS and Orbitrap use a pair of electrodes for ion detection without the ion directly impinging on a sensitive surface, as in the electron multiplier. Rather, ions are sensed as they oscillate near the electrodes to produces a weak AC image current. This type of detection can be very sensitive as a single ion can be sensed (counted) many times.

3.0 Therapeutic drug monitoring of antiretroviral drugs in human plasma by LCMS

3.1 Overview

While chapters 4-6 of this thesis deal specifically with MS based methods for quantitation and biochemical analysis of peptide and protein analytes, in this chapter, the implementation of a high throughput method for multiplexed therapeutic drug monitoring (TDM) of antiretroviral drugs in human plasma is described. Nine months of experimental work were dedicated to the content of this chapter and the general approach can also be adapted to peptide and protein analysis. As such, the author feels that the content of this chapter is sufficiently in line with the theme of this thesis and provides a useful perspective of a current MS based analysis of small molecule analytes in the clinical laboratory setting. Therapeutic drug monitoring (TDM) is used in the clinical chemistry laboratory to measure blood concentrations of medicines that have a narrow therapeutic index. Using TDM, the effectiveness of drug treatments can be optimised for individual patients. In this study an analytical method was developed and validated for TDM of antiretroviral drugs used to treat HIV infected patients. An ideal method would monitor simultaneously any combination of the commonly used anti-retroviral (ARV) drugs. The method should also possess adequate specificity, precision and offer high-throughput sample analysis. In our program, the determination of eleven compounds from heparinized human plasma was required. Sample workup for the validated method involved protein precipitation with acetonitrile

containing an internal standard. Supernatant from precipitated samples was extracted online using HPLC with solvent switching. A triple quadrupole tandem MS was used as a detector, operated in the MRM mode. The instrumentation used was an early generation tandem MS (PE Sciex API III) operated in the positive mode for all analytes. Analytical performance was comparable to that of more recent instruments. Detection by tandem MS is far superior in sensitivity and specificity to that of HPLC with UV detection. The method developed was linear for all analytes over a quantitation range spanning two orders of magnitude. Within-run precision (CV) for the various analytes varied from 2.0 to 12.5 % while CVs for between-run data varied from 2.1 to 13.4 %. Instrumental analysis required just 5 minutes per sample. The robustness of the system was tested as over 900 analyses were performed in triplicate from over 450 patient samples.

3.2 Introduction

The human immunodeficiency virus (HIV) is a retrovirus that can lead to acquired immunodeficiency syndrome (AIDS) where the immune system eventually fails, leading to fatal health complications resulting from infection by opportunistic pathogens and neoplastic complications.

The virus infects mainly immune cells such as T-helper cells, macrophages and dendritic cells. HIV infection results in a decreased number of these cells by three principle mechanisms: direct viral killing of the cells, increasing the rate of apoptosis in these cells (Badley, Pilon et al. 2000), and indirectly through increased recognition and killing of infected cells by CD8+ lymphocytes. Once T

cells fall bellow a critical titre (less than 200 cells per μL), the ability of macrophages, natural killer cells, and T-cells to become activated and secrete cytokines in response to antigens is lost.

The world health organization estimates that more than 25 million people have succumbed to HIV since 1981, making it one of the worse pandemics in recent history. Incidence of HIV is highest in South African countries and poor developing countries in South America. Increasingly, HIV infected women in resource rich countries are living with their illness and consciously choosing (with the support of the medical establishment) to bare children. This trend is mainly due to the introduction of effective retroviral therapies that dramatically increase the life expectancy of infected individuals and reduce the probability of viral transmission from mother to enfant (McGowan and Shah 2000). HIV can be transferred from mother to child in utero, during labour our during breast feeding. However, most cases of HIV transmission from mother to child occur during delivery. HIV infects T-cells by directly attaching and fusing to the cell membrane or by endocytosis. Hence, maternal HIV-1 plasma viral concentration during delivery is the most important predictor of mother to child transmission (McGowan and Shah 2000). For this reason, efforts to interrupt vertical transmission have focused on the use of highly active anti-retroviral therapy (HAART). HAART drug therapy during pregnancy reduces the risk of mother-to-child HIV transmission by 70%. Effective treatment and prevention of vertical transmission is optimised by TDM.

3.2.1 Anti-retroviral therapy

Currently used HAART inhibits either HIV's reverse transcriptase or its protease enzymes (Kilby and Eron 2003). These two therapies target HIV virus at different points of its life cycle. Briefly, HIV virus binds to CD4 and chemokine receptors on the surface of T-cells, undergoes T-cell membrane fusion, and then unloads copies of its RNA genome into the T-cell cytoplasm. The viral reverse-transcriptase enzyme transcribes viral RNA into double stranded DNA that can be integrated into the genome of the host T-cell. The reverse-transcriptase inhibitor drugs were the first agents approved for the treatment of HIV-1. Reverse-transcriptase inhibitor drugs commonly used in Quebec include delavirdine, efavirenz and nevirapine. Following reverse transcription, the viral integrase catalyzes the integration of proviral DNA into the host genome before replication. Integrase inhibitor drugs are currently under early clinical trials. When the infected host cells synthesises new proteins, the integrated proviral DNA is also translated into viral protein components required for new viral progeny. The viral components then assemble on the cell surface and bud out as immature viral particles. The final maturation step requires the HIV-1 protease to digest the immature virus into an infectious virion. Several protease inhibitors (PI) including amprenavir, atazanavir, indinavir, lopinavir, nelfinavir, ritonavir, saquinavir and tipranavir are currently used in Quebec as part of HAART.

The human immunodeficiency virus (HIV-1) is capable of acquiring a number of mutations which it can accommodate without significantly impairing viral fitness (Palmer, Kearney et al. 2005). The mutations are known to occur as a result of

nucleotide mis-incorporation by the error-prone viral reverse transcriptase (Menendez-Arias 2002). Pre-existing sub-populations of ARV resistant HIV may be selected upon initiation of drug treatment. Thus, HIV can develop resistance to antiretroviral drugs through replication and acquisition of drug resistance mutations.

The goal in treating an HIV infected patient is to keep blood levels of the virus as low as possible for as long as possible. In order to achieve this goal, several ARVs are commonly used simultaneously in HAART. The approach is more effective in controlling a number of HIV genetic sub-populations than using a single drug. Despite using HAART, viral resistance does occur and a change to the ARV regimen would then be indicated. Adjustment of ARV doses to optimal levels can be complicated by a number of factors including drug-drug interactions and inter-individual variability in pharmacokinetics and drug metabolism. In order to optimize dosages and monitor patient compliance, therapeutic drug monitoring (TDM) has been implemented in several laboratories around the world.

Over the last several years, there has been a steady improvement in the TDM of ARVs as summarized in Table 3.1. In 2000, an HPLC method with UV detection for the TDM of the 5 protease inhibitors approved by the United States Food and Drug Administration was reported (Poirier, Radembino et al. 2000). The method required a 1 mL plasma sample applied to an extraction cartridge, with a lengthy 45 min. HPLC run-time. Analytes (amprenavir, indinavir, nelfinavir, ritonavir, saquinavir) were monitored over a validated range of 25-5000 ng/mL.

Table 3.1 Overview of method developments in HIV antiretroviral TDM

Plasma volume (μL)	Number of drugs monitored	Sample analysis time (min.)	Instrumental setup	Reference
1000	8	45	LC-UV	(Titier, Lagrange et al. 2002)
250	8	30	LC-UV	(Tribut, Arvieux et al. 2002)
100	5	5	LC-MS-MS	(Chi, Jayewardene et al. 2002)
80	15	4.5	LC-MS-MS	(Volosov, Alexander et al. 2002)
100	7	5.5	LC-MS-MS	(Crommentuyn, Rosing et al. 2003)
250	7	4.2	LC-MS-MS	(Frerichs, DiFrancesco et al. 2003)
100	9	5	LC-MS	(Egge-Jacobsen, Unger et al. 2004)
20	17	< 4	LC-MS-MS	(Gu and Soldin 2007)

In 2002, an HPLC UV method for the determination of the six approved protease inhibitors (PI) as well as the two most widely used non-nucleoside reverse transcriptase inhibitors (NNRTI) in a single analysis was reported (Titier, Lagrange et al. 2002). The method involved a liquid-liquid extraction of 1 mL plasma and the use of gradient elution on a reversed phase column with a 45 min run-time. A modest improvement to ARVs quantitation was reported in the same year (Tribut, Arvieux et al. 2002). The method describes the simultaneous quantitation of six PIs (including the M8 active metabolite of nelfinavir) and two NNRTIs with a run-time of less than 30 min with isocratic elution and UV detection requiring a 250 µL plasma sample.

The principle disadvantages of HPLC methods with UV detection are lengthy runtime (> 20 min.) and the reliability on retention time for compound identification.(Droste, Verweij-Van Wisse et al. 2003; Keil, Frerichs et al. 2003). Their main advantages are simple and affordable instrumentation (HPLC with UV detection).

A major improvement in ARV quantitation, using LCMS, was reported by (Volosov, Alexander et al. 2002). The method provided a means to simultaneously monitor any combination of the 15 ARV drugs approved for HIV treatment available at the time. Sample analysis required 80 µL of plasma with a convenient protein precipitation followed by a rapid (4.5-min) LCMS analysis using atmospheric pressure chemical ionization (APCI) with MRM. This method was refined (Ghoshal and Soldin 2003) to extend the standard curve range to cover the most commonly encountered patient sample concentrations. Curve

ranges were established from 100-10,000 ng/mL. In addition, the matrix for calibration standards was changed from methanol to blood serum. A further improvement to the method was report in 2007 where two additional analytes, atazanavir and tipranavir, were added to expand the panel of ARV drugs monitored to 17 (Gu and Soldin 2007).

Other groups have also reported methods for ARV quantitation using LCMS. An LCMS method for the determination of five PIs using gradient elution with TurboIon spray was reported by (Chi, Jayewardene et al. 2002). The run-time was 5 min. with a calibration curve range from 5 - 10,000 ng/mL.

Two methods were reported in 2003 for the LCMS analysis of the 6 licensed PIs and the M8 active metabolite of nelfinavir. Both methods use gradient elution with 5 min run-times, with TurboIon spray on Sciex API 3000 triple-quadrupole mass spectrometers. While the method of (Crommentuyn, Rosing et al. 2003) uses a protein precipitation of 100 µL plasma, the method of (Frerichs, DiFrancesco et al. 2003) uses a liquid–liquid extraction of 250 µL plasma. The methods afford comparable performance with curve ranges similar to those reported by (Ghoshal and Soldin 2003) and (Chi, Jayewardene et al. 2002).

An LCMS method using ESI with a single quadrupole MS (used for selected ion monitoring (SIM)) for the simultaneous quantification of six PIs and three NNRTIs was described by (Egge-Jacobsen, Unger et al. 2004). A 100 µL plasma sample was treated by protein precipitation with a 5 min sample run-time. Single quadrupole instruments are less expensive than tandem mass spectrometers. The

singe quadrupole instrument used is an appropriate option for monitoring analytes present at relatively high concentration, as is the case with ARVs.

In this chapter, an LCMS method for the TDM of eleven commonly used ARVs in Quebec (Canada) is presented. Our method and system were validated by blind analysis of proficiency test samples obtained from the Netherlands International Quality Control Program for therapeutic Drug Monitoring in HIV Infection (University Medical Centre Nijmegan, Department of Clinical Pharmacy, 6500 HB Nijmegan, The Netherlands). The method was subsequently used for the analysis of over 450 patient samples spanning several months of sample analysis. The instrument employed was an early generation PE/Sciex API III tandem MS operated in the positive mode for all analytes. Despite its older design and limitations, the API III provided suitable analytical performance comparable to more recent triple quadrupole instruments (API2000, 3000 series) (Chi, Jayewardene et al. 2002; Volosov, Alexander et al. 2002; Crommentuyn, Rosing et al. 2003; Frerichs, DiFrancesco et al. 2003; Ghoshal and Soldin 2003; Egge-Jacobsen, Unger et al. 2004).

3.3 Materials and methods

Standards of amprenavir, atazanavir, efavirenz, indinavir, lopinavir, nelfinavir, nevirapine, ritonavir, saquinavir, tipranavir were obtained from the AIDS reagent reference program (McKessonHBOC BioServices, Rockville, MD). Delavirdine was purchased from Biomol (Plymouth Meeting, PA). Cimetidine was purchased from Sigma Aldrich (St. Louis, MO). Purified water, MilliQ or equivalent was

obtained in house. Acetonitrile and methanol were purchased from EMD chemicals (Gibbstown, NJ) and were of HPLC grade. Ammonium acetate was purchased from BDH (Toronto, Canada) and was reagent grade.

3.4 Standard curves and Quality Control sample preparation

Stock solutions of each of the ARVs were prepared at a concentration of 2 mg/mL in methanol. The potency and purity of each compound were considered in the calculation of stock solution concentrations. Calibration curves were prepared in pooled lithium-heparinized human blank plasma. A curve range with five non-zero standard points (0.1, 1.0, 2.5, 5.0, 10.0 µg/mL) was used for all analytes. A stock solution containing all the ARVs was prepared at 10 times the concentration of the upper limit calibration standard. The mixture was diluted into plasma to prepare the upper limit calibration standard. Additional calibration standards were prepared by serial dilution. A sufficient volume of calibration curve samples was prepared and 110 µL aliquots were stored frozen at -15°C. A similar procedure was used for the preparation of quality control samples at 0.4, 4.0, 8.0 µg/mL. QC samples were prepared with a separate weighing of reference standards.

3.5 Sample treatment

Before proceeding with sample analysis, all HIV infected samples were heated for 30 min. at 56°C to deactivate the HIV virus (Dasgupta, Wells et al. 1999) (Figure 3.1). Sample preparation consisted of aliquoting 100 µL of heparinized plasma

into a 1.5 mL eppendorf tube. Four hundred μL of acetonitrile containing 1 μg of cimetidine internal standard was added to each sample. Tubes were capped and vortexed vigorously for 30 s and centrifuged at 14,000 g for 10 min. Supernatants were transferred to HPLC vials for LCMS analysis. Ten μL of supernatant from each protein-precipitated sample was used for analysis. Calibration standards, QC samples and study samples were injected in a randomized fashion to ensure that the analytical system was performing properly.

3.6 Instrumental setup

Liquid chromatography was set up with a switching valve allowing the sample to be loaded onto the analytical column and washed with a mobile phase of 15-mM ammonium acetate in water (Figure 3.2). After 2.5 min of washing, the switching valve was activated and the sample was eluted with methanol in a chromatographic peak containing all analytes (Figure 3.3). Analytes were co-eluted within 1 minute of solvent switching. The setup requires 2 HPLC pumps (one pumping 15mM ammonium acetate, the other pumping methanol). The MS employed (PE/Sciex API III) was operated in the positive mode for all analytes with APCI. Adequate sensitivity was observed for all analytes over the concentration range used (0.1-10 μg/mL) with a 10 μL injection of protein precipitated supernatant. The analytical column was a Waters X-Terra MS C18 3.5 μm 4.6 x 50mm. A 6 port Valco Instrument Co. switching valve was used for instantaneous solvent switching from wash solvent (15 mM ammonium acetate)

Figure 3.1 Flow-diagram for sample preparation procedure

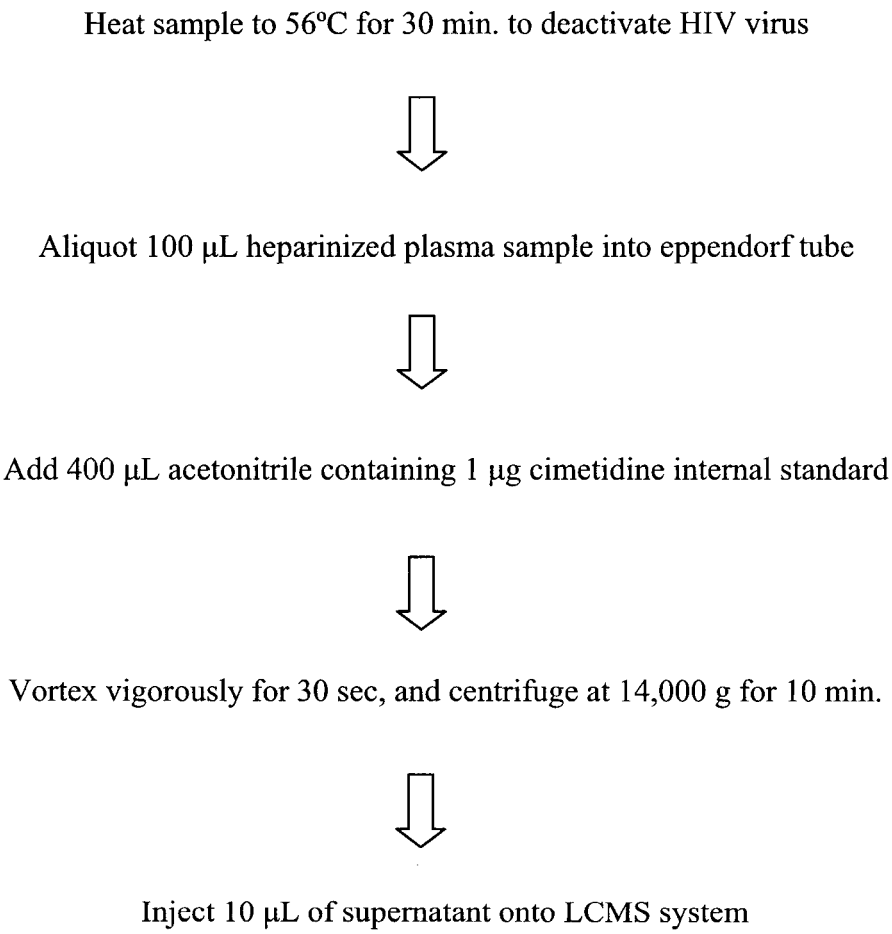
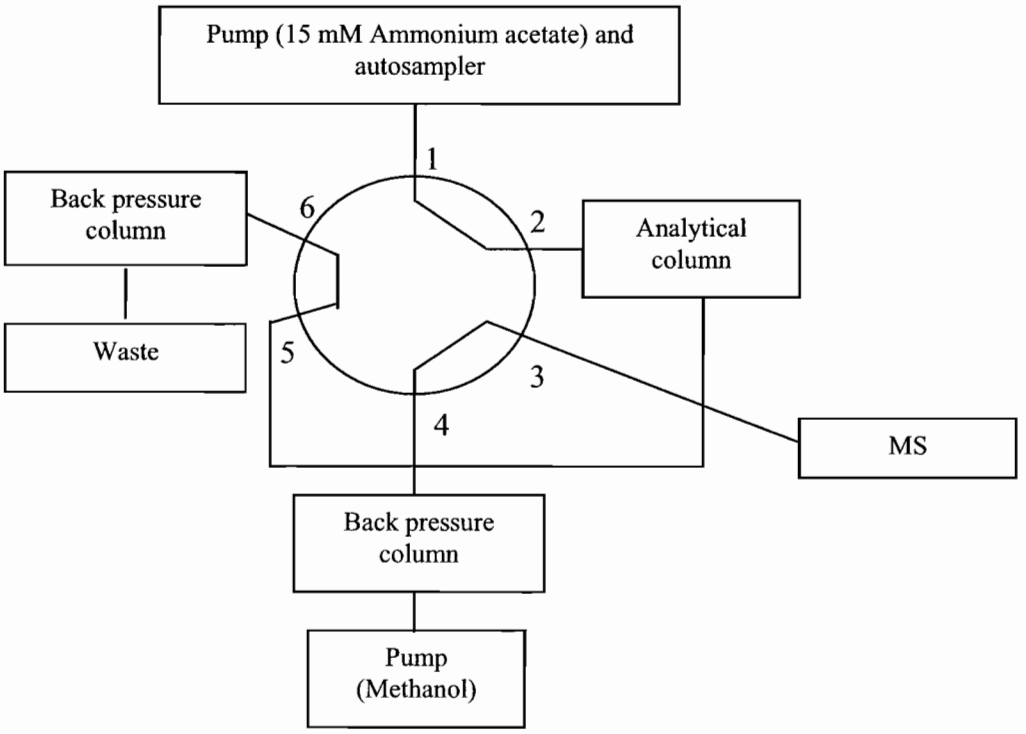


Figure 3.2 Schematic representation of 6 port column switching valve setup

Inject Position:



Load Position:

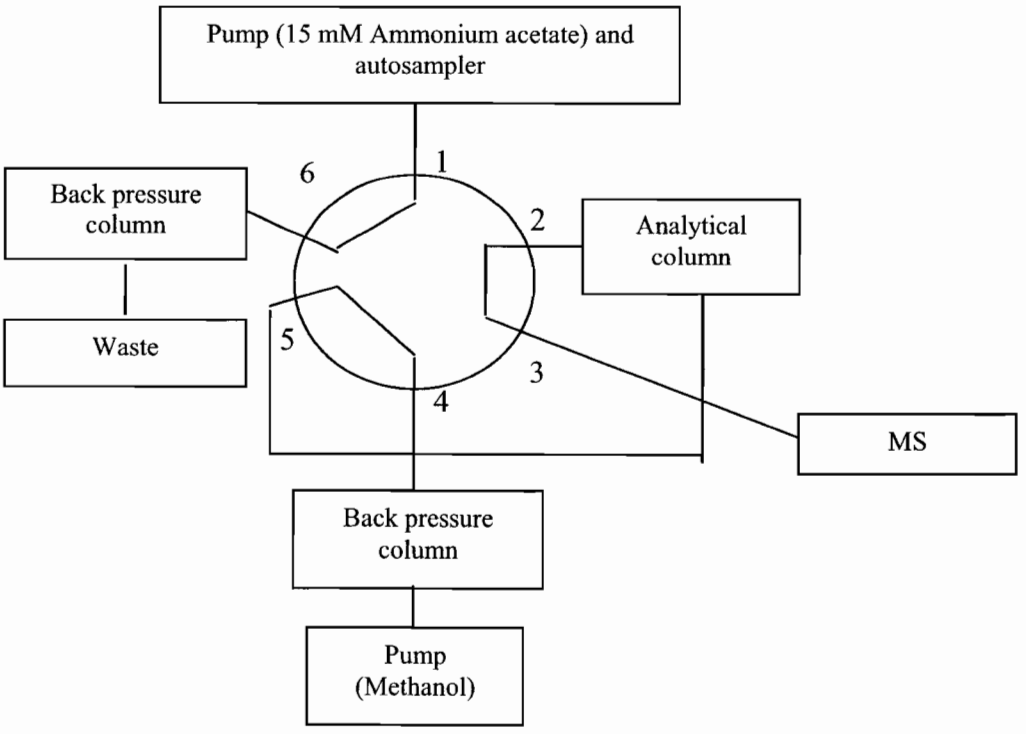


Figure 3.3

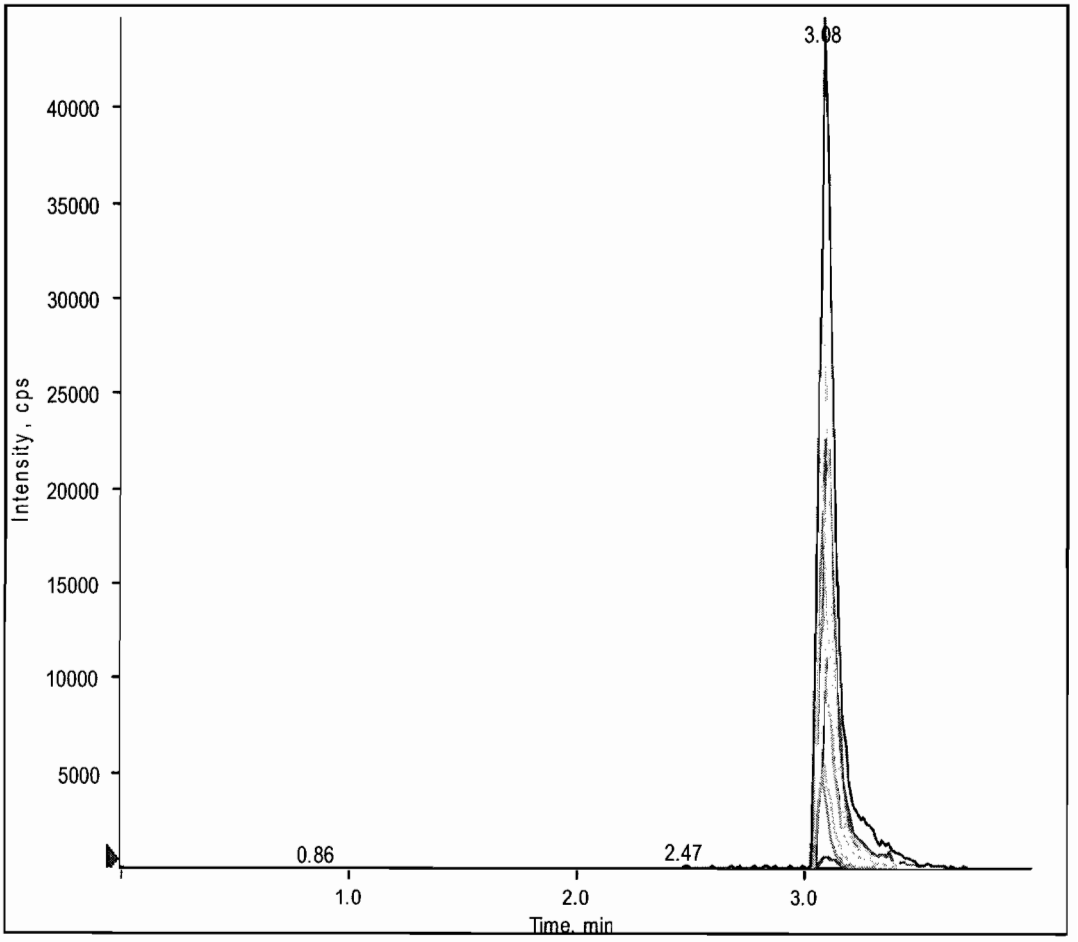

3.08
40000
35000
30000
25000
20000
15000
10000
5000
Intensity, cps
0.86
2.47
1.0
2.0
3.0
Time, min

Figure 3.3 Representative chromatogram showing responses of ARV analytes. Each drug trace is presented in a different color, 10 ng each drug injected. Samples were loaded onto the analytical column, washed with a mobile phase of 15-mM ammonium acetate in water for 2.5 min, the switching valve was activated and the ARV drugs were eluted with methanol in one chromatographic peak. Analytical instrumentation requires 2 HPLC pumps (Figure 3.2; one pumping 15mM ammonium acetate, the other pumping methanol). The MS employed (PE/Sciex API III) was operated in the positive mode for all analytes with atmospheric pressure chemical ionization. Analytes were monitored in the multiple reaction monitoring mode.

to elution solvent (methanol) with switching events at 2.5 min. and 4 min. Mobile phases (15-mM Ammonium acetate, Methanol) were pumped at 1 mL/min. The MS was operated in the MRM mode, monitoring the ion transitions indicated in Table 3.2 and using the instrument settings as outlined in Table 3.3. Data acquisition time was 4.0 min and the injection cycle time was 5.0 min. Instrument calibration, data acquisition and analysis was performed with Perkin-Elmer SCIEX software applications: Tune 2.5, RAD 2.6 and MacQuan 1.3.

3.7 Results and discussion

Our method was generally adapted from (Volosov, Alexander et al. 2002). However, there were several important improvements and modifications. The particular instrument adapted in this study (PE/Sciex API III) is an older model of tandem MS that did pose some analytical challenges. Despite the challenges, suitable analytical performance was established. Calibration curves were analysed by linear regression with a 1/concentration weighting. A correlation coefficient (R^2) of > 0.99 was established for quantitation of each analyte (Figure 3.4). Within run precision (CV) varied from 2.0 to 12.5 % (Table 3.4), whilst between run CVs varied from 2.1 to 13.4 % (Table 3.5). For comparison the method of Volosov et al., 2002, where data had been collected on a newer API2000 tandem MS, reported R values ranging from 0.911 – 0.993, within-run precision for all analytes CV < 7% and between-run precision of CV < 10%. Both of these systems can be obtained on the aftermarket for a fraction of the cost of current

Table 3.2 MRM transitions used to monitor antiretroviral drugs

Compound	Chemical formula	Protonated molecular weight (MH^+)	Fragment (m/z)
Amprenavir	$C_{25}H_{35}N_3O_6S$	506.3	245.2
Atazanavir	$C_{38}H_{52}N_6O_7$	705.4	168.1
Delavirdine	$C_{22}H_{28}N_6O_3S$	457.2	221.1
Efavirenz	$C_{14}H_9ClF_3NO_2$	316.0	168.0
Indinavir	$C_{36}H_{47}N_5O_4$	614.5	421.3
Lopinavir	$C_{37}H_{48}N_4O_5$	629.3	447.3
Nelfinavir	$C_{32}H_{45}N_3O_4S$	568.3	330.1
Nevirapine	$C_{15}H_{14}N_4O$	267.2	226.1
Ritonavir	$C_{37}H_{48}N_6O_5S_2$	721.3	268.2
Saquinavir	$C_{38}H_{50}N_6O_5$	671.4	570.3
Tipranavir	$C_{31}H_{33}F_3N_2O_5S$	603.1	175.1
Cimetidine (IS)	$C_{10}H_{16}N_6S$	253.0	159.0

Table 3.3 Mass spectrometric settings (PE/Sciex API III)

Parameter	Setting
Discharge needle current (DI)	3.0 μA
Ionspray voltage (ISV)	6000.0 (linked)
Interface plate voltage (IN)	650
Orifice plate voltage (OR)	65
Q0 rod offset voltage (RO)	30
Q1 rod offset voltage (R1)	28.0
lens element 7 voltage (L7)	6.0
Q2 rod offset voltage (R2)	1.0
lens element RX voltage (RX)	-9.0
Q3 rod offset voltage (R3)	-4.0
Lens element 9 (L9)	-50
Faraday plate voltage (FP)	-50
Channel electron multiplier voltage (MU)	-4700
Count control (CC)	1
Collision gas (CG)	Argon
Collision gas thickness (CGT)	250
Heated nebulizer temperature	480 °C
Interface heater temperature	55 °C
Source delta P	1.0 Inches WC
Auxiliary gas flow	5.0 L/min
Nebulizer pressure	60 psi
Curtain gas (Nitrogen)	1.0 L/min

Figure 3.4a

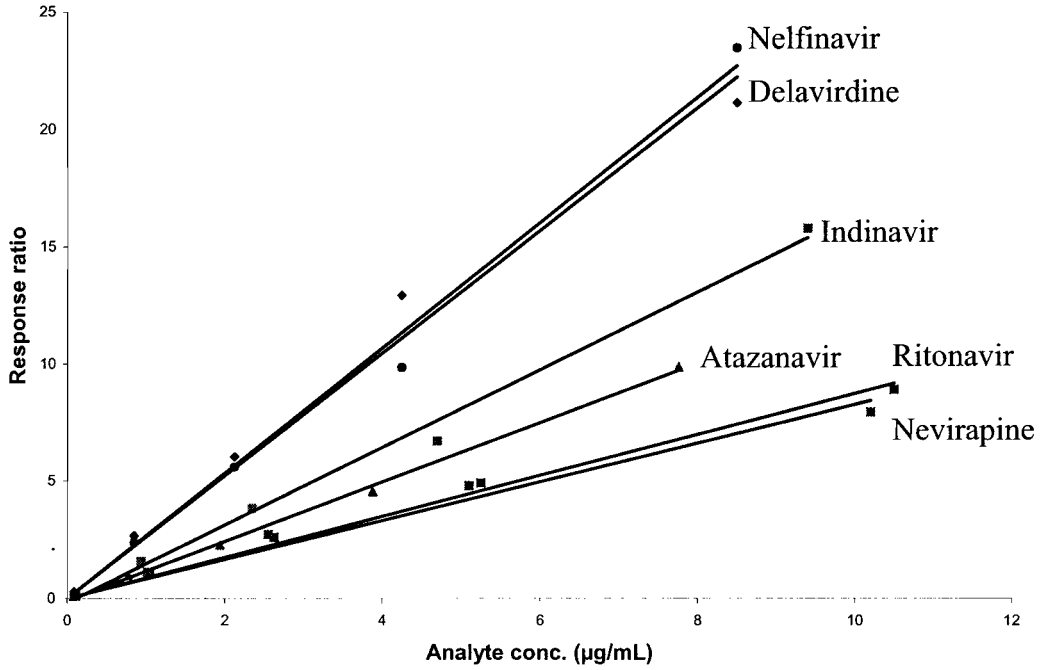

Nelfinavir
Delavirdine
Indinavir
Atazanavir
Ritonavir
Nevirapine
Response ratio
Analyte conc. (µg/mL)
0
5
10
15
20
25
0
2
4
6
8
10
12

Figure 3.4a Representative ARV standard curves (High response range: Nelfinavir, Delavirdine, Indinavir, Atazanavir, Ritonavir, Nevirapine). Calibration curves were analysed by linear regression with a 1/concentration weighting. A correlation coefficient (R^2) of > 0.99 was established for quantitation of each analyte. Note the difference in MS response for the various ARV drugs.

Figure 3.4b

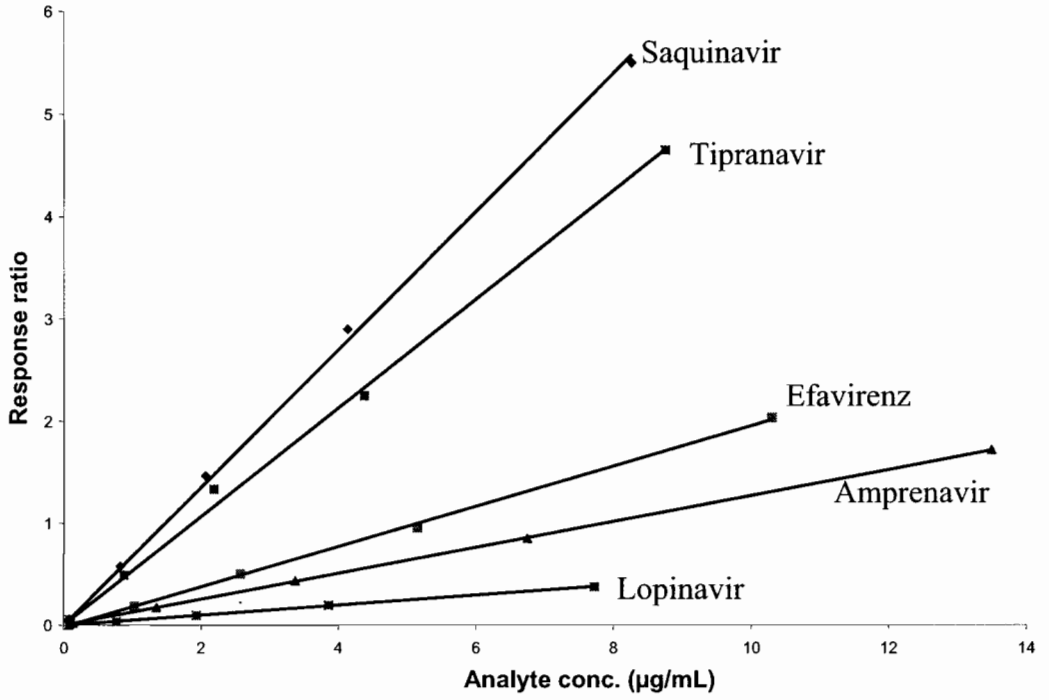

Saquinavir
Tipranavir
Efavirenz
Amprenavir
Lopinavir
Response ratio
Analyte conc. (µg/mL)
0
1
2
3
4
5
6
0
2
4
6
8
10
12
14

Figure 3.4b Representative ARV standard curves (Low response range: Saquinavir, Tipranavir, Efavirenz, Amprenavir, Lopinavir). Calibration curves were analysed by linear regression with a 1/concentration weighting. A correlation coefficient (R^2) of > 0.99 was established for quantitation of each analyte. The MS response for Nelfinavir (Fig. 3.4a) is approximately 50 fold that of Lopinavir.

Table 3.4 Within-run coefficient of variation for antiretroviral drugs

		% CV		
Compound	**n**	**0.10 µg/mL**	**1.0 µg/mL**	**10.0 µg/mL**
Amprenavir	20	10.6	8.1	4.4
Atazanavir	20	4.1	4.1	3.2
Delavirdine	20	3.9	3.8	3.5
Efavirenz	20	12.5	5.9	6.4
Indinavir	20	5.3	2.4	3.4
Lopinavir	20	9.5	2.5	3.7
Nelfinavir	20	2.6	3.2	9.1
Nevirapine	20	6.3	2.8	4.4
Ritonavir	20	7.0	2.0	3.1
Saquinavir	20	3.8	4.0	3.0
Tipranavir	20	9.3	4.8	9.3

Table 3.5 Between-run coefficient of variation for antiretroviral drugs

		% CV		
Compound	**n**	**0.40 µg/mL**	**4.0 µg/mL**	**8.0 µg/mL**
Amprenavir	24	5.9	7.6	5.8
Atazanavir	35	9.5	9.9	7.7
Delavirdine	3	13.2	2.1	2.3
Efavirenz	28	11.7	5.7	7.6
Indinavir	11	10.6	8.5	8.2
Lopinavir	23	13.4	9.1	6.4
Nelfinavir	19	8.2	7.8	10.6
Nevirapine	15	4.4	4.8	6.7
Ritonavir	38	10.8	8.7	6.3
Saquinavir	25	9.7	9.2	6.5
Tipranavir	8	10.3	13.0	5.9

generation tandem MS instruments. In both cases, the analyses were performed in a high-throughput fashion, requiring only inexpensive reagents for sample treatment and analysis. No other analytical technique can offer equivalent multiplexed high-throughput analysis. Implementation of tandem MS technology in prospective research or clinical biochemistry laboratories need not be prohibitively expensive as an increasing number of aftermarket instruments in good working condition can be acquired.

On the API III, one set of instrument parameters (Tables 3.2 and 3.3) were used for all compounds which creates a challenge for obtaining similar MS responses for each drug at a given concentration. Ideally, drugs of the same concentration would produce similar MS signals. The API III provides approximately 3.5 orders of linear response. We were able to overcome dynamic range difficulties associated with variable drug responses by two considerations. a) Limiting our calibration curve concentration range to two orders of magnitude for all analytes b) by loading just enough analyte to the LCMS to observe a reproducible signal for the lower limit of quantitation standard for the drug with the least MS signal (lopinavir) while not saturating the MS signal for the upper limit of quantitation for the drug with the strongest MS signal (Nelfinavir). These considerations are easily overcome on more recent instruments (like the API 2000 used by (Volosov, Alexander et al. 2002)) as they can accept optimized settings separately for each drug in a single experiment. This enables the operator to easily obtain similar MS responses for different drugs at the same concentration.

An important improvement of our method relative to that of (Volosov, Alexander et al. 2002) was the addition of two ARV drugs analysed (Atazanavir and Tipranavir). It should be noted that in 2007, the group of Dr. Soldin also reported the addition of the same two drugs to their list of ARVs monitored (Gu and Soldin 2007).

Ionization sources in MS can produce cationic and/or anionic gas phased analytes, depending on their chemical characteristics. Most nitrogen containing compounds such as peptides, or drugs (ARVs) can be observed as cations for MS analysis in the positive mode. However some compounds (e.g. organic acids) can also be observed as anions in the negative MS mode with a stronger signal. An important adaptation of our method was the analysis of all analytes in the positive MS mode which is a requirement of MRM analysis on the API III. This is in contrast to the method of (Volosov, Alexander et al. 2002) where analysis of several ARVs is performed in the positive mode and a few in the negative mode.

The model of instrument employed in this study (API III) is available on the aftermarket throughout North America for less than $ 10,000 US. Technical support is also available from several companies in the US and Canada. Other previously owned tandem MS instruments (such as the API 2000) can also be purchased at a fraction of the cost of the latest generation equipment. Thus, implementing tandem MS technology in a research facility or clinical chemistry laboratory need not be an exceedingly expensive endeavour. These considerations are especially relevant for health organizations with limited resources, typical of developing countries or impoverished areas of the world where HIV/AIDS is a

serious health problem affecting a large segment of the population. In order to benefit these patients, antiretroviral medications must be made available along with suitable technology and methods for optimizing dosages, adjusting for viral resistance and monitoring patient compliance.

3.8 Conclusion

This report describes the adaptation and implementation of a high throughput method for TDM of ARV's in a clinical chemistry laboratory using LCMS with MRM. In our study eleven ARVs approved for HIV treatment in Quebec were simultaneously monitored in over 450 patient samples spanning nine months of analyses. Results from these analyses were crucial in allowing physicians to optimise and adjust dose regimens appropriately. The method is sensitive, highly specific and requires 100 μL sample volume. The sample volume could have been reduced to just 10 μL, which would still provide sufficient sample supernatant for multiple 10 μL injections. Sample analysis is high-throughput, requiring just 5-min per sample. When set up and maintained properly, the equipment and method described herein are reliable and analytically robust. The model of instrument employed in this study and other (more recent) instrument models providing similar performance can be affordably obtained on the aftermarket. As such, implementation of tandem LCMS technology to a prospective research or clinical research laboratory need not be an exceedingly expensive endeavour.

4.0 Sensitive capillary chromatography mass spectrometric methods for the determination of salcatonin in human biological matrices.

Aguiar, M., B. F. Gibbs, Masse R (2005). Journal of Chromatography B **818**(2): 301-308.

4.1 Connecting text

Chapter 4 presents the development of a novel approach for the quantitation of a clinically useful peptide hormone in human biological matrices. Currently, clinical assessment of most peptide hormones and proteins involve immunoassays and other analytical techniques ie. Do not use LCMS technology. The goal of this chapter is to demonstrate, using a practical example, that current μLCMS instrumentation can provide a versatile alternative to traditional assays for quantitative analysis of a peptide hormone in a complex biological background. While only a single peptide is determined in the current assay, the method could readily be adapted to simultaneously monitor many more peptides with similar analytical performance. Novel aspects of the approach presented include the use of capillary liquid chromatography in combination with high resolution time-of-flight mass spectrometry (μLC-TOF/MS) as an analytical platform. To our knowledge, such a platform has not yet been implemented in a clinical chemistry laboratory for routine sample analysis. The same platform is again employed in the next chapter for the determination of tryptic peptides derived from urinary C-reactive protein. The capillary chromatography employed is well suited to low microliter sample volumes for high sensitivity detection, especially when coupled to nano-electrospray MS. The high-resolution TOF mass analyzer can be operated

in the selected ion monitoring mode for sensitive and specific analysis without the need for tandem MS. Our results demonstrate that this approach can be used to rapidly generate quantitative data about the absolute concentration of polypeptides in biological matrices. The approach may be directly and dependably applicable to many current bioanalytical needs in pharmacokinetic studies, degradation product assessment and other areas related to biology, clinical chemistry and proteomics.

4.2 Abstract

New methods employing capillary liquid chromatography in combination with time-of-flight mass spectrometry (μLC-TOF/MS) were developed for the rapid determination of salcatonin in human urine and plasma. The approaches presented utilize $^{13}C_6$-leucine (19)-labeled salcatonin as internal standard, small matrix volumes and simple sample preparation procedures. They allow TOF/MS to be used as a highly selective detector for providing accurate quantitation of salcatonin. Data acquisition was performed in enhanced mode optimizing the signal for the triply charged species of salcatonin and its internal standard. We demonstrate that the determination of salcatonin is straightforward and reliable and can be performed with excellent linearity ($R2 > 0.999$), precision and accuracy over the concentration ranges of 2.9–290 pmol/mL in human urine, and 7.3–730 pmol/mL in human plasma.

4.3 Introduction

Calcitonin is a hypocalcaemia factor secreted from the parafollicular "C" cells of the thyroid gland originating from the neural crest (Silverman 2003). Many vertebrates express calcitonin and secretion is regulated by subtle changes in serum calcium levels. Gastrointestinal peptides, estrogens and Vitamin D also regulate its secretion. The physiological role of endogenous calcitonin in calcium homeostasis is not completely understood. Human calcitonin (hCT) is a useful biomarker in the diagnosis and monitoring of medullary thyroid carcinoma (Martinetti, Seregni et al. 2003). Salcatonin (sCT) is the salmon variety of calcitonin whose precursor is a 136-residue polypeptide. In vivo processing of the precursor results in a 32-residue active peptide, with a molecular weight of 3429.71 Da whose features include a disulfide bridge and C-terminal amidation (Fig. 4.1). Salcatonin is used to reduce pain from Pagets' disease (Nagant de Deuxchaisnes 1983) and bone malignancies (Szanto, Jozsef et al. 1986) by direct action on the central nervous system. The determination of drugs and biomolecules from complex biological matrices may be performed by a variety of analytical techniques. Typically, the most sensitive bioanalytical quantitative methods commonly used are radioimmunometric and enzyme-linked immunosorbent assays. However, the antibody-based methods are time consuming and costly to implement, and may suffer from cross-reactivity towards other antigens. Recently, several reports have appeared advising that caution be exerted in interpreting results from immunoassays for the determination of hCT from serum (Martinetti, Seregni et al. 2003). The report by

Figure 4.1.

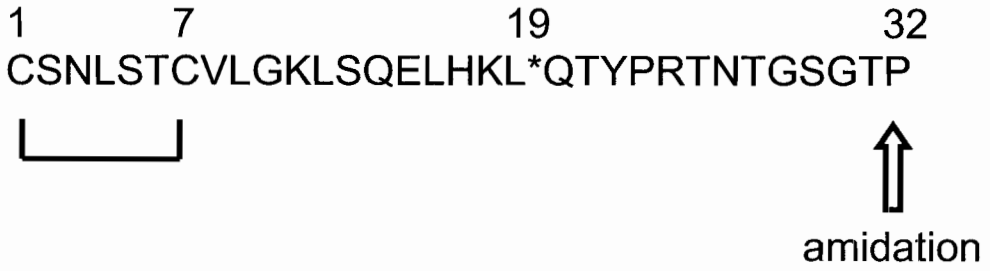


Figure 4.1 Calcitonin is a hypocalcaemic peptide hormone expressed by fishes, reptiles, birds and mammals. Salmon calcitonin (Salcatonin) is used clinically to treat metabolic bone disease. Salcatonin is more potent and has a longer half life than human calcitonin. Processing of the salcatonin precursor protein yields a biologically active 32 amino-acid peptide with a 1-7 bisulphide bridge and C-terminal amidation. A $^{13}C_6$-labelled analog of salcatonin, labelled Leu19, was used as internal standard.

(Martinetti, Seregni et al. 2003) concluded that the analytical accuracy of hCT is flawed even if new, highly specific antibodies were utilized. LC methods with ultraviolet detection have been reported for the determination of sCT, but these are inadequate for trace detection (Buck and Maxl 1990; Lee, Pollack et al. 1991). Mass spectrometric techniques can be applied towards the high sensitivity qualitative and quantitative study of many compounds, especially proteins and peptides. Developments in mass spectrometer design have resulted in improved detection of biomolecules approaching the levels routinely achieved with immunoassays without the corresponding shortcomings. Recently, several LC ESI/MALDI TOF methods incorporating stable-labelled isotope tags have been developed to examine relative protein concentration (Gygi, Rist et al. 1999; Cagney and Emili 2002). There has been limited use of stable-labelled proteolytic peptides or polypeptides for analytical assays of proteins (Gerber, Rush et al. 2003). A recent review provides further information on application of stable isotope techniques for protein and peptide determination (Julka and Regnier 2004). Qualitative mass spectrometric approaches have been used to characterize human or salmon calcitonin in different biological matrices (Lee, Moon et al. 1999; Kobayashi, Yamamura et al. 2000). A LC-MS method was reported for the quantitative determination of sCT in rat and dog serums without internal standard, which makes accurate quantitation more challenging than with our approach (Song, An et al. 2002). Here we describe the first μLC-TOF/MS assays employing a labelled internal standard for the determination of sCT in microliter volumes of human urine or human plasma with a low fmol lower limit-of-

quantitation (LLOQ). Data acquisition was performed in enhanced mode optimizing the signal for the triply charged species of salcatonin and its internal standard. We demonstrate that the determination of salcatonin is straightforward and reliable and can be performed with excellent linearity ($R^2 > 0.999$), precision and accuracy over the concentration ranges of 2.9–290 pmol/mL in human urine, and 7.3–730 pmol/mL in human plasma.

4.4 Materials and methods

4.4.1 Reagents and chemicals

All solvents and reagents were HPLC grade and were used without further purification. Salcatonin was purchased from Bachem, King of Prussia, PA, USA. Synthetic $^{13}C_6$-labelled salcatonin was purchased from SynPep (Dublin, CA, USA). The purity of the synthetic labelled peptide was assessed by μLC with UV and mass spectrometric detection. No extraneous peak was detected in the UV and total ion mass chromatograms. These data and complementary information provided by the supplier indicated that the purity of both compounds was >99.9%. Human urine and EDTA salcatonin-free plasma, previously screened for infectious pathogens, were purchased from Biological Specialty Corporation (Colmar, PA, USA).

4.4.2 Calibration standards and quality control samples

Stock solutions of salcatonin and $^{13}C_6$-salcatonin were prepared in a mixture of acetonitrile: 0.1% TFA 1:1 (v/v) and stored at -20°C. Prior to use, blank urine and

plasma samples were screened for the presence of potential interfering compounds at the retention time and *m/z* of salcatonin and $^{13}C_6$-labelled salcatonin using the extraction methods and LCMS analysis procedures described below. Calibration standards and quality control (QC) samples were freshly prepared using the following procedure: blank human urine and plasma samples were allowed to thaw on ice. Two hundred microliter aliquots were placed in individual 1.5mL eppendorf tubes. Samples were spiked with appropriate amounts of salcatonin spiking solutions prepared by serial dilution of the stock solutions. The calibration standards and QC samples were used to assess precision and accuracy and determine the LLOQ. Sets of five calibration standards ranging from 10 to 1000 ng/mL (urine) and from 25 to 2500 ng/mL (plasma) were prepared in triplicate. For the urine assay, QC samples at low (30 ng/mL), medium (400 ng/mL) and high (700 ng/mL) concentrations were prepared as described above. In the case of the plasma assay, the concentration of the QC samples was 75, 1000 and 1750 ng/mL, respectively.

4.5 Sample preparation procedures

4.5.1 Urine

In a typical experiment, 20_L of the internal standard (ISTD) solution spiked at a concentration of 10 μg/mL was added to 200 μL aliquots of freshly prepared calibration standard and QC samples in blank human urine so as to obtain a final ISTD concentration of 910 ng/mL. The resulting mixture was vortexed and centrifuged at 16,000 rpm for 1 min. A portion of the supernatant was transferred

to a standard HPLC vial containing a 100 µL glass insert. One µL was applied directly to the µLCMS. For the 10 ng/mL LOQ sample, this corresponds to an on-column injection of 9.1 pg or 2.65 fmol of sCT.

4.5.2 Plasma

Fifty microliters of a 10 µg/mL ISTD spiking solution was added to 200 µL aliquots of freshly prepared calibration standard and QC samples so as to obtain a final ISTD concentration of 2.0 µg/mL. Proteins were precipitated by the addition of 200 µL acetonitrile and the mixture was centrifuged at 16,000 rpm for 1 min. The supernatant was transferred into a 1.5mL eppendorf tube and lyophilized. The dry residue was reconstituted in 5 µL of a mixture of acetonitrile:water 0.1% TFA (1:1, v/v), and the resulting solution transferred into a HPLC vial containing a 100 µL glass insert. One microliter was applied directly to the µLCMS. If we assume that the recovery of sCT is quantitative, this corresponds to an on column injection of 1.0 ng or 291.5 fmol for the 25 ng/mL LLOQ sample.

4.6 Capillary liquid chromatography conditions and instrumentation

Gradient HPLC methods were used for chromatographic separation on an Agilent 1100 capillary liquid chromatography system equipped with an in-line degasser and a diode array detector (Agilent Technologies, Palo Alto, CA, USA) set to detect at 206 and 280 nm. A Zorbax SB-C18 300Å 250mm×0.3mm i.d. capillary column was used. The gradients employed two mobile phases (A and B). Mobile phase A was a 0.1% TFA in water and mobile phase B consisted of 0.1% TFA in acetonitrile. All samples were chromatographed at a flow rate of 5 µL/min.

Chromatographic conditions were optimized separately for urine and plasma samples. Urine samples were chromatographed using a 10 min linear gradient where mobile phase B was increased from 10 to 70% B and maintained for 10 min to ensure that all major components in the sample had eluted, as revealed by the UV signal at 206 nm. Only then was the solvent composition reset to the starting conditions (i.e. 90% A/10% B) and the system allowed to re-equilibrate. For plasma samples, the same 10–70% B gradient was extended over a period of 30 min for adequate chromatographic resolution of the analyte of interest from extraneous peaks.

4.7 Mass spectrometric analysis

The effluent from the LC column was directly coupled to the electrospray source of a QSTAR Pulsar *i* hybrid tandem time-of-flight mass spectrometer (Applied Biosystems/ MDS Sciex, Concord, Ontario, Canada) operated in the enhanced TOF-MS mode, scanning positive ions from *m/z* 800–1300 Da. Typically, a voltage of 5.5 kV was applied to the electrospray needle for ionization, while declustering potentials were adjusted to 50V and curtain gas was set at 25 psig. The mass spectrometer was mass calibrated prior to sample analysis using cesium iodide (Cs+ *m/z* 132.9054) and an octapeptide (*m/z* 829.5398). MS parameters were optimized by infusing a reference solution of the analyte.

4.8 Quantitation

Extracted ion chromatograms for the mass ranges of *m/z* 1144–1145 (salcatonin) and *m/z* 1146–1147 ($^{13}C_6$-labelled salcatonin) were obtained for all samples. The mass spectrum of the peak of interest in the extracted ion chromatograms was used to verify that the signal was as a result of the triply charged $[M + 3H]^{3+}$ ions. Quantitation was based on the peak height of the signal for the triply charged species of salcatonin and its $^{13}C_6$-labelled analog used as internal standard. Standard calibration curves were constructed by plotting the corresponding peak height ratios against five standard concentrations of salcatonin spiked in blank human urine or human plasma and analysed by least square linear regression using a Microsoft Excel spreadsheet.

4.9 Results and discussion

4.9.1 Mass spectrometry of salcatonin and $^{13}C_6$-leucine-labelled salcatonin

Salcatonin and its $^{13}C_6$-leucine-labelled synthetic analog were examined by capillary LC TOF/MS to confirm their identity by high mass accuracy and the expected mass increment due to the stable $^{13}C_6$-labeling of salcatonin. As illustrated in Fig. 4.2a and b, both peptides have similar mass spectral profiles dominated by a triply-charged ion at 1144.82 and 1146.53 Da, respectively. The high relative intensity of the $[M + 3H]^{+3}$ ion and the absence of extensive product ions was achieved by selecting appropriate mass spectrometric operating parameters. The distribution of the isotopic peaks for both $[M + 3H]^{+3}$ ions (Fig.

Figure 4.2

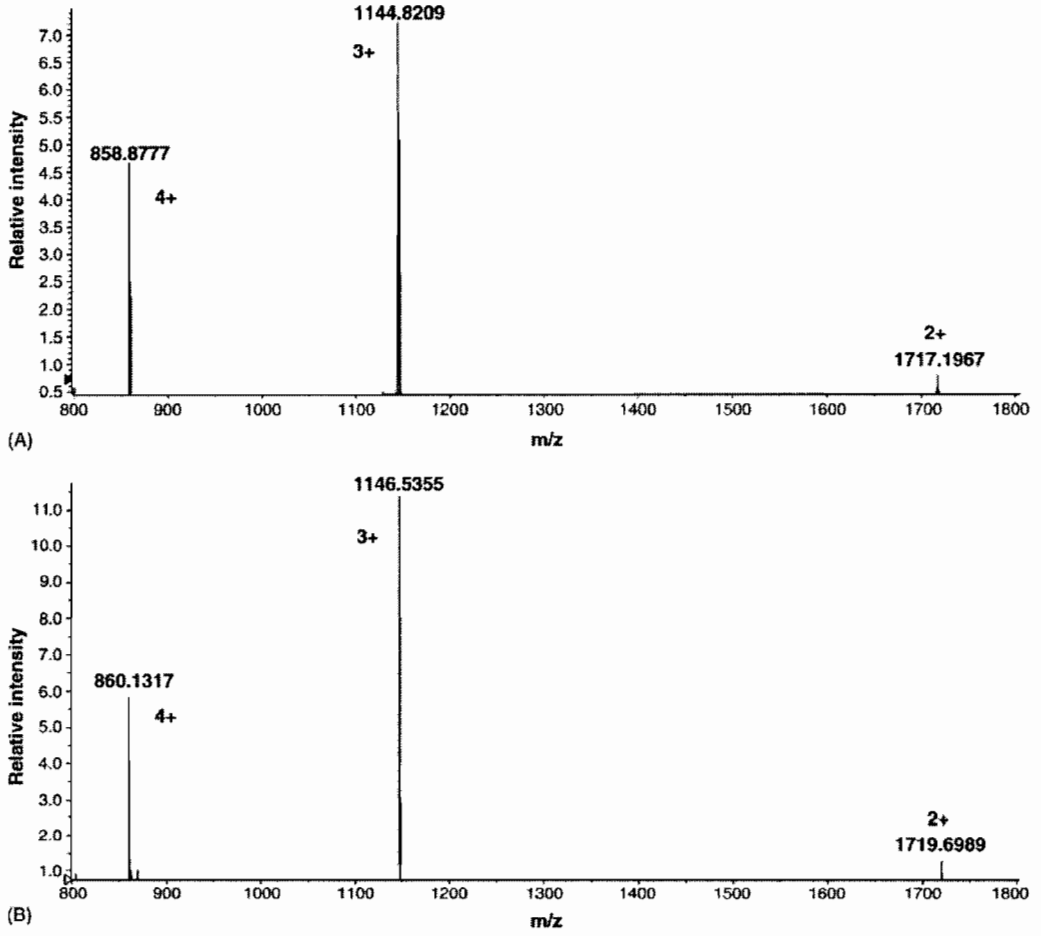

1144.8209
3+
858.8777
4+
2+
1717.1967
Relative intensity
m/z
(A)
1146.5355
3+
860.1317
4+
2+
1719.6989
Relative intensity
m/z
(B)

Figure 4.2 ESI TOF/MS of (A) salcatonin and (B) $^{13}C_6$-salcatonin obtained by μLC-TOF/MS analysis of a reference solution. Data displays m/z range from 800 to 1800, encompassing multi-charged species of interest. The triply charged species at m/z 1144.82 and 1146.54 yielded the strongest MS signals. These signals were selected for quantitation using extracted ion chromatograms.

Figure 4.3

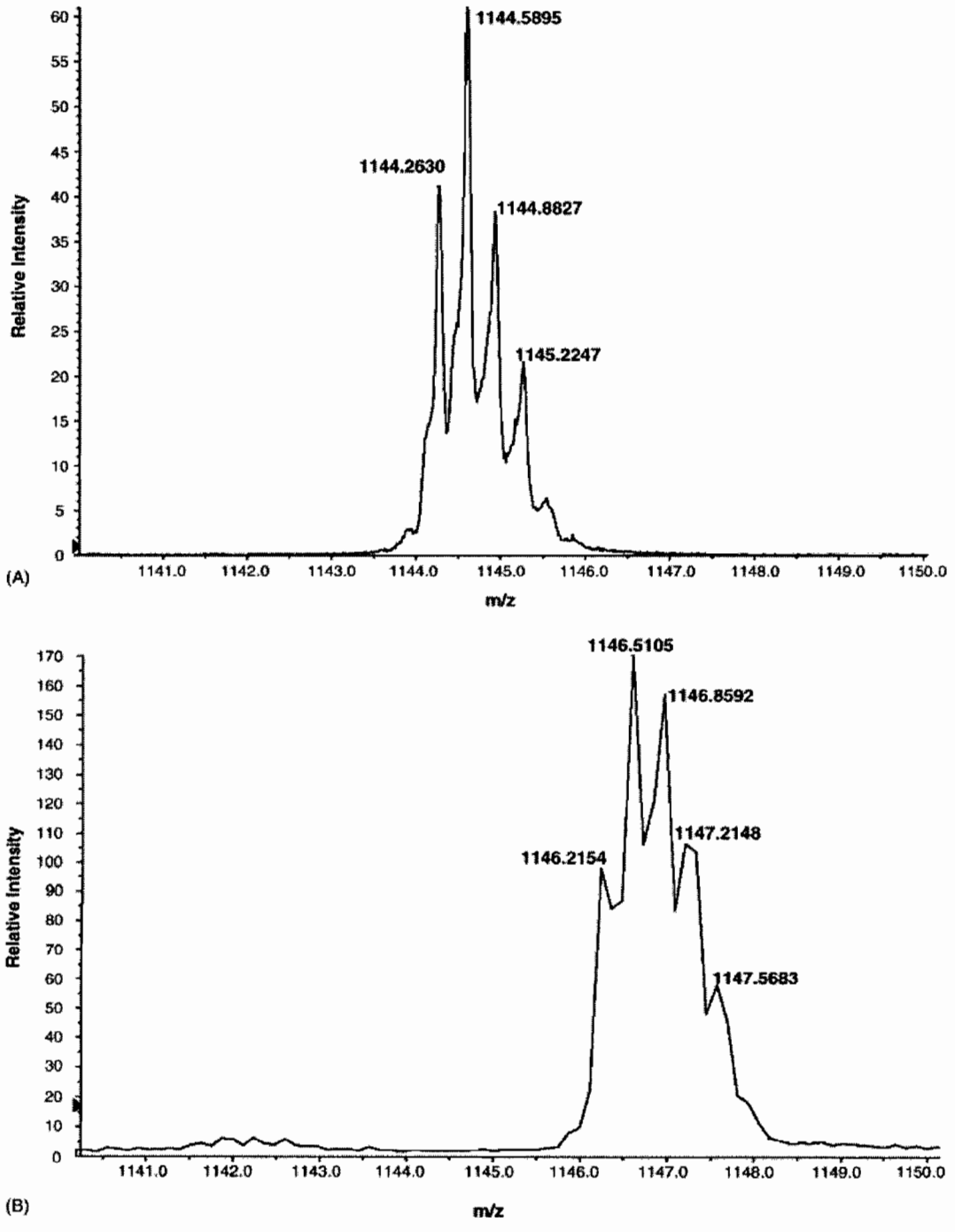

1144.5895
1144.2630
1144.8827
1145.2247
Relative Intensity
m/z
(A)
1146.5105
1146.8592
1147.2148
1146.2154
1147.5683
Relative Intensity
m/z
(B)

Figure 4.3 Isotopic distribution of the triply charged species of (A) salcatonin and (B) $^{13}C_6$-leucine labelled salcatonin internal standard. The monoisotopic ions at m/z 1144.2630 and m/z 1146.2154 were measured with accuracies of 22.2 and 19.4 ppm respectively. Isotopic peaks of unlabelled and labelled salcatonin demonstrate that there is no mass overlap between the two and that the labelled peptide is isotopically pure.

4.3a and b) verifies their charge state and indicates the absence of any mass overlap between the two compounds, thus confirming that the isotopic purity of the $^{13}C_6$-labelled analogue was >99.9%. Although tandem MS multiple reaction monitoring (MRM) experiments provide better signal to noise ratios with crude samples than TOF/MS methods, the high mass resolution of the latter technique provides for unambiguous identification of the analyte and unique selectivity of detection, particularly in complex biological matrices.

4.9.2 Human urine method

Our minimal sample preparation approach was ideal since it minimizes the degradation of the analytes. The μLC QSTAR combination provides sufficient resolving power for the clean separation and identification of the analytes. Specificity, linearity, accuracy and precision of salcatonin quantitation were assessed for the 10–1000 ng/mL concentration range with a 1μL on-column sample requirement which correspond to the injection of 2.65 fmol for the LLOQ sample. Specificity was assessed in three different batches of blank urine, in which the ionic background noise at the retention time of salcatonin was less than 25% of the height of the LLOQ calibration standard peak. A representative total ion chromatogram of a 10 ng/mL LLOQ sample is shown in Fig. 4.4a. The corresponding salcatonin extracted ion chromatogram at *m/z* 1144–1145 (Fig. 4.4b) shows the peak of interest with a signal to noise ratio of about 10:1, whereas that of the internal standard at *m/z* 1146–1147 is displayed in Fig. 4.4c. The lower limit of detection was approximately 3.6 ng/mL or 3.6 pg (1.05 fmol) on-column. The fact that salcatonin and its ^{13}C-labelled analog co-elute greatly minimized

Figure 4.4

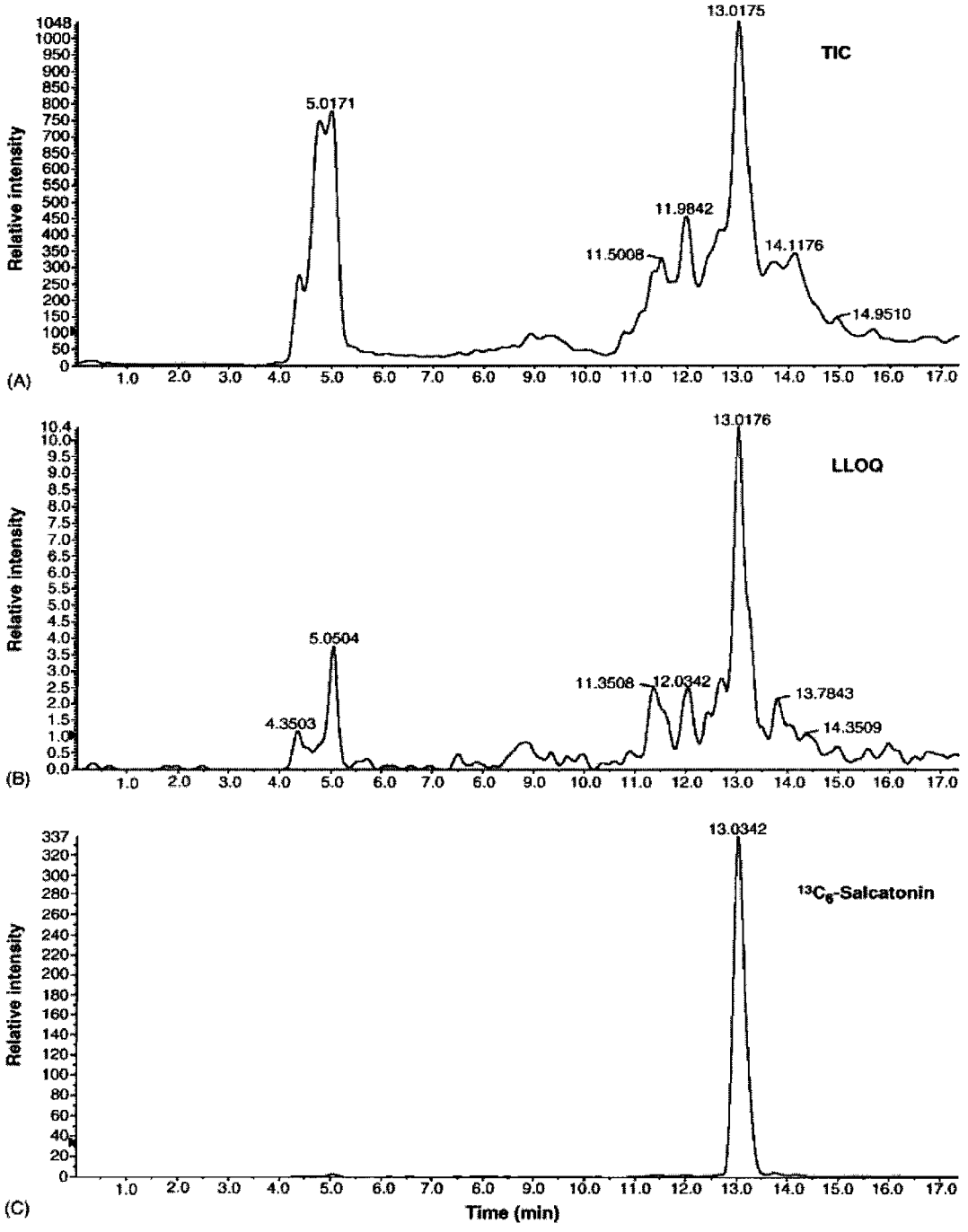

13.0175
TIC
5.0171
11.9842
11.5008
14.1176
14.9510
Relative intensity
(A)
13.0176
LLOQ
5.0504
11.3508
12.0342
13.7843
4.3503
14.3509
Relative intensity
(B)
13.0342
$^{13}C_6$-Salcatonin
Relative intensity
(C)
Time (min)

Figure 4.4 Analysis of a lower limit of quantitation (10 ng/mL) for salcatonin in human urine: (A) μLC TOF/MS total ion chromatogram obtained by scanning positive ions from m/z 800 - 1300, (B) extracted ion chromatogram for salcatonin over m/z range 1144–1145 and (C) extracted ion chromatogram for the $^{13}C_6$-leucine labelled salcatonin internal standard over the m/z range of 1146–1147. Conditions: mobile phase A was a 0.1% TFA solution in water and mobile phase B consisted of 0.1% TFA in acetonitrile, a 10 min linear gradient was used at a flow rate of 5 μL/min where mobile phase B was increased from 10 to 70% B and maintained for 10 min.

Table 4.1 Precision and accuracy data for human urine and human plasma calibration standards and QC samples.

Calibration standards			**QC samples**			
Conc. (ng/mL)	**Calculated conc. (ng/mL)[a]**	**Accuracy (% bias)[b]**	**Conc. (ng/mL)**	**Calculated conc. (ng/mL)[a]**	**% CV[c]**	**Accuracy (% bias)[b]**
Human urine						
10	10.6	106.4	30	31.7	9.5	105.7
20	20.4	102.1	400	447.2	2.3	111.8
500	502.6	100.5	700	773.2	1.8	110.5
800	805.8	100.7				
1000	1009.1	100.9				
Human plasma						
25	19.1	76.4	75	73.9	1.5	98.5
50	41.9	83.8	1000	1129.1	0.8	112.9
1250	1284.1	102.7	1750	1841.1	0.8	105.2
2000	2002.1	100.1				
2500	2497.0	99.9				

[a] Concentration results were rounded to one decimal place.
[b] % Bias is the calculated salcatonin concentration expressed as a percentage of its nominal concentration.
[c] % CV was calculated from three individual results at each salcatonin concentration.

potential differences in ionization efficiency (e.g. matrix effect) which are likely to arise in non-isotope dilution methods where the internal standard and the analyte of interest display different chromatographic behaviour. This specific feature of our approach maximizes the precision and accuracy of the measurements. A linear regression expressed by the equation $y = 0.00011x + 0.0157$ gave an excellent fit for the detector response to concentration relationship with a coefficient of correlation (R^2) of 1.0000. Calibration standards were measured with an accuracy (% bias) ranging from 100.5 to 106.4%. Three replicates of QC samples afforded within-run % bias of 105.7, 111.8 and 110.4% for low, med and high QC concentrations respectively (Table 4.1). These results demonstrate that salcatonin can be measured with good precision and accuracy in human urine using a crude sample preparation procedure combined with μLC-TOF/MS.

4.9.3 Human plasma method

The determination of salcatonin in plasma was more challenging due to the very complex nature of this matrix. Although the sample preparation procedure was straightforward, chromatographic resolution of salcatonin from abundant endogenous matrix components was achieved using an extended gradient μLC method. A representative total ion chromatogram of a 25 ng/mL (7.3 pmol/mL) LLOQ sample is shown in Fig. 4.5a, along with the corresponding extracted ion chromatograms of salcatonin (Fig. 4.5b) and its $^{13}C_6$-leucine-labelled analog (Fig. 4.5c). Even with the extended gradient, a 10-fold decrease was observed in the detection of internal standard signal of the plasma extracts relative to the urine

Figure 4.5

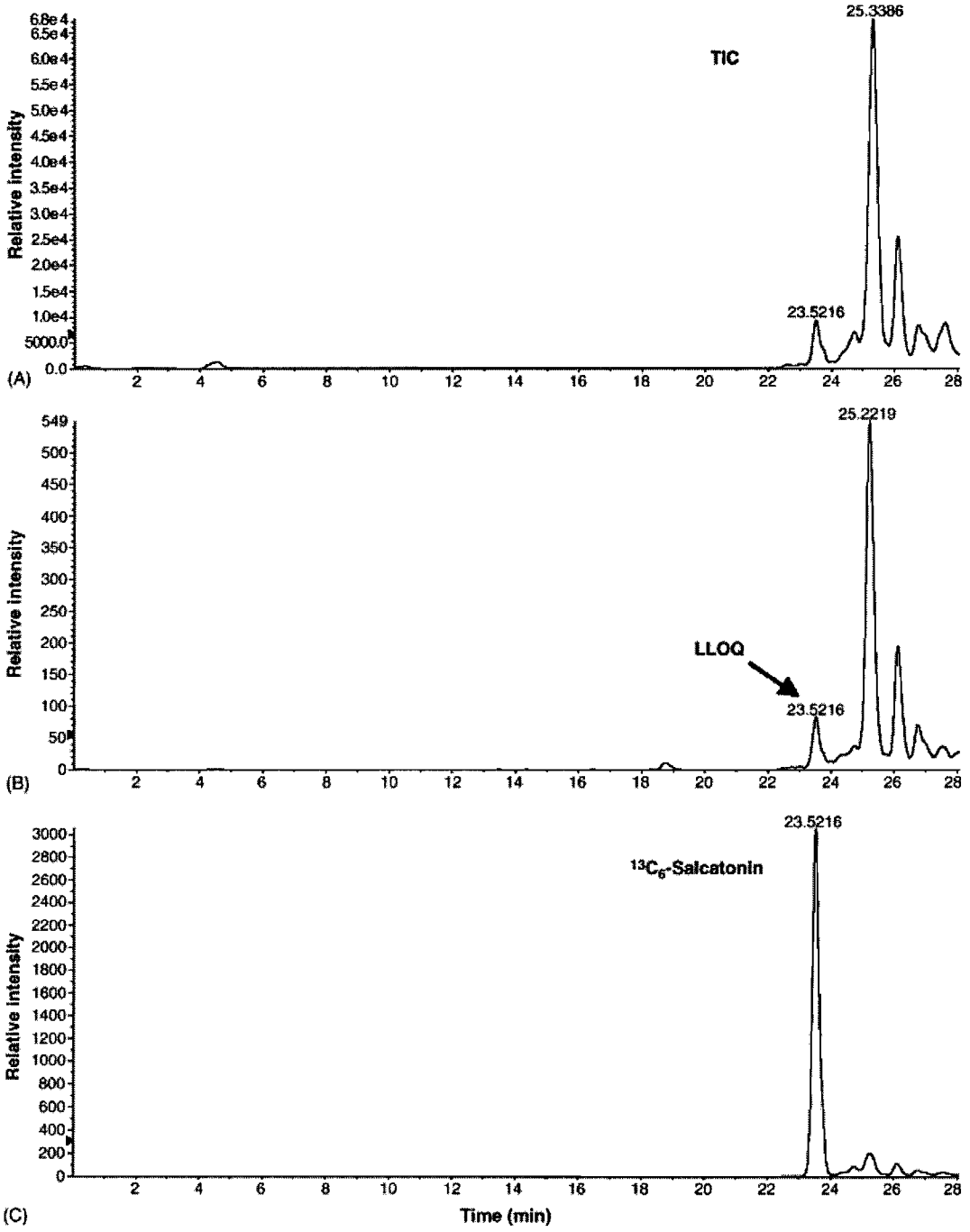

25.3386
TIC
23.5216
Relative intensity
(A)
25.2219
LLOQ
23.5216
(B)
23.5216
$^{13}C_6$-Salcatonin
Relative intensity
(C)
Time (min)

Figure 4.5 Analysis of a lower limit of quantification (25 ng/mL) for salcatonin in human plasma: (A) μLC-TOF/MS total ion chromatogram obtained by scanning positive ions from m/z 800 - 1300, (B) extracted ion chromatogram of salcatonin over the m/z range 1145-1146. (C) extracted ion chromatogram for the $^{13}C_6$-leucine labelled salcatonin internal standard over the m/z range 1146–1147. Conditions: mobile phase A was a 0.1% TFA solution in water and mobile phase B consisted of 0.1% TFA in acetonitrile, a 30 min linear gradient was used at a flow rate of 5 μL/min where mobile phase B was increased from 10 to 70% B and maintained for 10 min.

samples as illustrated in Figs. 4.4c and 4.5c where 1 and 100 ng of internal standard was applied to the system, respectively. This difference was most likely due to severe ion suppression by the complex matrix of the precipitated plasma samples. The intensity of the background noise arising from co-eluting material in blank plasma samples was found to be lower than 25% that of the LLOQ sample so that a signal to noise ratio of 5/1 or greater was routinely achieved. The normalized peak heights were shown to be linear with concentration over the 25–2500 ng/mL range and the corresponding relationship is expressed by the equation $y = 0.0109x + 0.0179$ with a coefficient of correlation (R^2) equal or better than 0.9998. The mean back-calculated concentrations of the calibration standards were between 76.3 and 100.1% and QC samples prepared at 75, 1000 and 1750 ng/mL salcatonin were analyzed with excellent precision and accuracy (Table 4.1) as indicated by their respective % standard deviation values ranging from 0.77 to 1.49% and % bias varying between 98.6 and 112.9%. Our μLC-TOF/MS method compared advantageously with that reported by (Song, An et al. 2002) where on-column injection of 200 pg (58.3 fmol) salcatonin was necessary to achieve a LLOQ of 10 ng/mL in rat and dog serum.

4.10 Conclusion

The combination of capillary column chromatography with the use of a $^{13}C_6$-labelled salcatonin analog as an internal standard permits the development of versatile and straightforward assays. Optimal sensitivities were obtained with minimal fragmentation and quantitation was based on the triply charged species

of salcatonin and its internal standard. Sample volume requirements were in the microliter range.

The data generated from both the plasma and the urine assays afforded similar precision and accuracy. The analytical approach employed can be used to monitor salcatonin in human urine and plasma since no significant interference by endogenous peptides or proteins were observed. Also, it could be applied to assess salcatonin purity and its degradation products in pharmaceutical preparations and in the course of stability studies. Whilst the instrumentation used can afford detection in the attomol range, the LLOQs presented in this paper were essentially limited by the intensity of chemical background, which was pronounced in plasma extracts. Affinity extraction methods such as immuno-precipitation combined with capillary or nano LC may provide solutions for the removal of the excessive chemical background noise observed in plasma samples, thus allowing the quantitation of salcatonin in the low fmol/mL range. The specificity of a mass spectrometric method with good sensitivity is an appropriate alternative to established methods for the measurement of salcatonin.

5.0 Mass spectrometric quantitation of C-reactive protein using labelled tryptic peptides.

Aguiar, M., Masse, R., Gibbs, B.F. (2006). Analytical Biochemistry **354**(2): 175-181.

5.1 Connecting text

Chapter 5 presents the development of a novel approach for the absolute quantitation of urinary proteins. We applied our method to the quantitation of rat C-reactive protein and used the method for quantitation in a model of drug induced nephrotoxicity. Analytical performance was suitable for implementation in a clinical setting. In particular, the approach makes use of capillary liquid chromatography in combination with time-of-flight mass spectrometry (μLC-TOF/MS) as an analytical platform. Capillary chromatography is ideal for low microliter sample volumes and offers high specificity and sensitivity when coupled to an appropriate detector. The approach is generally applicable to the quantitation of any urinary protein and may be suitable for protein quantitation in other biological matrices.

5.2 Abstract

Given the extensive efforts applied toward proteomics and research in biomarkers, methods for the simultaneous measurement of proteins, peptides, metabolic intermediates, hormones, etc. in a complex sample may be required in the foreseeable future. Assays based on mass spectrometric detection may be suitable for meeting the demands of such complex samples with sensitivity and

specificity. An analytical method for the quantitation of C-reactive protein (CRP), a well-known marker of inflammation, is described. Exact quantities of two synthetic ^{13}C-labeled CRP tryptic peptides were added as internal standards directly to the sample prior to chemical treatment, trypsinization, and liquid chromatography/mass spectrometry quantitation. C-reactive protein levels based on isotopic response ratios were measured. Intact C-reactive protein was spiked into blank rat urine for chemical and enzymatic treatment, producing linear response ratios of labeled to unlabeled peptides. For rigorous quantitation, standard curves, and quality control samples were prepared in rat urine with highly purified labeled and unlabeled peptides over the 50 pg–5 ng/μL concentration range. Using the same chemical and enzymatic treatment used for digestion of intact CRP, data from these samples demonstrated excellent analytical performance. The method was successfully applied toward the quantitation of urinary C-reactive protein from a study of drug-induced nephrotoxicity.

5.3 Introduction

C-reactive protein (CRP) (Fig. 5.1) is a well-conserved protein with homologs in many species that participates in the inflammatory response (Black, Kushner et al. 2004). Increased concentration of plasma CRP, observed within hours after injury or infections, has been used diagnostically in the clinic for many years (Ablij and Meinders 2002). During the acute phase of the inflammatory response, circulating CRP levels are greatly increased, reaching levels over 500 ng/μL or more

Figure 5.1

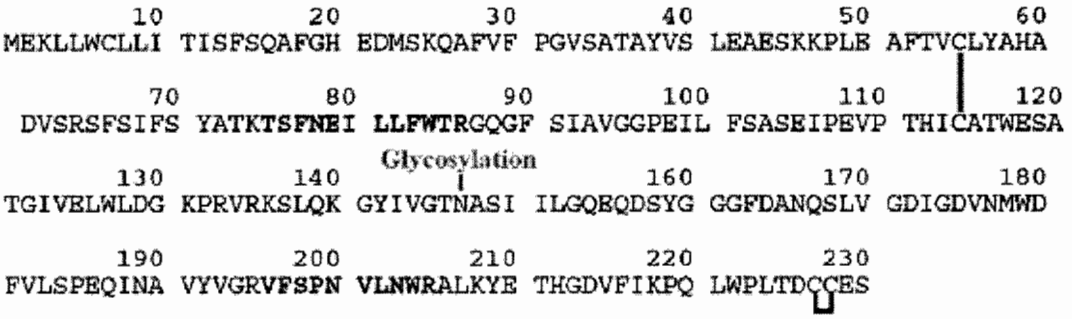

10 20 30 40 50 60
MEKLLWCLLI TISFSQAFGH EDMSKQAFVF PGVSATAYVS LEAESKKPLE AFTVCLYAHA
70 80 90 100 110 120
DVSRSFSIFS YATKTSFNEI LLFWTRGQGF SIAVGGPEIL FSASEIPEVP THICATWESA
Glycosylation
130 140 160 170 180
TGIVELWLDG KPRVRKSLQK GYIVGTNASI ILGQEQDSYG GGFDANQSLV GDIGDVNMWD
190 200 210 220 230
FVLSPEQINA VYVGRVFSPN VLNWRALKYE THGDVFIKPQ LWPLTDCCES

Figure 5.1 C-reactive protein (CRP) is a 25 kDa glycoprotein. The CRP amino acid sequence is shown with tryptic peptides selected for quantitation indicated in boldface. Disulfide bonds are shown along with N-linked glycosylation site.

(Steel and Whitehead 1994). The increased levels are mainly due to synthesis and secretion from the liver (Hurlimann, Thorbecke et al. 1966). CRP is also expressed in neurons, atherosclerotic plaques, monocytes, and lymphocytes (Kuta and Baum 1986; Jialal, Devaraj et al. 2004). Recent studies have correlated slightly elevated CRP levels, from less than 1 ng/μL blood to greater than 3 ng/μL, with the risk of developing cardiovascular disease (Ablij and Meinders 2002). Several ligands are recognized by CRP including phosphocholine, histones, polycations, phosphoethanolamine, chromatin, fibronectin, small ribonucleoproteins, and laminin (Szalai, Agrawal et al. 1999; Black, Agrawal et al. 2003). Upon ligand recognition, CRP activates the classical complement pathway and can stimulate phagocytic cells, thus playing a crucial role in the innate host immune defence (Ablij and Meinders 2002). Studies performed with ^{125}I-labelled CRP have shown that the kidney is the primary organ for excretion of CRP in mice and humans (Vigushin DM 1993; Motie, Schaul et al. 1998). Hence, high levels of urinary CRP can potentially be correlated with elevated plasma CRP concentration and used to monitor physiological states known to be associated with increased CRP concentrations. Protein levels in a complex biological sample can be measured by several analytical techniques. For the determination of CRP, clinical samples are usually measured by immunonephelometric or immunoturbidimetric assays (Rifai, Tracy et al. 1999). The versatility of mass spectrometric (MS) techniques encourages its use for various applications. MS methods are increasingly being used for the sensitive and specific quantitation of many types of molecules. The methods are relatively quick to implement and

offer the possibility of monitoring many analytes in a single analysis. In contrast, immunoassays require antibodies and time for implementation and are typically used to detect only one analyte per assay. Although their sensitivity and specificity can be excellent, the cost of immunoassay equipment, antibodies and other reagents may be prohibitive.

A number of studies have demonstrated the use of stable-labelled isotope tags for monitoring relative protein concentration (Schneider and Hall 2005). Since the tagged analytes are chemically identical, differing only in the isotopic abundance, sample recovery differences and variation in detection are essentially eliminated with these approaches. Alternatively, labelled internal standards may be added to a sample for the analytical determination of peptides and proteins (Gerber, Rush et al. 2003; Aguiar, Gibbs et al. 2005).

An assay for serum CRP in patients with rheumatoid arthritis using multiple reaction monitoring MS and ^{13}C-labelled peptide standards has been reported (Kuhn, Wu et al. 2004). The approach utilized 1 mL of starting serum with extensive sample preparation including depletion of abundant serum proteins, size exclusion chromatography, and concentration and filtration in two steps of centrifugation with addition of labelled peptides after overnight tryptic digestion. Given the losses associated with several purification steps before the addition of labelled internal standards, accurate and reliable quantitation of CRP could be problematic.

A method for the determination of rat urinary CRP using labelled tryptic peptide internal standards added directly to the starting sample, with subsequent in situ

denaturation, reduction, alkylation and trypsinization, is reported. The method was successfully applied to the determination of urinary CRP concentrations in a study of drug-induced nephrotoxicity. The approach may find applicability in several areas including bioanalytical and clinical chemistry, biology, and proteomics.

5.4 Materials and methods

5.4.1 Reagents and chemicals

All solvents and reagents employed were HPLC grade and were used without further purification. Sequencing-grade modified porcine trypsin was purchased from Princeton Separations Inc., NJ, USA. CRP purified from rat serum was purchased from Life Diagnostics Inc., PA, USA at an approximate concentration of 2.11 μg/μL (Bradford assay vs. bovine serum albumin) with ⩾95% purity. Synthetic rat CRP tryptic peptides (Thr-Ser-Phe-Asn-Glu-Ile-Leu-Leu-Phe-Try-Thr-Arg and Val-Phe-Ser-Pro-Asn-Val-Leu-Asn-Try-Arg; hereafter referred to as TS peptide and VF peptide, respectively, in lieu of their first two residues) and their [$^{13}C_6$]leucine-labelled analogs each containing one labelled leucine residue were obtained from the Sheldon Biotechnology Center, McGill University, Que., Canada. The crude peptides were purified by reversed-phase preparatory liquid chromatography on an octadecyl silane column. Purified synthetic peptides were tested by liquid chromatography with ultraviolet (UV) and MS detection. Product ion spectra confirmed their identities. No extraneous peaks were detected in the

purified peptides, and the final purity was estimated to be greater than 99% by MS.

5.4.2 Demonstration of intact CRP digestion in rat urine with a linear response of digestion products

Pooled blank rat urine, previously tested and determined not to contain CRP, was allowed to thaw on ice. Ten micrograms of intact rat CRP was mixed into 10 µL of blank rat urine in a microfuge tube. One microgram of intact rat CRP was mixed into 10 µL of blank rat urine in a second microfuge tube. Five hundred nanograms of labelled TS and VF internal standard peptides were added to each sample. The samples were mixed with 10 µL each of trifluoroethanol. Four microliters of 100 mM dithiothreitol in 100 mM ammonium bicarbonate was added to each sample for a 45-min disulfide bond reduction at 56 °C, followed by alkylation with 4 µL of 550 mM iodoacetamide in 100 mM ammonium bicarbonate for 30 min in the dark. Samples were then diluted to 200 µL with 100 mM ammonium bicarbonate and digested overnight at 37 °C with 200 ng modified porcine trypsin (0.4 µL addition of trypsin solution in water previously stored in aliquots at −15 °C at a concentration of 0.5 µg/µL). The following day samples were lyophilized, reconstituted with 10 µL of formic acid:methanol:water (5:5:90) desalted on Millipore C18 Z-tips, eluted with 10 µL formic acid:methanol:water (5:60:35), and applied to the MS by infusion.

Stock solutions of the labelled and unlabelled TS and VF peptides were prepared in a mixture of acetonitrile: 0.1% TFA (1:1) and stored at −20 °C. Calibration standards and quality control (QC) samples were prepared using the following

procedure. One-hundred-ninety-six-microliters aliquots of blank rat urine were placed in individual 0.2-mL PCR tubes. Calibration standards and QC samples were spiked with 4 μL of appropriate spiking solutions containing both unlabelled peptides; spiking solutions were prepared by serial dilution of stocks diluted in acetonitrile:0.1% TFA (1:1). Calibration standards and QC samples were used to assess precision and accuracy and determine the lower limit of quantitation (LLOQ). Five calibration standards ranging from 50 to 5000 pg/μL (Table 5.1) were used to define the standard curve. QC samples at low (150 pg/μL), medium (1000 pg/μL), and high (3500 pg/μL) concentrations were prepared as described above and analyzed in triplicates.

5.4.3 Treatment of calibration curve and QC samples for LCMS analysis

Ten-microliter aliquots of calibration standards and QC samples were placed in microfuge tubes. Internal standards (5 ng) of the two [$^{13}C_6$]leucine-labelled peptides dissolved in 5 μL of acetonitrile:water (1:1) were added to each 10-μL sample. Samples were then denatured (with trifluoroethanol), reduced (with dithiothreitol), and alkylated (with iodoacetamide) as described above for treatment of intact CRP. Samples were then diluted to 200 μL with 100 mM ammonium bicarbonate and digested overnight at 37 °C with 200 ng modified porcine trypsin. The digestion mixture was lyophilized and reconstituted with 10 μL of acetonitrile:water (1:1) and transferred to an HPLC vial. One microliter of each sample was applied to the μLCMS for analysis.

Table 5.1 Treatment of calibration curve and QC samples for LCMS analysis

Calibration standards			**QC samples**			
Concentration (pg/µL)	**Calculated concentration (pg/µL)[a]**	**Accuracy (% bias)[b]**	**Concentration (pg/µL)**	**Calculated concentration (pg/µL)[a]**	**% CV**	**Accuracy (% bias)[b]**
TS peptide						
50	53	110	150	140	4.2	93
100	92	92		140		93
2500	2600	100		130		87
4000	4200	110	1000	870	3.8	87
5000	4800	96		860		86
				810		81
			3500	3200	1.8	91
				3100		89
				3200		91
VF peptide						
50	56	110	150	120	4.7	80
100	82	82		120		80
2500	2700	110		130		87
4000	4000	100	1000	920	4.2	92
5000	4900	98		1000		100
				970		97
			3500	3400	2.9	97
				3300		94
				3500		100

[a] Concentration results were rounded to two significant figures.
[b] % Bias is the calculated peptide concentration expressed as a percentage of its nominal concentration.

5.4.4 Capillary liquid chromatography conditions and instrumentation

Samples were chromatographed on an Agilent 1100 capillary chromatography system with gradient elution. The system was equipped with a vacuum degasser and a diode array detector (Agilent Technologies, Palo Alto, CA, USA). UV signals at 206 and 280 nm were monitored throughout the chromatographic separations. Chromatography was performed with a Zorbax SB-C18 300 Å 3.5-μm 150 × 0.3-mm i.d. capillary column. Gradients were established with a two-solvent system (A and B) at a 5-μL/min flow rate. Solvent A was 0.1% TFA (trifluoroacetic acid) in water while solvent B consisted of 0.1% TFA in acetonitrile. Ten-minute linear gradients from 10 to 70% solvent B were developed to provide adequate chromatography within a 20-min run time. At the end of each run the solvent composition was held at 70% B until all major components had eluted, as revealed by the UV signal at 206 nm.

5.4.5 Mass spectrometric analysis

The effluent of the μLC system was directed into a QSTAR Pulsar *i* hybrid tandem time-of-flight (TOF) mass spectrometer (Applied Biosystems/MDS Sciex, Concord, Ont., Canada). Mass spectrometric settings were optimized for the detection of the peptides of interest. Briefly, the mass spectrometer was configured for electrospray ionization in the positive mode. The instrument was set to scan using the TOF mass analyzer with resolution set at >4000 and mass accuracy <10 ppm. Ionspray voltage was set at ~4.5 kV. Nebulizer and curtain gas settings and ion optics lenses were adjusted for optimal system performance.

5.4.6 Quantitation method

Peptide quantitation was based on signals derived from extracted ion chromatograms of the peptides of interest. Extracted ion chromatograms for the doubly charged unlabelled and [$^{13}C_6$]leucine-labelled TS peptide, over the mass ranges *m/z* 763.5–765.5 and *m/z* 766.5–768.5 (Fig. 5.2) were obtained for all samples. The mass spectrum of the chromatographic peaks of interest were verified to confirm the charge state $[M + 2H]^{2+}$ and exact mass of each peptide (Fig. 5.3). Peak height ratios of the unlabelled over the labelled internal standard peptide signals were calculated for each sample. A calibration curve based on the peak height ratios of five concentrations of the CRP tryptic peptides was plotted (Fig. 5.4). The same analysis was applied to the unlabelled and labelled VF peptide using extracted ion chromatograms with *m/z* 616–618 (doubly charged unlabelled peptide) and *m/z* 619–621 (doubly charged [$^{13}C_6$]leucine-labelled peptide) (Fig. 5.5 and Fig. 5.6) for calibration curve preparation (Fig. 5.7). Least square linear regression and statistical analysis were performed using an Excel spreadsheet (Table 5.1).

5.5 Nephrotoxicity study design and collection of rat urine samples

A rodent species acceptable to the regulatory agencies was selected for this study. Male Sprague-Dawley rats were obtained from Charles River Laboratories, Domaine des Oncins, Saint Germain sur l'Arbresle, France. The animals were inspected for ill health upon arrival and allowed to acclimatize for 7 days before the beginning of treatment. Rats were 6 weeks old at initiation of treatment and

Figure 5.2

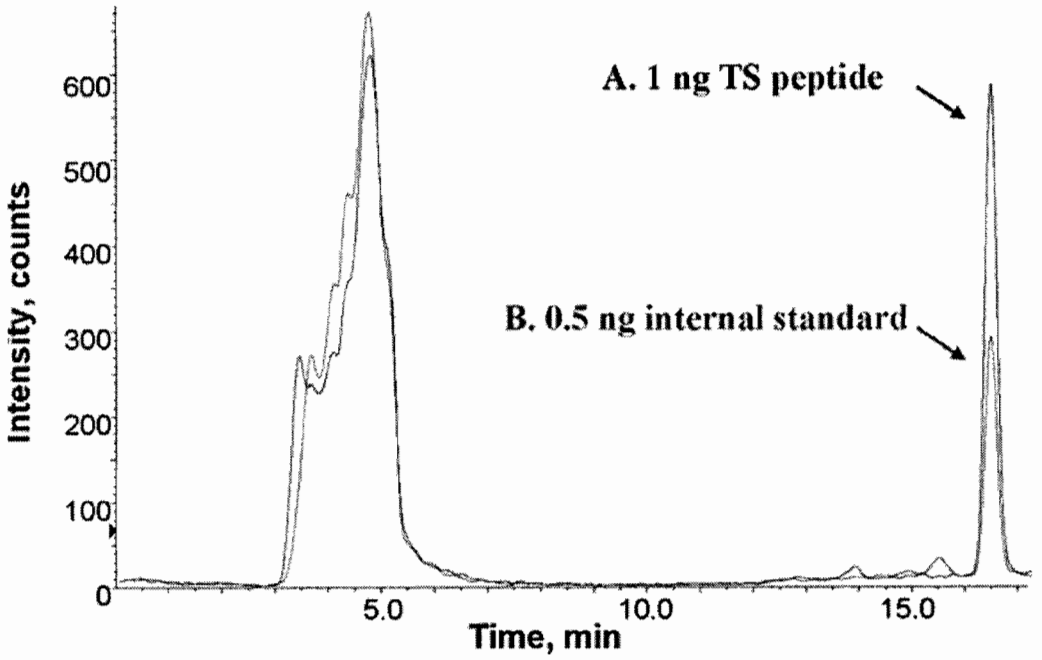

A. 1 ng TS peptide
B. 0.5 ng internal standard
Intensity, counts
600
500
400
300
200
100
0
5.0
10.0
15.0
Time, min

Figure 5.2 Representative LCMS extracted ion chromatogram of CRP tryptic peptide in rat urine. (A) Extracted ion chromatogram of Thr-Ser-Phe-Asn-Glu-Ile-Leu-Leu-Phe-Try-Thr-Arg peptide over mass range 763.5-765.5; signal obtained from 654 fmol of peptide, (B) extracted ion chromatogram of internal standard over mass range 766.5-768.5. The petide is well resolved from contaminants in the solvent front. Unlabelled and labelled species co-elute at 16.5 min.

Figure 5.3

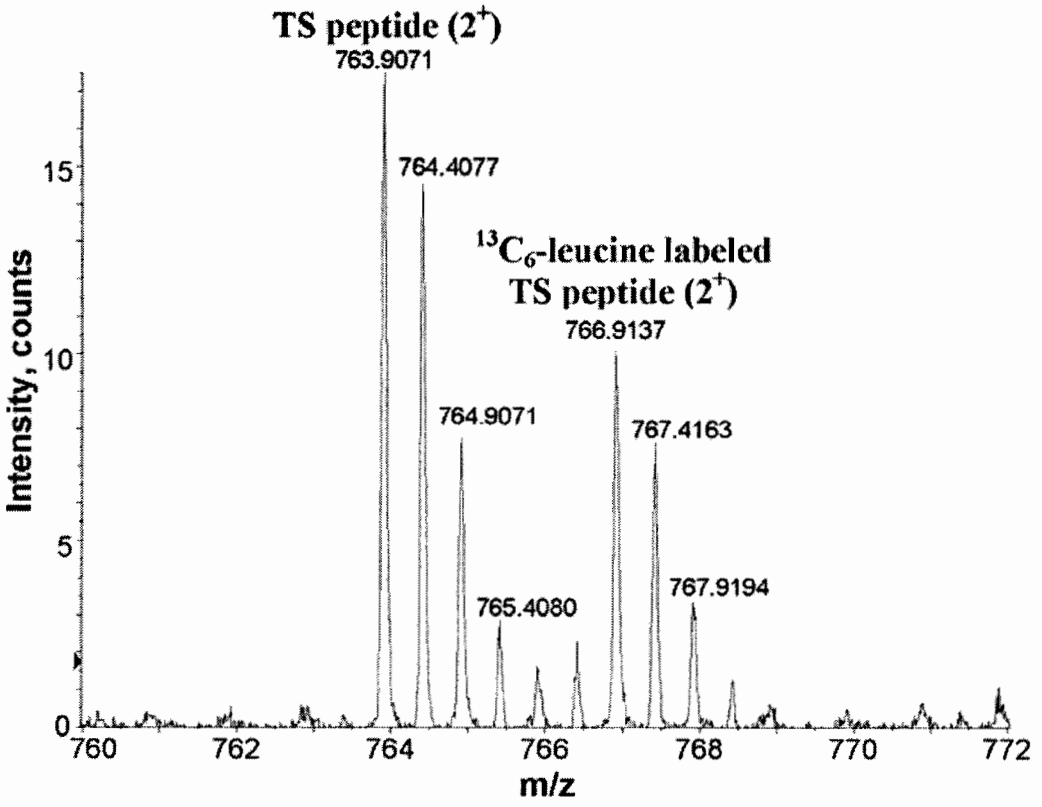

TS peptide (2+)
763.9071
764.4077
13C6-leucine labeled
TS peptide (2+)
766.9137
764.9071
767.4163
765.4080
767.9194
Intensity, counts
0
5
10
15
760
762
764
766
768
770
772
m/z

Figure 5.3 Isotopic distribution of the doubly charged species of tryptic CRP peptide Thr-Ser-Phe-Asn-Glu-Ile-Leu-Leu-Phe-Try-Thr-Arg and $^{13}C_6$-leucine-labeled internal standard. The monoisotopic ions at m/z 763.9071 and m/z 766.9137 were measured with an accuracy of 3.9 and 0.5 ppm, respectively.

Figure 5.4

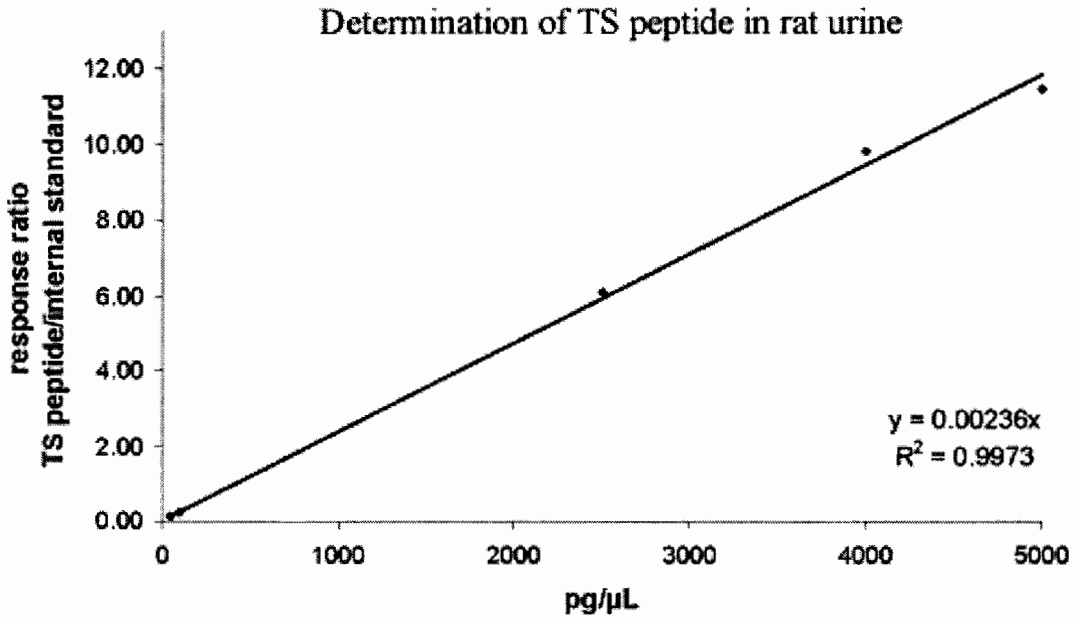

Determination of TS peptide in rat urine
response ratio
TS peptide/internal standard
12.00
10.00
8.00
6.00
4.00
2.00
0.00
0
1000
2000
3000
4000
5000
pg/µL
y = 0.00236x
R2 = 0.9973

Figure 5.4 Determination of CRP tryptic peptide Thr-Ser-Phe-Asn-Glu-Ile-Leu-Leu-Phe-Try-Thr-Arg in rat urine standard curve. Blank rat urine was spiked with purified unlabeled and labelled peptides, reduced, alkylated, trypsinized, and applied to μLCMS for analysis.

Figure 5.5

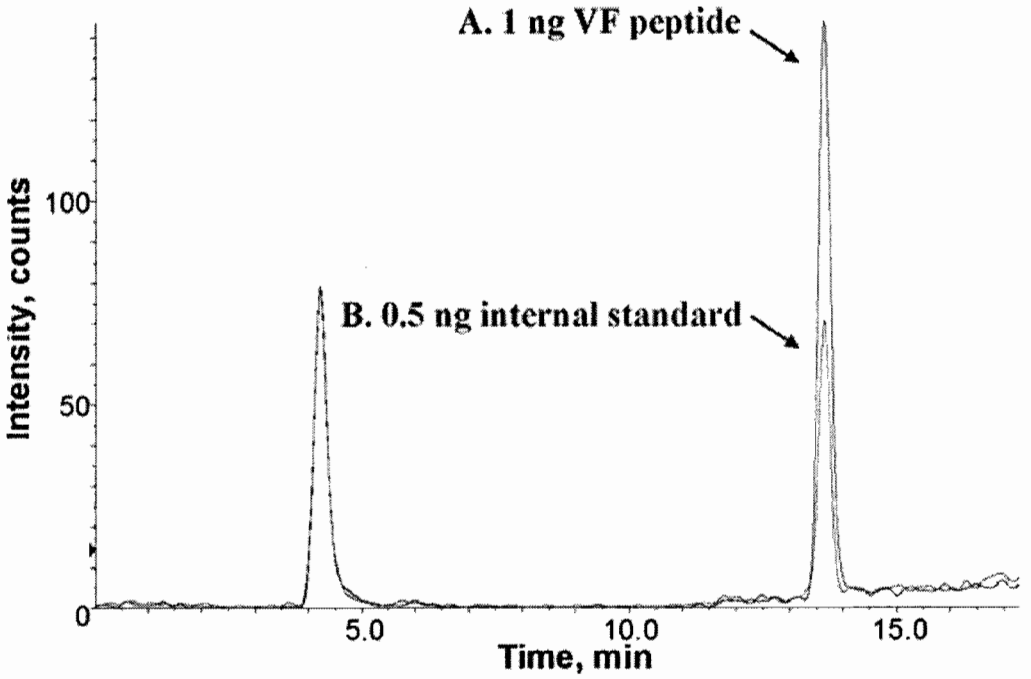

A. 1 ng VF peptide
B. 0.5 ng internal standard
Intensity, counts
100
50
0
5.0
10.0
15.0
Time, min

Figure 5.5 Representative standard of CRP tryptic peptide in rat urine. (A) Extracted ion chromatogram of Val-Phe-Ser-Pro-Asn-Val-Leu-Asn-Try-Arg peptide over m/z range 616–618, (B) extracted ion chromatogram of internal standard over m/z range 619–621.

Figure 5.6

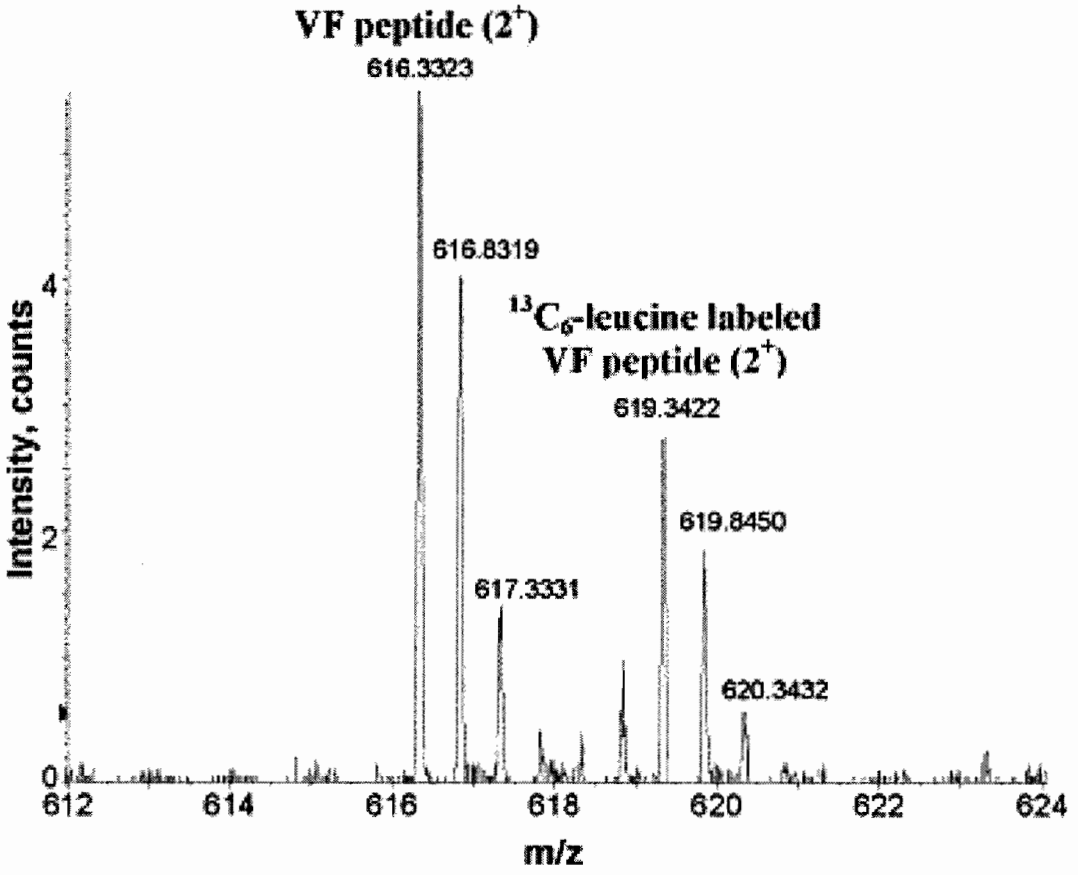

VF peptide (2+)
616.3323
616.8319
13C6-leucine labeled
VF peptide (2+)
619.3422
619.8450
617.3331
620.3432
Intensity, counts
0
2
4
612
614
616
618
620
622
624
m/z

Figure 5.6 Isotopic distribution of the doubly charged species of tryptic CRP peptide Val-Phe-Ser-Pro-Asn-Val-Leu-Asn-Try-Arg and $^{13}C_6$-leucine-labeled internal standard. The monoisotopic ions at m/z 616.3323 and m/z 619.3422 were measured with an accuracy of 1.1 and 1.4 ppm, respectively.

Figure 5.7

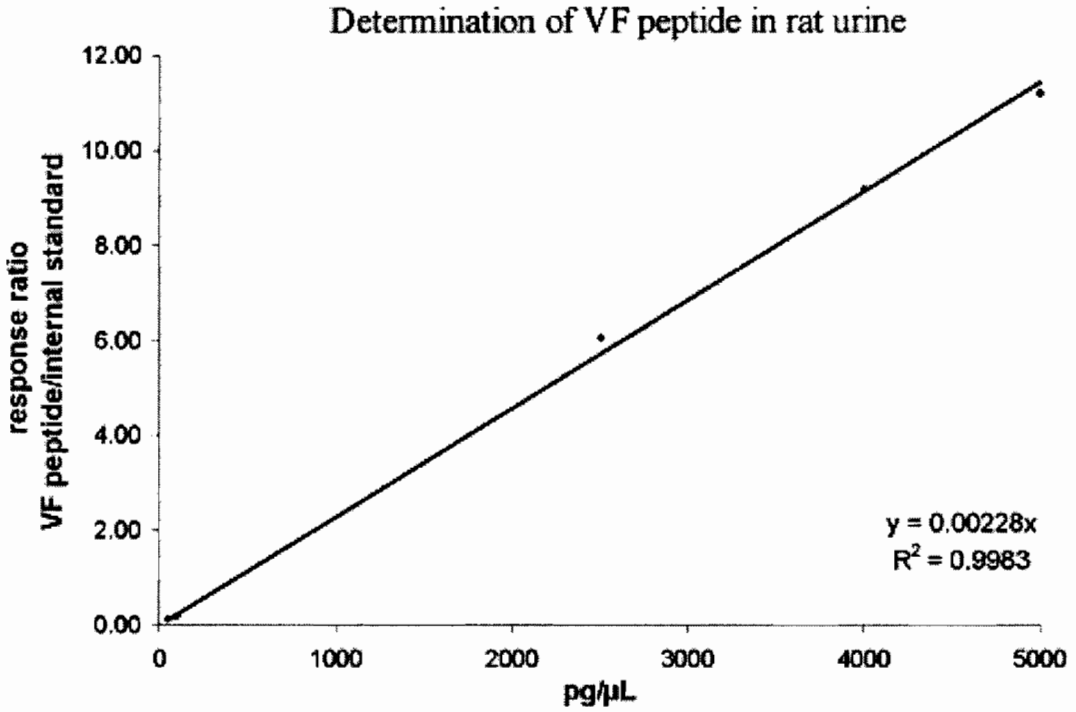

Determination of VF peptide in rat urine
response ratio
VF peptide/internal standard
12.00
10.00
8.00
6.00
4.00
2.00
0.00
0
1000
2000
3000
4000
5000
pg/µL
y = 0.00228x
R2 = 0.9983

Figure 5.7 Determination of CRP tryptic peptide Val-Phe-Ser-Pro-Asn-Val-Leu-Asn-Try-Arg in rat urine standard curve. Blank rat urine was spiked with purified unlabeled and labelled peptides, reduced, alkylated, trypsinized, and applied to μLCMS for analysis.

body weight range at initiation of the study varied from 170 to 220 g. Each animal was identified with an ear tattoo. Animals were housed in groups of five in plastic cages in an air-conditioned building (barrier protected unit) at 22 ± 2 °C, 55 ± 15% relative humidity, with more than eight air changes per hour and 12 h light (artificial)/12 h dark. A pelleted complete diet was provided ad libitum (Diet reference A04-C10). The feed was sterilized by irradiation and analyzed for the absence of chemical and bacteriological contaminants. Drinking water, free of chemical and bacterial contaminants, was also supplied ad libitum.

Ten animals were treated with a single bolus injection of puromycin aminonucleoside (100 mg/kg), introduced into a tail vein, to induce nephrotoxicity with accompanying proteinuria (Lannigan, Kark et al. 1962). Animals were fasted the night before sample collection. A gavage of drinking water was given to each animal (20 mL/kg) before the beginning of the collection period. Urine samples were collected from all animals on days 2, 17, and 29 in metabolism cages for 16 h. Samples were stabilized with a phenol solution (50 µL phenol 5% added in collection vials before the beginning of the collection period). Samples were placed on ice just after collection and aliquots of minimum 1 mL were stored at −80 °C for subsequent analysis. To accommodate rat to rat variability, equal volumes of rat urine samples from 3 animals experiencing proteinuria were pooled from days 2, 17, and 29 with subsequent sample analysis. Collected rat urine samples were treated according to the protocol used for calibration curve and QC samples. Determined CRP concentrations were back-calculated against the calibration curves.

5.6 Results

5.6.1 Effective in situ digestion of rat urinary CRP

To demonstrate the utility of our approach, initial experiments were performed with intact rat CRP (approximately 25 kDa as observed on one-dimensional SDS gel) isolated from rat serum. Blank rat urine samples spiked with a 10-fold concentration difference of intact rat CRP, each sample containing the same amount of labelled internal standard peptides, were treated according to the sample preparation method described above for MS analysis. Results from these experiments revealed that the method effectively cleaved the intact CRP in situ, consistently producing the anticipated unlabelled tryptic peptides. The response ratio of unlabelled over labelled tryptic peptides varied linearly with concentration for both the TS and the VF peptides over the 0.1- to 1-μg/μL CRP concentration range.

5.6.2 Analytical performance

Calibration standards and QC samples were prepared and treated as described above. Analysis of the resulting samples revealed excellent analytical characteristics (Table 5.1, Fig. 5.2, Fig. 5.3, Fig. 5.4, Fig. 5.5, Fig. 5.6 and Fig. 5.7). Blank rat urine samples were devoid of interferences >25% of the LLOQ samples. Back-calculated concentrations of calibration standards were within 82–110% accuracy, and QC samples were analyzed with precision and accuracy as shown in Table 5.1. Standard curves yielded R^2 values >0.997 for the 50 pg–5 ng/μL concentration range employed for both peptides (Fig. 5.4 and Fig. 5.7).

This peptide concentration range corresponds to CRP concentration of 820 pg–82 ng/μL for the TS peptide and 1.0–100 ng/μL for the VF peptide.

5.6.3 Determination of CRP from urinary samples collected from a nephrotoxicity study

Study rats given a single dose of puromycin aminonucleoside exhibited nephrotoxicity with accompanying proteinuria as revealed by one-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis with Coomassie blue staining (data not shown). When subjected to the method described above, samples collected at day 2 post puromycin aminonucleoside administration contained undetectable levels of CRP. However, samples collected on days 17 and 29 contained 1.4 and 1.1 ng/μL CRP, respectively (Table 5.2). Our results clearly indicated that CRP is one of the urinary proteins whose levels are increased as part of the nephrotoxicity response. Furthermore, the results are consistent with literature reports indicating that the kidney is the main organ responsible for the elimination of circulating levels of CRP (Vigushin DM 1993; Motie, Schaul et al. 1998).

5.7 Discussion

5.7.1 Selection of peptides for quantitation

An important aspect of the approach described is the selection of tryptic peptides to be used as labelled internal standards, which should be based on several criteria. The TS and VF peptides employed for his study were both readily produced and consistently observed by MS upon several tryptic digestions of CRP. It is strongly

Table 5.2 Determined urinary CRP concentrations from rat nephrotoxicity samples. Refer to section on nephrotoxicity study design and collection of rat urine samples for further details.

Day	CRP concentration (ng/μL)
2	Not detectable
17	1.4
29	1.1

advisable to perform test digestions of the protein of interest to determine which peptides are readily produced and easily detected in the digestion mixture prior to committing resources to peptide synthesis and purification.

Given the choice, it is preferable to select peptides that do not contain residues that can be oxidized. Oxidizable residues in the intact protein may result in an underestimation of the protein content. The two tryptic peptides selected for CRP quantitation each contained one tryptophan residue. However, the presence of the tryptophan residues did not affect the analytical characteristics of the method as oxidation products of TS or VF peptides were not observed. The overall sensitivity of the determination will depend on several factors including the strength of the signal produced by a given peptide. A peptide selected for quantitation should be of a suitable molecular mass for specific and sensitive detection. Ideally, a tryptic peptide of 1 to 2 kDa molecular mass, producing a good signal as a doubly charged species located in a portion of the spectrum with relatively quiet chemical background, should be selected. The TS and VF peptides, 1.5 and 1.2 kDa, respectively, fitted that criteria and produced strong MS signals that were consistently observed.

As the digestion of CRP was carried in rat urine, producing a final sample of significant ionic strength and moderate complexity, a one-dimensional reversed-phase chromatographic separation was employed to resolve the peptides of interest from the solvent front and interfering species. For optimal analytical performance, a given peptide should behave well chromatographically (symmetric

peaks with adequate capacity factor). As shown in Fig. 5.2 and Fig. 5.5, the TS and VF peptides demonstrated suitable chromatographic performance.

Finally, a database search of a candidate peptide should be performed to confirm that it will not be produced from the digestion of another potentially interfering protein. A database search (all taxa) of the TS and VF peptides on several commercial databases revealed that they were found to occur only in rat CRP. For human CRP detection, the analogous tryptic undecapeptide CRP (77-87) can be used since it is not present in any other protein of any other taxa.

5.7.2 Linear response of digested CRP and purified CRP tryptic peptides

Initially, experiments were performed with two concentrations of intact CRP treated in situ to ensure that the procedure was effectively producing the target peptides and that they varied in a linear fashion with concentration. In optimizing the method, measures were taken to favour the complete proteolysis of CRP by performing an effective denaturation, reduction, and alkylation prior to overnight enzymatic digestion with modified trypsin. These precautions allowed us to observe a linear response for the digestion of intact CRP across the 0.1–1 μg/μL range.

Accurate quantitative experiments were performed with standards and QCs prepared with highly purified tryptic CRP peptides. Calibration standards and QC samples were prepared and treated according to the protocol described above, resulting in good analytical performance as shown in Table 5.1. Back-calculated concentrations of standards and QCs were all less than 20% deviation from

nominal. These results demonstrate that the protocol can be used for analytical quantitation in a robust fashion.

5.7.3 Determination of CRP in rat urine from a drug-induced nephrotoxicity study

The analytical method described was used to determine CRP in urine samples collected from a puromycin-aminonucleoside-induced nephrotoxicity study. Nephrotoxicity was accompanied by proteinuria and CRP was observed as one of the urinary proteins whose levels increased with the onset of toxic effects of the drug. Previous studies have shown the kidney to be the principal organ involved in the excretion of circulating CRP (Vigushin DM 1993; Motie, Schaul et al. 1998). The results of this study indicate that urine samples collected on days 17 and 29 post puromycin administration had elevated CRP levels (Table 5.2). These results provide a concrete example of the utility of the approach for the non-invasive assessment of nephrotoxicity which could potentially be extended to the assessment of the acute inflammatory response in humans.

5.8 Conclusions

The use of appropriately selected synthetic labelled internal standard peptides, in situ chemical treatment, and tryptic digestion with μLCMS analysis provides a useful approach for urinary CRP quantitation. A 1-μL sample requirement was used to establish a LLOQ for two tryptic peptides at 50 pg/μL. The specificity of a liquid chromatography mass spectrometric method with high sensitivity is a useful alternative to established methods for the measurement of CRP.

6.0 Mass spectrometric study and search for posttranslational modifications of human aromatase

6.1 Overview

In this chapter, we report on our analysis of human estrogen synthase (aromatase) which was intensively studied with the aim of identifying novel modifications. Estrogens are crucial regulators of many bodily functions including development of reproductive systems, bone metabolism, central nervous system function, sexual behaviour and potentially cardio-protective effects (Sanchez, Nguyen et al. 2002). Sixty percent of breast cancers are estrogen receptor positive and respond to antiestrogen therapy (Biswas, Cruz et al. 2000). Due to the widespread physiological affects of estrogens, the discovery of novel aromatase modifications may be clinically diagnostic.

Our strategy was to obtain complete sequence coverage of human aromatase with the hope of identifying any novel modifications that may be present. Protein digestion followed by MS analysis of resulting peptides yielded 90.2% sequence coverage. Tandem MS sequencing of the N-terminal peptide suggests the presence of glutamine at residue 3 rather than glutamic acid, indicating a new sequence variant. Treatment of the enzyme with N-glycosidase F followed by MALDI analysis confirms the presence of N-glycosylation as previously suggested.

6.2 Introduction

Advances in DNA sequencing technologies in the last few years have yielded complete genome sequences for many species including our own. However, the gene products of most genes have not been characterized. Proteins which have received biochemical study have largely not been considered in terms of posttranslational modifications. Such modifications can be critical for proper cellular function while deregulated modification may be correlated with disease. A number of protein-based biopharmaceuticals including hormones (e.g. Insulin), antibodies (eg. Herceptin), blood factors, interferons, gonadotropins are currently used clinically with many more in development. Most bear some form of posttranslational modification that can dramatically affect chemical and therapeutic properties (Walsh and Jefferis 2006). In addition, many small molecule drugs target signal transduction pathways that affect critical intracellular proteins through modifications such as phosphorylation (Cohen 2002).

6.2.1 Background on Cytochrome P450 enzymes

Cytochrome P450s are a family of over 6,000 enzymes expressed in many species. Humans express 57 putatively functional enzymes with a variety of critical physiological roles. They are involved in the metabolic oxidation, peroxidation, and reduction of numerous endogenous and exogenous compounds including xenobiotics, steroids, bile acids, fatty acids, eicosanoids, environmental pollutants, and carcinogens (Nelson, Kamataki et al. 1993). Many illnesses including cancer, cardiovascular disease and endocrine dysfunction are linked to

P450 enzymes. Several levels of regulation, including transcription, translation, and posttranslational modification, participate in maintaining proper P450 function. Modifications including phosphorylation, glycosylation, nitration, and ubiquitination have been described for P450s. Physiological significance of the modifications include modulation of enzyme activity, targeting to specific cellular compartments, and tagging for proteasomal degradation (Aguiar, Masse et al. 2005). Relatively few P450 enzymes are known to harbour posttranslational modifications. In many cases, there is only enough evidence to suggest the occurrence and a possible role for the modification. Thus, many P450 enzymes have not been fully characterized. With the introduction of current proteomics tools, we are primed to answer many important questions regarding regulation of P450 in response to a posttranslational modification.

6.2.2 Posttranslational modification

A posttranslational modification may be defined as "any difference between a functional protein and the linear polypeptide sequence encoded between the initiation and the termination codons of its structural gene" (Han and Martinage 1992). Examples of non-covalent modifications include incorporation of cofactors such as heme, protein folding, and the association of subunits to form an oligomeric protein. Allosteric phenomena manifested as deviations from Michaelis-Menten kinetics have been demonstrated for numerous P450 enzymes. Various components known to interact with P450, including substrates, inhibitors, membrane lipids, and redox partners like Cytochrome-b5 are homotropic and

heterotropic effectors (Hlavica and Lewis 2001). P450 2E1 is stabilized by one of its substrates (ethanol), leading to increased cellular levels of the P450 (Roberts, Song et al. 1995). Covalent modifications, including cleavage of a signal peptide, formation of disulfide bonds, and an array of modifications to amino acid residues, including glycosylation, phosphorylation, ubiquitination, nitration etc. have been described for many proteins.

6.2.3 Phosphorylation

"Protein phosphorylation regulates most aspects of cell life, whereas abnormal phosphorylation is a cause or consequence of disease." (Cohen 2002). Cascades that activate the production of cyclic adenosine monophosphate (cAMP), leading to activation of protein kinase A (PKA) in eukaryotic cells illustrate a remarkable example. Once activated, PKA can phosphorylate many target proteins, resulting in an array of cellular responses, including regulation of gene transcription, modulation of enzyme activity, targeting for protein degradation, and targeting to various intracellular locations.

6.2.4 Modulation of P450 activity by phosphorylation

A number of reports over the last two decades describe the phosphorylation of over twenty P450 enzymes in microsomes, intact hepatocytes, cell culture, and in vivo (Table 6.1). Whereas some studies demonstrate stimulation of phosphorylation by addition of hormones and intracellular second messengers, other reports correlate phosphorylation with modulation of enzyme activity

Table 6.1 Phosphorylated P450 enzymes

P450	Species	Source	Reference
P450 1A2	Rat	Liver microsomes	(Pyerin, Taniguchi et al. 1987)
	Rabbit	Liver microsomes	(Pyerin, Taniguchi et al. 1987)
P450 2B1	Rat	Liver microsomes	(Jansson, Curti et al. 1990)
		Hepatocytes	(Oesch-Bartlomowic, Richter et al. 2001)
		Liver *(in vivo)*	(Oesch-Bartlomowic, Richter et al. 2001)
P450 2B2	Rat	Liver microsomes	(Pyerin, Taniguchi et al. 1987)
		Hepatocytes	(Oesch-Bartlomowic and Oesch 1990)
		Liver *(in vivo)*	(Koch and Waxman 1989)
P450 2B4	Rabbit	Liver microsomes	(Epstein, Curti et al. 1989)
P450 2C6	Rat	Liver microsomes	(Epstein, Curti et al. 1989)
		Liver *(in vivo)*	(Koch and Waxman 1989)
P450 2C7	Rat	Liver microsomes	(Epstein, Curti et al. 1989)
P450 2C11	Rat	Liver microsomes	(Pyerin, Taniguchi et al. 1987)
P450 2C12	Rat	Liver microsomes	(Epstein, Curti et al. 1989)
P450 2E1	Mouse	COS7	(Freeman and Wolf 1994)
		V79	(Oesch-Bartlomowic, Padma et al. 1998)
	Rat	Liver microsomes	(Menez, Machu et al. 1993)
		Hepatocytes	(Oesch-Bartlomowic, Padma et al. 1998)
P450 3A1	Rat	Liver *{in vivo)*	(Koch and Waxman 1989)
		Hepatocytes, Liver microsomes	(Eliasson, Mkrtchian et al. 1994)
P450 3A4	Human	*E-coli* expressed	(Wang, Medzihradszky et al. 2001)
P450 3A6	Rabbit	Liver microsomes	(Pyerin, Taniguchi et al. 1987)
P450 7A1	Rat	Liver microsomes	(Tang and Chiang 1986)
		E-coli expressed	(Nguyen, Shefer et al. 1996)
	Human	*E-coli* expressed	(Nguyen, Shefer et al. 1996)
P450 11A1	Bovine	Corpus luteum mitochondria	(Caron, Goldstein et al. 1975)
		Adrenal cortex mitochondrial inner membrane	(Vilgrain, Defaye et al. 1984)
P450 11B1	Bovine	Adrenal cortex mitochondrial inner membrane	(Defaye, Monnier et al. 1982)
P450 17A1	Human	NCI-H295, COS-1, Kin 8 expressed, Adrenal microsomes	(Zhang, Rodriguez et al. 1995)
	Rat	Testis microsomes	(Lohr and Kuhn-Velten 1997)
	Human	NCI-H295R, NCI-H295R expressed	(Biason-Lauber, Zachmann et al. 2000)
P450 19A1	Human*	Placenta	(Bellino and Holben 1989)
		MCF-7	(Yue, Wang et al. 2003)
		HeLa	(Beausoleil, Villen et al. 2006)
	Quail	Brain	(Balthazart, Baillien et al. 2001)
			(Balthazart, Baillien et al. 2001)
P450 51	Rat	Liver microsomes	(Sonoda, Amano et al. 1995)
P450 27A1**	Chicken	Kidney mitochondria	(Ghazarian and Yanda 1985)

*Putatively phosphorylated.

**Chicken 25-OH Vitamin D 1-hydroxylase sequence not yet reported.

(Koch and Waxman 1989; Pyerin and Taniguchi 1989; Oesch-Bartlomowic, Padma et al. 1998; Oesch-Bartlomowic, Richter et al. 2001). Much of the work has focused on members of family 1-3 P450s. Unfortunately, studies regarding regulation by posttranslational modification of the major drug metabolizing P450s, with the exception of P450 3A4 and P450 2E1 (i.e., P450 1A2, P450 2B6, P450 2C8, P450 2C19, P450 2C9, P450 2D6), have not been reported in the literature. However, evidence for the modification and regulation of P450 enzymes involved specifically in cholesterol and steroid homeostasis has been reported.

6.2.5 Estrogen Biosynthesis, Neuromodulation, and Cancer

P450 19A1 is the enzyme responsible for biosynthesis of estrogens from androgens. Catalysis results in the aromatization of the steroidal A-ring, which provides the origin of the common name for the enzyme (aromatase). The reaction involves 3 consecutive oxidation steps resulting in elimination of the androgen C19 methyl group (Figure 6.1). P450 19A1 is expressed in tissues associated with primary steroid synthesis, as well as a variety of other peripheral tissues, including gonads, brain, adipose, placenta, blood vessel, skin, and bone. Estrogens signal through the estrogen receptor, a transcription factor that regulates target gene expression (Mangelsdorf, Thummel et al. 1995). Selective estrogen receptor modulators (antiestrogens) are estrogen receptor antagonists designed to inhibit the mitogenic effects of estrogens. An alternative therapeutic strategy is to inhibit estrogen synthesis with an aromatase inhibitor.

Figure 6.1

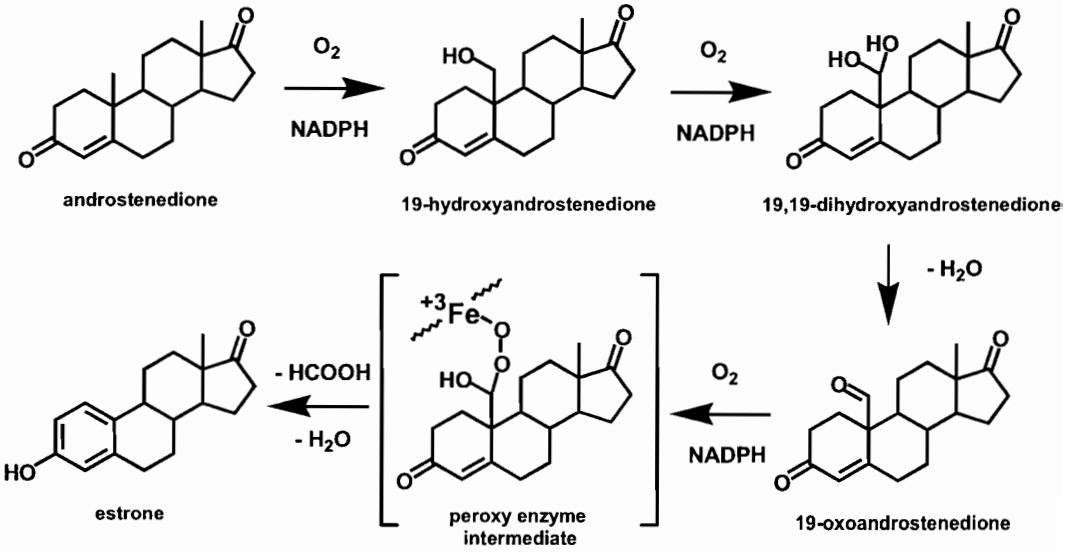

androstenedione
O_2
NADPH
19-hydroxyandrostenedione
O_2
NADPH
19,19-dihydroxyandrostenedione
$-H_2O$
19-oxoandrostenedione
O_2
NADPH
peroxy enzyme intermediate
- HCOOH
$-H_2O$
estrone

Figure 6.1 Aromatase reaction mechanism. P450 19A1 is the enzyme that catalyzes the formation of estrogens from androgens. The reaction involves 3 moles O_2 and NADPH, with elimination of the androgen C19 methyl group. The steroidal A-ring becomes aromatic in the formation of estrogens.

Transcriptional regulation of aromatase is well characterized and involves at least 10 different promoters, many of which regulate tissue specific expression (Simpson 2004). In addition to well-established transcriptional regulation, evidence supporting the regulation of P450 19A1 by posttranslational phosphorylation has been reported (Bellino and Holben 1989; Balthazart, Baillien et al. 2001; Balthazart, Baillien et al. 2001; Balthazart, Baillien et al. 2003; Yue, Wang et al. 2003; Beausoleil, Villen et al. 2006). Initial support came from a study employing microsomes isolated from human term placenta, where it was demonstrated that P450 19A1 activity could be maintained in phosphate buffer or by the inhibition of phosphatase activity with tartaric acid or EDTA in a phosphate-free buffer. The authors hypothesized that phosphorylation may play a role in regulation of P450 19A1 activity (Bellino and Holben 1989). P450 19A1 phosphorylation has since been implicated in aromatase activity modulation in relation to several different physiological phenomena. Reports in recent years have demonstrated the neuromodulatory effects of estrogenic metabolites [referenced in (Balthazart, Baillien et al. 2001)]. For example, a study with castrated sexually experienced male rats illustrates that estrogen administration rapidly activates sexual behaviour (within minutes), consistent with a non-genomic mechanism (such as rapid and reversible phosphorylation); gene transcription and translation requires hours to days for an observable effect (Cross and Roselli 1999). In another study using quail hypothalamic homogenates, rapid and pronounced changes in P450 19A1 activity were induced with kinase

activators and inhibitors (Balthazart, Baillien et al. 2001; Balthazart, Baillien et al. 2001). Stimulation of kinase activity by addition of normal intracellular concentrations of Ca^{2+}, ATP, and Mg^{2+} resulted in decreased P450 19A1 activity. The reduced activity could be completely abolished by the addition of a Ca^{2+} chelator (glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA). The authors noted, however, that the study had been performed with total hypothalamic homogenates, which raised the possibility that intermediary proteins interacting with P450 19A1 were phosphorylated rather than P450 19A1 directly. Conclusive experiments linking P450 19A1 phosphorylation with altered enzymatic activity were subsequently reported (Balthazart, Baillien et al. 2003). Aromatase from quail preoptic area homogenates was immunopurified, and phosphorylated Ser, Thr, and Tyr residues were detected by Western analysis employing phospho-amino acid specific antibodies. Together, these results demonstrate that the local production of estrogens in quail brain can be rapidly altered by calcium-dependent P450 19A1 phosphorylation.

P450 19A1 activity is also a key factor in the progression of estrogen-dependent diseases such as breast, endometrial, and ovarian cancers. Like P450 17A1, P450 19A1 represents an important target in the treatment of such diseases. Hormone-dependent breast cancer can be treated by surgical removal of the affected area or by endocrine treatment with a selective estrogen receptor modulator such as tamoxifen. Unfortunately, disease progression is only delayed by 12-18 months with endocrine treatment. Subsequent treatment with an aromatase inhibitor blocks disease progression in half the patients who relapse, suggesting an

adaptation to therapy that involves aromatase. Several mechanisms may account for the adaptation. It has been demonstrated that long-term estrogen deprivation causes hypersensitivity of cultured MCF-7 breast cancer cells to the mitogenic effects of estradiol, with an associated activation of the MAP kinase and PI3 kinase pathways. It was also discovered that aromatase activity is elevated in long-term estrogen deprivation (Yue, Santen et al. 1999). In order to establish a link between the kinase cascades and activation of aromatase, MCF-7 cells deprived of estrogens were treated with inhibitors of the kinase pathways. A significant decrease in aromatase activity was observed in just 2 hours, suggesting a non-genomic regulation of aromatase, possibly by P450 19A1 dephosphorylation (Yue, Wang et al. 2003). The authors concluded that more detailed studies would be required to understand the mechanisms of the kinase pathway inhibitors. Interestingly, a recent phosphoproteomic study of nocodazole arrested HeLa cells has identified phosphorylation of aromatase at residue Thr162 (Beausoleil, Villen et al. 2006). The role of aromatase phosphorylation in cancer biology has yet to be clearly established.

6.2.6 Glycosylation of P450

Protein glycosylation is an important posttranslational modification involved in cell adhesion, protein targeting, and protection from proteolytic attack. As illustrated in Table 6.2, a number of P450s have been identified as glycoproteins. The majority of these enzymes have not been characterized for the modification, and little is known about the underlying significance of their glycosylation. Two

Table 6.2 Glycosylated P450 enzymes

P450	Species	Tissue	Reference
P450 1A2*	Mouse	Liver (*in vivo*)	(Negishi, Jensen et al. 1981)
P450 2B2	Rabbit	Liver (*in vivo*)	(Haugen and Coon 1976)
P450 2B4	Rabbit	Liver (*in vivo*)	(Haugen and Coon 1976)
P450 11AI	Bovine	Adrenal mitochondrial	(Ichikawa and Hiwatashi 1982)
P450 17A1	Pig	cortex (*in vivo*)	(Nakajin, Hall et al. 1981)
P450 I9A1	Human	Testis (*in vivo*)	(Shimozawa, Sakaguchi et al. 1993)
	Human	COS1 expressed	(Amarneh, Corbin et al. 1993)
	Horse	Testis (*in vivo*)	(Moslemi, Vibet et al. 1997)
P450 21AI	Bovine	Adrenal cortex (*in vivo*)	(Hiwatashi and Ichikawa 1981)
P450 27B1	Bovine	Liver (*in vivo*)	(Hiwatashi and Ichikawa 1980)

*Putatively glycosylated.

enzymes, P450 11A1 and P450 19A1, have been studied in an effort to define the relationship between glycosylation and modulation of enzyme activity (Ichikawa and Hiwatashi 1982; Sethumadhavan, Bellino et al. 1991; Amarneh, Corbin et al. 1993). P450 11A1 demonstrates that glycosylation can modulate the catalytic activity of a P450. Treatment with neuramidase resulted in the inability to receive reducing equivalents from the reducing system. The authors concluded that the sugar moiety of glycosylated P450 11Al was essential for electron transport from reduced redoxin (Ichikawa and Hiwatashi 1982).

6.2.7 Aromatase glycosylation

Aromatase glycosylation has received attention in several reports. Human placental P450 19A1 is an N-linked glycoprotein (Sethumadhavan, Bellino et al. 1991). Its carbohydrate side chain can be selectively cleaved by endoglycosidases F and H, which hydrolyze N-linked glycans in a canonical N-X-S/T sequence. The site of glycosylation was determined as Asn12, which is consistent with core glycosylation (glycosylation occurring in the lumen of the ER) of an N-terminal portion of the protein (Amarneh, Corbin et al. 1993; Shimozawa, Sakaguchi et al. 1993). The hydrophobic N-terminal region of P450s comprises a signal recognition particle-dependent signal that directs insertion of P450 into the membrane of the endoplasmic reticulum (Bar-Nun, Kreibich et al. 1980; Sakaguchi, Mihara et al. 1987). Unfortunately, the significance of the core glycosylation has not been clarified. In terms of activity modulation,

glycosylation of P450 19A1 does not significantly alter catalytic activity (Amarneh, Corbin et al. 1993).

6.3 Materials and methods

6.3.1 Reagents and chemicals

Eighty micrograms of chemically pure (>95% as observed on SDS PAGE) and catalytically active (80.61 nmol/min/mg) human placental aromatase was obtained from the Hauptman-Woodward Institute, Buffalo NY. Sequencing grade (modified) porcine trypsin and chymotrypsin were purchased from Princeton Separations Inc., NJ, USA. Sequencing grade endoproteinases Asp-N, Glu-C and N-Glycosidase F were purchased from Roche Molecular Biochemicals, Laval QC, Canada. Bovine stomach mucosal pepsin was purchased from Cabiochem, EMD Chemicals Inc., Darmstadt, Germany. All solvents and analytical reagents employed were HPLC grade and were used without further purification.

6.3.2 Enzymatic digestions

In an attempt to maximize sequence coverage, multiple proteolytic enzymes (Trypsin, Chymotrypsin, Asp-N, Glu-C, Pepsin) were used for liquid and in-gel digestion. Enzymatic deglycosylation with N-Glycosidase F was performed directly on a surface enhanced laser desorption ionization (SELDI) NP20 chip for a MALDI-TOF analysis of native and deglycosylated aromatase.

6.3.2.1 Liquid Digestion

Liquid digestion of aromatase was performed as follows. Two μg aromatase was reduce for 30 min at 56°C with 20 μL of 10mM Cleland's reagent dithiothreitol (DTT)/100 mM ammonium bicarbonate. Cysteine residues were alkylated for 45 min (dark) at room temperature with 20 μL 55 mM iodoacetamide/100 mM ammonium bicarbonate. Digestions were performed for 12 hours with 1-4% protease at 37°C. Pepsin digestions were performed at pH 3.5 in 5% acetic acid. Digested samples were centrifuged to sediment particulates and the supernatant was applied to capillary LCMS. Peptides were chromatographically separated and identified on a Sciex QSTAR Pulsar *i* hybrid tandem MS.

6.3.2.2 In-Gel Digestion

In-gel digestions were performed essentially as described by (Shevchenko, Wilm et al. 1996) for tryptic digestion. Appropriate modifications were implemented for digestion with other proteases. Prior to in-gel digestion, aromatase was applied to 1-D sodium dodecylsulfate (SDS) PAGE on a 12% gel. Aromatase was visualized with Coomassie Blue staining. Protein bands were excised and washed for 15 min. with 100 μL water. Gel bands were cut into 1mm cubes and washed again with 100 μL water, then 100 μL 1:1 water:acetonitrile. Gel cubes were dehydrated with acetonitrile until they became white and sticky. Dehydrated gel material was then incubated with 100 μL 100 mM ammonium bicarbonate for 5 min (until gel cubes become fully translucent). Gel material was dried in a centrifugal vacuum apparatus (speedvac) until gel material was no longer elastic (resembling grains of

sand). Gel material was then chemically reduced for 30 min at 56°C with sufficient volume of 10mM DTT/100 mM ammonium bicarbonate to completely swell and cover gel cubes. Reducing solution was replaced with sufficient volume of 55 mM iodoacetamide/100 mM ammonium bicarbonate to alkylate cysteine residues for 30 min (dark) at room temperature. Gel material was washed with 100 µL of 100 mM ammonium bicarbonate (with 5% acetic acid for protein to be digested with pepsin) for 15 min. and then dried again in a speedvac. Once dry, gel material was incubated for 45 min. on ice (4°C) with a sufficient volume of protease solution (12ng/µL in 100 mM ammonium bicarbonate (pH 8.0)) to completely swell and submerge gel cubes. Sample digestion was performed at 37°C on a block heater for 12 hours. The digestion was then acidified with sufficient volume of 2% TFA to a final pH of approximately 3.0. Peptides were then extracted with 3 x 50µL 1:1 0.1% TFA: acetonitrile. Pooled extracts were evaporated to dryness in a speedvac, reconstituted with 5µL 1:1 0.1% TFA: acetonitrile for analysis by µLCMS.

6.3.2.3 Aromatase deglycosylation

Deglycosylation of aromatase was performed by depositing 1 µg of aromatase on a NP20 chip to which a 1 unit/µL solution of N-glycosidase F was added followed by incubating the array in a 37 °C water bath for 2 h. To deglycosylated aromatase, 1 µL of saturated sinapinnic acid prepared in 50% aqueous acetonitrile containing 0.5% TFA was added for SELDI-TOF analysis.

6.3.3 Proteomics tools

Analytical equipment used for aromatase sequencing and analysis included an Agilent 1100 capillary LC with a UV diode array detector. The MS was coupled to the LC with an ESI source. A QSTAR tandem hybrid mass spectrometer was used for peptide sequencing in the information dependent acquisition (IDA) mode. The QSTAR offers high mass accuracy (< 10 ppm error) and mass resolution (> 10,000), sufficient to unambiguously assign the charge state of multiply charged peptides. Doubly and triply charged peptides eluting throughout the chromatographic separation were automatically selected for tandem MS analysis. Database searches of tandem MS data were performed using Mascot (matrix science, UK). Appropriate search parameters (enzyme specificity, number of miscleavages, fixed modifications (e.g. carbamidomethylation), variable modifications (e.g. Met oxidation), precursor peptide mass tolerance, fragment ion mass tolerance, sequence database) were defined for searching peptide mixtures produced with different proteases. Dedicated searches were also performed in an attempt to identify phosphorylated residues by selecting phosphorylation as a variable modification. Peptides identified by Mascot were manually verified to ensure correct identifications.

A SELDI-TOF PBS 2c MS was used to measure the molecular mass of native and N-deglycosylated aromatase. The instrument was calibrated externally with adrenocorticotropin 2465 Da and bovine insulin at 5733 Da. The average mass accuracy after external calibration of PBSII-c is 2000 ppm (0.2%) for proteins of 10-300 kDa.

6.3.4 Capillary liquid chromatography conditions and instrumentation

Samples were chromatographed on an Agilent 1100 capillary chromatography system with gradient elution. The system was equipped with a vacuum degasser and a diode array detector (Agilent Technologies, Palo Alto, CA, USA). UV signals at 206 and 280 nm were monitored throughout the chromatographic separations. Chromatography was performed with a Zorbax SB-C18 300 Å 3.5μm 150x 0.3-mm i.d. capillary column. Gradients were established with a two-solvent system (A and B) at a 5μL/min flow rate. Solvent A was 0.1% TFA in water while solvent B consisted of 0.1% TFA in acetonitrile. A thirty-minute linear gradient from 10 to 70% solvent B was used for chromatographic resolution of peptides.

6.3.5 Mass spectrometric analysis

6.3.5.1 Tandem MS instrumentation and settings

Mobile phase from the μLC system was directed into a QSTAR Pulsar *i* hybrid tandem MS (Applied Biosystems/MDS Sciex, Concord, Ont., Canada). Mass spectrometric settings were optimized for detection and identification of proteolytic peptides. Briefly, the MS was configured for ESI in the positive mode. The instrument was set to scan using the TOF mass analyzer with resolution set at >8000 and mass accuracy <10 ppm. Ionspray voltage was set at 4.5 kV. Nebulizer and curtain gas settings and ion optics lenses were adjusted for optimal system performance. Multiply charged peptides above a defined signal threshold were automatically selected for tandem MS fragmentation. Peptides selected for

tandem MS were dynamically excluded from multiple consecutive tandem MS analyses in order to maximize the number of peptides sequenced.

6.3.5.2 SELDI-TOF instrumentation and settings

Demonstration of aromatase N-linked glycosylation was performed on a Ciphergen PBS2c chip reader. The instrument is essentially a MALDI-TOF designed to read SELDI chips with specialized surface chemistries (Merchant and Weinberger 2000). Mass spectra were collected in the positive-ion mode using a PBS2c ProteinChip reader (Ciphergen Biosystems Inc, Fremont, CA).

6.4 Results and discussion

Our efforts to obtain complete sequence coverage of aromatase involved a multi-pronged approach using several proteases for solution and in-gel digestion. We exploited the high mass accuracy and resolution of the QSTAR MS for sequencing and peptide identification; including peptides arising from non-specific cleavage. Nearly all of the sequence coverage was obtained using tryptic digestion of aromatase applied to gel electrophoresis. In addition to a well-defined cleavage specificity, tryptic peptides have the inherent advantage of an N-terminus that can conveniently be protonated as well as a C-terminal basic Lys/Arg whose side chain can also be protonated. As such, tryptic peptides carry a charge at acidic pH on both ends and will consequently be observed as a prominent doubly charged species by ESI. These doubly charged peptides are readily dissociated by CID to form characteristic product ions that can carry a

charge from either end, producing a series of information rich b and y" type ions that provide redundant and complementary sequence information. Thus, tryptic peptides are especially well suited for peptide sequencing by tandem MS. The other proteases used (chymotrypsin, Asp-N, Glu-C, pepsin) do not all have well defined cleavage specificities and do not produce peptides with a basic residue at the C-terminus. Thus, peptides resulting from cleavage with these enzymes are not as well suited to tandem MS sequence. Digestion with chymotrypsin and pepsin provided limited additional sequence coverage as compared to trypsin. Endoproteases Asp-N and Glu-C did not yield any additional sequence coverage. In-gel digestion is especially well suited for analysis of integral membrane proteins (such as aromatase and other P450 enzymes) because these are readily solubilized and denatured in Laemli buffer prior to gel electrophoresis. Residual SDS and other chemical contaminants are then rinsed away and the protein is efficiently trypsinized in-situ. Proteolytic peptides in an ideal "clean" chemical background are then extracted and analysed by LCMS. Solution digestions of equivalent samples are less ideal as proteolysis may not proceed effectively in the presence of detergents and solubilizing agents, which are necessary to solubilize the membrane protein. Once digested, the detergents must be removed removed prior to MS analysis without discarding the peptides of interest, which is not a trivial task. Other reports on proteomic studies of integral membrane proteins have also demonstrated the advantages of 1-D gel separation followed by in-gel trypsinization for maximizing the number of integral membrane proteins identified (Chung, Ng-Thow-Hing et al. 2007). Figure 6.2 illustrates a sample

Mascot search result identifying multiple aromatase peptides derived from a tryptic digestion. This single search covered 48% of the sequence of aromatase. While most of the peptides identified in this particular search were true tryptic peptides (cleaving after Lys and Arg except before Pro), several peptides derived from non-specific tryptic activity were also unambiguously identified. Using the QSTAR MS, precursor peptide masses were consistently measured with less than 100 ppm error. In addition, nearly complete b and y" fragment ions series were observed to provide confident peptide sequence assignments, even for peptides resulting from non-specific proteolysis.

Interestingly, the N terminal peptide spanning residues 1-10 uncovered a novel amino acid substitution (Figures 6.3 and 6.4). While the aromatase genomic sequence indicates Glu3, we observed Gln3 suggesting a novel sequence variant. Overall 90.2% sequence coverage was obtained (as shown in Figure 6.5). Aromatase is well conserved among vertebrates (Figure 6.6). Human and Japanese quail aromatase share 71% amino acid identity. Japanese quail hypothalamic aromatase is phosphorylated at Ser, Thr, Tyr residues (Balthazart, Baillien et al. 2003) and is rapidly and reversibly regulated by phosphorylation (Balthazart, Baillien et al. 2003). Conceivably, human aromatase may also be regulated by phosphorylation in a mechanism similar to that of the Japanese quail (Bellino and Holben 1989). Our tandem MS data were searched in an attempt to identify phosphorylated residues. We found no evidence of placental aromatase phosphorylation. However, human aromatase phosphorylation has been identified from nocodazole arrested HeLa cells (phospho Thr162)

Figure 6.2

1. gi|13904860 Mass: 58358 Score: 1105 Queries matched: 32
cytochrome P450, family 19 [Homo sapiens]
☐ Check to include this hit in error tolerant search

	Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
	32	450.26	898.51	897.60	0.91	0	52	11	3	K.AILVTLLR.R
☑	33	452.18	902.35	902.43	-0.08	0	46	56	1	E.MLNPIHY.N + Oxidation (M)
	49	494.25	986.48	986.56	-0.08	0	40	2.4e+02	7	F.ATELILAEK.R
	56	522.75	1043.48	1043.56	-0.09	0	45	64	4	K.EIQTVIGER.D
☑	64	550.76	1099.51	1099.61	-0.10	0	46	59	1	K.DAIEVLIAEK.R
	69	560.79	1119.56	1119.67	-0.11	0	48	32	2	L.LIKPDIFFK.I
☑	71	569.23	1136.46	1136.56	-0.10	0	50	21	1	R.MVTVCAESLK.T
☑	77	592.29	1182.56	1182.69	-0.12	0	62	1.3	1	R.IPLDESAIVVK.I
☑	83	618.27	1234.52	1234.64	-0.11	0	33	1.1e+03	1	R.TQPVVDLVMR.K + Oxidation (M)
	96	656.77	1311.53	1311.65	-0.12	0	53	11	2	R.RVMLDTSNTLF.L + Oxidation (M)
☑	106	695.78	1389.55	1389.69	-0.14	0	72	0.15	1	K.TLQGQCVESIQK.I
	110	702.79	1403.56	1403.70	-0.14	0	45	69	2	K.IHDLSLHPDETK.N
☑	117	717.29	1432.56	1432.71	-0.14	0	66	0.56	1	K.ALEDDVIDGVPVK.K
☑	119	717.75	1433.48	1433.61	-0.13	0	50	25	1	R.FLWMGIGSACNLY + Oxidation (M)
☑	118	722.80	1443.59	1443.75	-0.16	0	71	0.17	1	K.GIIFNNNPELWK.T
☑	120	484.92	1451.75	1451.87	-0.13	0	66	0.54	1	F.LRIPLDESAIVVK.I
☑	124	737.83	1473.64	1473.81	-0.17	0	53	13	1	R.VWISGEETLIISK.S
☑	126	492.24	1473.69	1473.81	-0.12	0	(46)	56	1	R.VWISGEETLIISK.S
☑	133	781.33	1560.64	1560.80	-0.16	0	76	0.06	1	K.ALEDDVIDGVPVKK.G
	134	521.23	1560.66	1560.80	-0.14	0	(44)	97	2	K.ALEDDVIDGVPVKK.G
☑	135	783.80	1565.58	1565.75	-0.17	0	(42)	1.5e+02	1	R.LEEVTNESGIVDVL.T
☑	137	783.80	1565.58	1565.75	-0.16	0	51	17	1	R.LEEVTNESGIVDVL.T
☑	150	890.84	1779.68	1779.88	-0.20	0	46	62	1	R.LEEVTNESGIVDVLTL.L
☑	152	947.38	1892.74	1892.96	-0.22	0	50	24	1	R.LEEVTNESGIVDVLTLL.R
	155	1036.41	2070.80	2070.05	0.75	0	33	1.2e+03	5	R.LEFFPKPNEFTLENFAK.N
☑	156	1094.91	2187.81	2188.06	-0.25	0	79	0.034	1	K.THLDRLEEVTNESGIVDVL.T
☑	159	730.29	2187.84	2188.06	-0.23	0	(64)	1.1	1	K.THLDRLEEVTNESGIVDVL.T
☑	160	730.29	2187.85	2188.06	-0.21	0	(58)	4.2	1	K.THLDRLEEVTNESGIVDVL.T
	163	760.62	2278.82	2279.05	-0.22	0	49	32	2	R.EHVNQCILEMLIAAPDTMSV.S + 2 Oxidation (M)
☑	167	801.66	2401.96	2402.20	-0.24	0	51	19	1	K.THLDRLEEVTNESGIVDVLTL.L
☑	168	839.34	2515.00	2515.28	-0.28	0	72	0.15	1	K.THLDRLEEVTNESGIVDVLTLL.R
	169	876.32	2625.94	2626.23	-0.29	0	35	8e+02	6	R.EHVNQCILEMLIAAPDTMSVSLF.F + 2 Oxidation (M)

Proteins matching the same set of peptides:
gi|28847 Mass: 58331 Score: 1104 Queries matched: 32
unnamed protein product [Homo sapiens]
gi|181213 Mass: 58362 Score: 1104 Queries matched: 32
cytochrome P-450-Arom

Figure 6.2 Mascot search result identifying multiple aromatase peptides derived from a tryptic digestion. This single search covered 48% of the sequence of aromatase. The majority of peptides identified were true tryptic peptides (cleaving after Lys and Arg except before Pro), but several peptides derived from non-specific tryptic activity were also unambiguously identified.

Figure 6.3

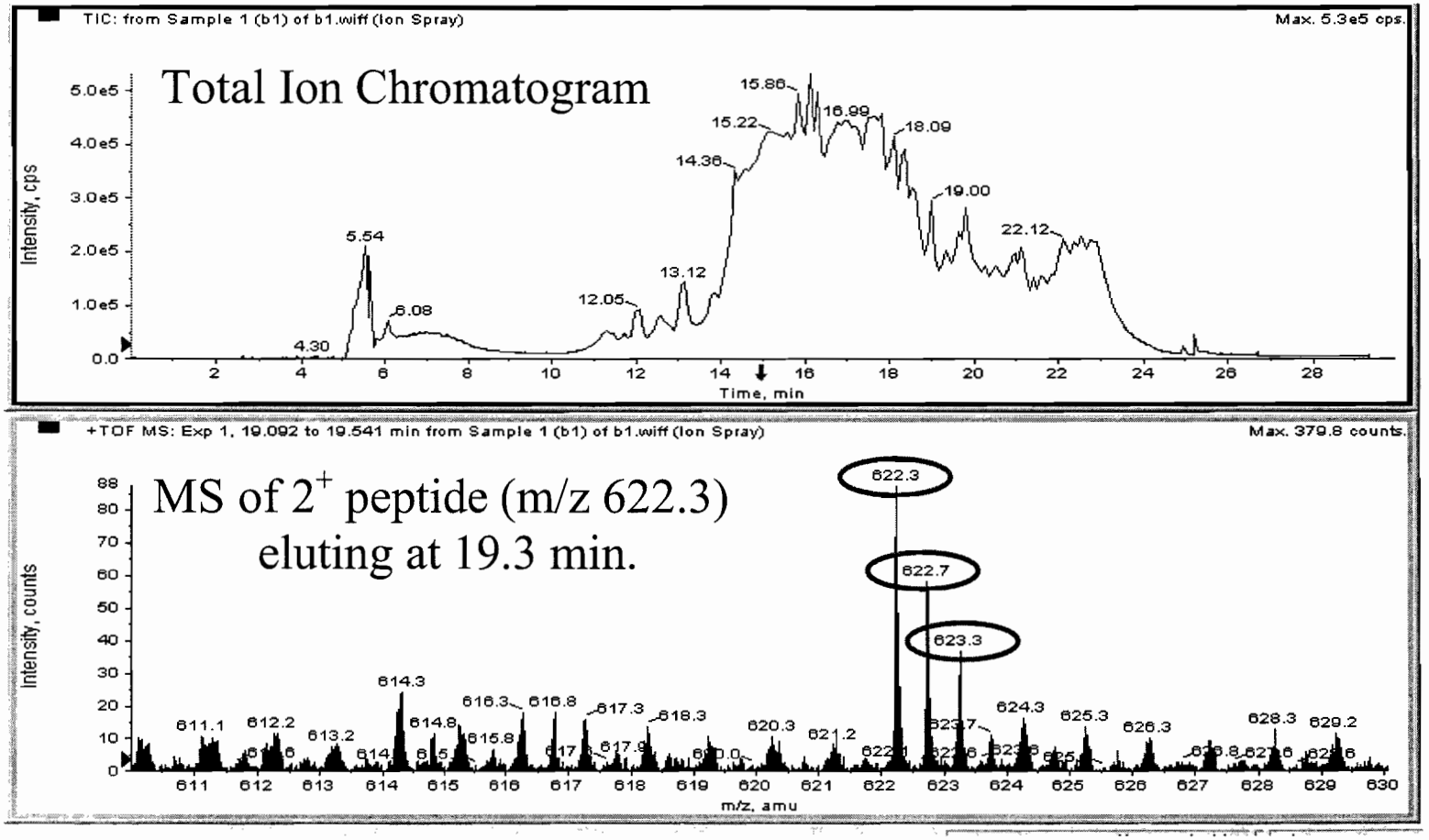

TIC: from Sample 1 (b1) of b1.wiff (Ion Spray)
Max. 5.3e5 cps.
Total Ion Chromatogram
Intensity, cps
Time, min
+TOF MS: Exp 1, 19.092 to 19.541 min from Sample 1 (b1) of b1.wiff (Ion Spray)
Max. 379.8 counts.
MS of 2+ peptide (m/z 622.3)
eluting at 19.3 min.
Intensity, counts
m/z, amu

Figure 6.3 Total ion chromatogram of in-gel digested aromatase. Hundreds of peptides were observed in the chromatographic separation. A doubly charged species was observed at m/z 622.3 corresponding to the N-terminal peptide. Sequencing of this peptide identified a novel amino acid substitution.

Figure 6.4

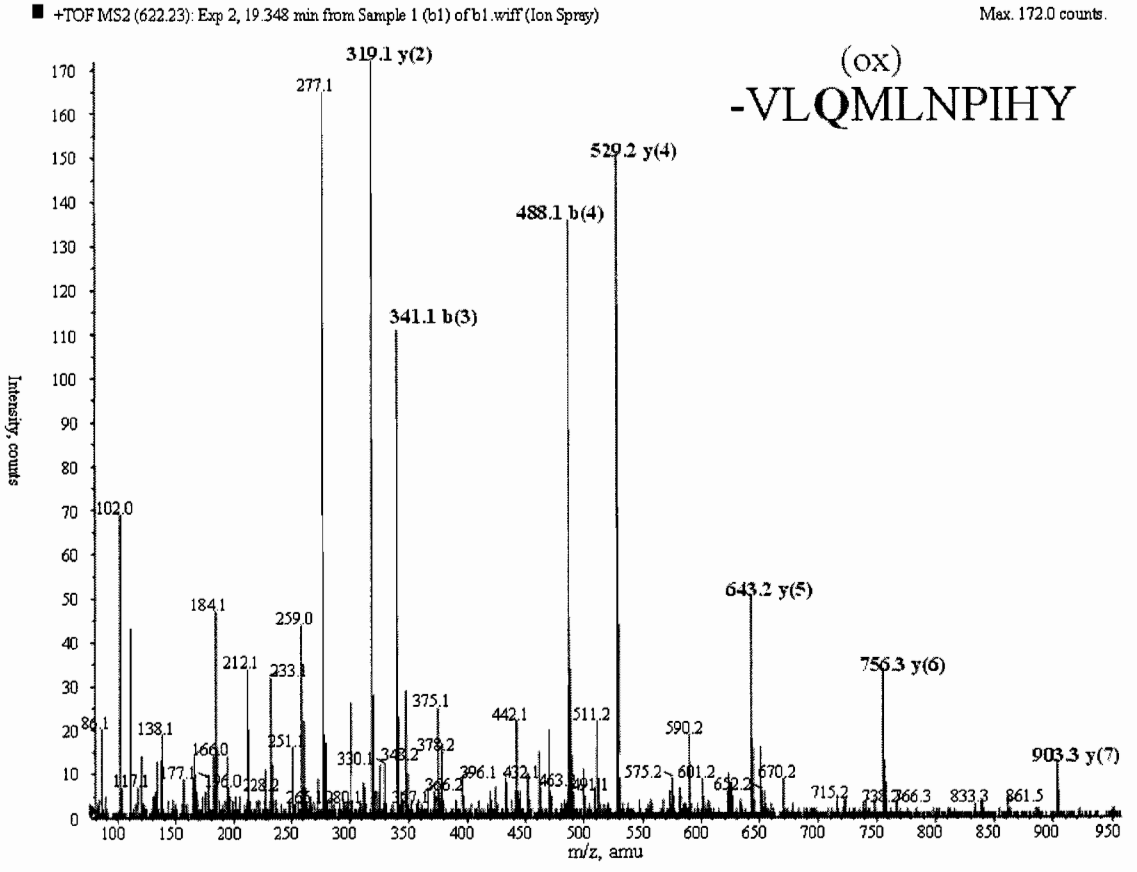

+TOF MS2 (622.23): Exp 2, 19.348 min from Sample 1 (b1) of b1.wiff (Ion Spray)
Max. 172.0 counts.
(ox)
-VLQMLNPIHY
319.1 y(2)
277.1
529.2 y(4)
488.1 b(4)
341.1 b(3)
643.2 y(5)
756.3 y(6)
903.3 y(7)
102.0
184.1
259.0
212.1
233.1
375.1
442.1
511.2
590.2
Intensity, counts
m/z, amu

Figure 6.4 Product ion spectrum of 2^+ peptide at m/z 622.23. While the aromatase genomic sequence indicates Glu3, Gln3 was observed as indicated by several b ions, indicating a novel sequence variant.

Figure 6.5

```
          10         20         30         40         50         60
VLQMLNPIHY NITSIVPEAM PAATMPVLLL TGLFLLVWNY EGTSSIPGPG YCMGIGPLIS
          70         80         90        100        110        120
HGRFLWMGIG SACNYYNRVY GEFMRVWISG EETLIISKSS SMFHIMKHNH YSSRFGSKLG
         130        140        150        160        170        180
LQCIGMHEKG IIFNNNPELW KTTRPFFMKA LSGPGLVRMV TVCAESLKTH LDRLEEVTNE
         190        200        210        220        230        240
SGYVDVLTLL RRVMLDTSNT LFLRIPLDES AIVVKIQGYF DAWQALLIKP DIFFKISWLY
         250        260        270        280        290        300
KKYEKSVKDL KDAIEVLIAE KRRRISTEEK LEECMDFATE LILAEKRGDL TRENVNQCIL
         310        320        330        340        350        360
EMLIAAPDTM SVSLFFMLFL IAKHPNVEEA IIKEIQTVIG ERDIKIDDIQ KLKVMENFIY
         370        380        390        400        410        420
ESMRYQPVVD LVMRKALEDD VIDGYPVKKG TNIILNIGRM HRLEFFPKPN EFTLENFAKN
         430        440        450        460        470        480
VPYRYFQPFG FGPRGCAGKY IAMVMMKAIL VTLLRRFHVK TLQGQCVESI QKIHDLSLHP
         490        500
DETKNMLEMI FTPRNSDRCL EH
```

Missing sequences in RED

Figure 6.5 Cumulative sequence coverage obtained (90.2 % coverage) for human P450 19A1. Despite extensive efforts to obtain complete sequence coverage, several amino acid sequences remained unaccounted for. "Missing" amino acid sequences are indicated in red.

Figure 6.6 Comparison of several aromatase amino acid sequences from multiple vertebrate species, showing significant amino acid conservation.

```
[Quail          ----------------MIPETLNPLNY-YTSLVPDLIPAATVPIIILICVLFLIWNHEG 42
[Alligator]     ----------------MILETLNPMHYNITNVVPEAMPAATVPILLLMGFLFLIWNYEE 43
[Human]         ----------------MVLEMLNPIHYNITSIVPEAMPAATMPVLLLTGLFLLVWNYEG 43
[Dog]           ----------------MLLEMLNPMHYNITSMMPEVMPVATMPILLLTGFLLLVWNYED 43
[Pig]           ----------------MVLEMLNPMHYKVTSMVSEVVPFASIAVLLLTGFLLLVWNYKN 43
[Horse]         ----------------MILEMLNPMHYNLTSMVPEVMPVATLPILLLTGFLFFVWNHEE 43
[Mouse]         ----------------MFLEMLNPMQYNVTIMVPETVTVSAMPLLLIMGLLLLIWNCES 43
[Rat]           ----------------MFLEMLNPMHYNVTIMVPETVPVSAMPLLLIMGLLLLIRNCES 43
[Frog]          ----------------MEALNPVQYNITEAVPTLAPATTLSLLLFIFVLIILWNQEE 41
[Halibut        MDRIPACDLAMTPVGLGAALGDLVSTSPNATAVRTPGISVASRTLILLVCVLLVAWSHTD 60
[Grouper        MDLISACERAMTPVGLDDMVADLATMSPNATAVGSPGISVATRLLILLICVLLAAWSHTD 60
                                 * .    *   .   . ::  :::: .::   .

[Quail          TSSIPGPGYCMGIGPLISHGRFLWMGVGNACNYYNKTYGEFVRVWFSGEETFIISKSSSV 102
[Alligator]     TSSIPGPCYCMGIGALISHGRFLWMGVGNACNYYNKIYGEFVRVWISGEETLIISKSSSV 103
[Human]         TSSIPGPGYCMGIGPLISHGRFLWMGIGSACNYYNRVYGEFMRVWISGEETLIISKSSSM 103
[Dog]           TSSIPGPGYCMGIGPLISHCRFLWMGIGSACNYYNKMYGEFMRVWICGEETLIISKSSSM 103
[Pig]           TSSIPGPGYFLGIGPLISYLRFLWMGIGSACNYYNKTYGEFIRVWIGGEETLIISKSSSV 103
[Horse]         TSSIPGPGYCMGIGPLISHLRFLWMGLGSACNYYNKMYGEFVRVWISGEETLVISKSSST 103
[Mouse]         SSSIPGPGYCLGIGPLISHGRFLWMGIGSACNYYNKMYGEFMRVWISGEETLIISKSSSM 103
[Rat]           SSSIPGPGYCLGIGPLISHGRFLWMGIGSACNYYNKMYGEFMRVWISGEETLIISKSSSM 103
[Frog]          TSLIPGPAYCMGLGPLISYGRFLLTGIGKAANYYNNMYGEFVRVWINGEETLIISKSSAT 101
[Halibut        RRTVPGPPFCLGLGPLLSYVRFIWTGIGTACNYYNKRYGDIVRVWIDGEETLILSRASAI 120
[Grouper        KKTVPGPSFCLGLGPLLSYVRFIWTGIGTASNYYNNKYGDIVRVWINGEETLVLSRASAV 120
                  :*** : :*:*.*:*: **:  *:*.*.****. **:::***: ****:::*::*:

[Quail          FHVMKHWNYVSRFGSKLGLQCIGMYENGIIFNNNPAHWKEIRPFFTKALSGPGLVRMIAI 162
[Alligator]     FYVMKHGHYVSRFGSKLGLQCIGMYENGIIFNNNPTLWKEMRPFFTKALSGPGLVRMISI 163
[Human]         FHIMKHNHYSSRFGSKLGLQCIGMHEKGIIFNNNPELWKTTRPFFMKALSGPGLVRMVTV 163
[Dog]           FHIMKHSHYSSRFGSKLGLQCIGMHENGIIFNNNPTLWKAIRPFFTKALSGPGLVRMVTV 163
[Pig]           FHVMKHSHYTSRFGSKPGLQFIGMHEKGIIFNNNPVLWKAVRTYFMKALSGPGLVRMVTV 163
[Horse]         FHIMKHDHYSSRFGSTFGLQYMGMHENGVIFNNNPAVWKALRPFFVKALSGPSLARMVTV 163
[Mouse]         FHVMKHSHYISRFGSKRGLQCIGMHENGIIFNNNPSLWRTIRPFFMKALTGPGLVRMVEV 163
[Rat]           VHVMKHSNYISRFGSKRGLQCIGMHENGIIFNNNPSLWRTVRPFFMKALTGPGLIRMVEV 163
[Frog]          FHIMKHSHYVSRFGSKLGLQCIGMNENGIIFNSNPSLWKVIRPYFIRALSGPGLMQTTEN 161
[Halibut        YHVLKNGHYTSRFGSKQGLSCIGMYERGIIFNNNVSLWKKIRTHFTRALTGPGLQKTVEV 180
[Grouper        HHVLKNGNYTSRFGSKQGLSCIGMNKRGIIFNNNVELWKKIRTYFSKALTGPGLQQTVEV 180
                 :::*: :* *****. **. :** :.*:***.*   *:  *..* :**:**.* :

[Quail          CVESTIVHLDKLEEVTTEVGNVNVLNLMRRIMLDTSNKLFLGVPLDENAIVLKIQSYFDA 222
[Alligator]     CVESTTDHLDKLEEVTTELGNINVLNLMRRIMLDTSNKLFLRIPLDENAIVLKIQNYFDA 223
[Human]         CAESLKTHLDRLEEVTNESGYVDVLTLLRRVMLDTSNTLFLRIPLDESAIVVKIQGYFDA 223
[Dog]           CVGSIITHLDRLEEVSNELGYVDVLTLMRRIMLDTSNILFLGIPLDESAIVVKIQGYFDA 223
[Pig]           CADSITKHLDKLEEVRNDLGYVDVLTLMRRIMLDTSNNLFLGIPLDEKAIVCKIQGYFDA 223
[Horse]         CVESVNNHLDRLDEVTNALGHVNVLTLMRRTMLDASNTLFLRIPLDEKNIVLKIQGYFDA 223
[Mouse]         CVESIKQHLDRLGEVTDTSGYVDVLTLMRHIMLDTSNMLFLGIPLDESAIVKKIQGYFNA 223
[Rat]           CVESIKQHLDRLGDVTDNSGYVDVVTLMRHIMLDTSNTLFLGIPLDESSIVKKIQGYFNA 223
[Frog]          CIRSTNHYLDNLSNVTNELGNVDVLKLMRLIMLDTSNNLFLRIPLDESEIVLKIQKYFDA 221
[Halibut        CVSSTQTHLDDLDG----LGHVDVLSLLRCTVVDISNRLFLDVPINEKELLVKILKYFDT 236
[Grouper        SVSATQTHLDDLDG----LGHVDVLSLLRCTVVNISNRLFLDVPVSEKELLLKILKYFDT 236
                .  :   :** *       * ::*:.*:*  ::: ** *** :*:.*. :: **  **::

[Quail          WQALLLKPDIFFKISWLCKKYEEAAKDLKGAMEILIEQKRQKLSTVEKLDEHMDFASQLI 282
[Alligator]     WQALLLKPDIFFKISWLYKKYEESVKDLKGAMDLLIQQKRQKLSTVEKLDEHVDFATQLI 283
[Human]         WQALLIKPDIFFKISWLYKKYEKSVKDLKDAIEVLIAEKRRRISTEEKLEECMDFATELI 283
[Dog]           WQALLLKPDIFFKISWLYKKYEKSVKDLKDAMEILIEEKRHRISTAEKLEDHMDFATELI 283
[Pig]           WQALLLKPDIFFKIPWLYRKYEKSVKDLKEDMEILIEKKRRRIFTAEKLEDCMDFATELI 283
[Horse]         WQALLIKPNIFFKISWLSRKHQKSIKELRDAVGILAEEKRHRIFTAEKLEDHVDFATDLI 283
[Mouse]         WQALLIKPNIFFKISWLYRKYERSVKDLKDEIAVLVEKKRHKVSTAEKLEDCMDFATDLI 283
[Rat]           WQALLIKPNIFFKISWLYRKYERSVKDLKDEIEILVEKKRQKVSSAEKLEDCMDFATDLI 283
[Frog]          WQALLLKPDIFFKISWLYKKYEKSANDLKEAIELLIEQKRQKLSSSEKLDEDMDFSSELI 281
[Halibut        WQTVLIKPDIYFKFDWIHQRHKAAVQELHDAIGDLVEQKRRDVEQADKLDN-INFTTGLI 295
[Grouper        WQTVLIKPDVYFKFDWIHQRHKTAAQELQDAIESLVEQKRRDMEQADKLDN-INFTAELI 295
                **::*:**:::**: *: :::: : ::*:  :  *  :**: :   :**:: ::*:: **
```

```
[Quail          FAQNRGDLTAENVNQCVLEMMIAAPDTLSVTLFIMLILIAEHPTVEEKMMREIETVMGDR 342
[Alligator]     FAQNRGDLTAENVNQCVLEMMIAAPDTLSVTLFFMLVLIAEHPKVEEEIMKEIETVMGDR 343
[Human]         LAEKRGDLTRENVNQCILEMLIAAPDTMSVSLFFMLFLIAKHPNVEEAIIKEIQTVIGER 343
[Dog]           FAEKRGDLTRENVNQCILEMLIAAPDTMSVSVFFMLFLIAKHPKVEESIMKEIQAVVGER 343
[Pig]           LAEKRGELTKENVNQCILEMLIAAPDTMSVTVFFMLFLIAKHPQVEEELMKEIQTVVGER 343
[Horse]         LAEKRGELTKENVNQCILEMMIAAPDTLSVTVFFMLCLIAQHPKVEEALMKEIQTVLGER 343
[Mouse]         FAERRGDLTKENVNQCILEMLIAAPDTMSVTLYFMLLLVAEYPEVEAAILKEIHTVVGDR 343
[Rat]           FAERRGDLTKENVNQCILEMLIAAPDTMSVTLYVMLLLIAEYPEVETAILKEIHTVVGDR 343
[Frog]          FAQNHGDLTAENVNQCILEMLIAAPDTMSVSLFFMLVLIAQHPKIEEGIMNEMDKVIGNR 341
[Halibut        FAQNHGELSAENVVQCVLEMVIAAPDTLSVSLFFMLLLLKQNPDVELQLLREIDTVVGER 355
[Grouper        FAQNHGELSAENVRQCVLEVVIAAPDTLSISLFFMLLLLKQNPDVELQLLQGIDTVVGER 355
                :*:.:*:*: *** **:**::******:*::::.** *: : * :*  ::. :. *:*:*

[Quail          DVQSDDMPNLKIVENFIYESMRYQPVVDLIMRKALQDDVIDGYPVKKGTNIILNIGRMHK 402
[Alligator]     DVQSDDMPNLKVVENFIYESMRYQPVVDLIMRKALQDDVIDGYPVKKGTNIILNIGRMHK 403
[Human]         DIKIDDIQKLKVMENFIYESMRYQPVVDLVMRKALEDDVIDGYPVKKGTNIILNIGRMHR 403
[Dog]           DIRIDDMQKLKVVENFIYESMRYQPVVNLVMRKALQDDIIDGYLVKKGTNIILNIGRMHR 403
[Pig]           DIRNDDMQKLEVVENFIYESMRYQPVVDLVMRKALEDDVIDGYPVKKGTNIILNIGRMHR 403
[Horse]         DLKNDDMQKLKVMENFINESMRYQPVVDIVMRKALEDDVIDGYPVKKGTNIILNIGRMHK 403
[Mouse]         DIKIEDIQNLKVVENFINESMRYQPVVDLVMRRALEDDVIDGYPVKKGTNIILNIGRMHR 403
[Rat]           DIRIGDVQNLKVVENFINESLRYQPVVDLVMRRALEDDVIDGYPVKKGTNIILNIGRMHR 403
[Frog]          DVESNDIPNLKILESFIYESMRYQPVVDLVMRKALEDDIIDGYYVKKGTNIILNLGRMHK 401
[Halibut        QLQNGDLQKLQVLESFINECLRFHPVVDFSMRRALSDDIIDGYRVPKGTNIILNTGRMHR 415
[Grouper        QLQNGDLQKLQVLESFINECLRFHPVVDFTMRRALSDDIIDGYRVPKGTNIILNTGRMHR 415
                ::.  *: :*:::*.** *.:*::***:: **:**.**:**** * ******** ****:

[Quail          LEFFPKPNEFSLENFEKNVPSRYFQPFGFGPRGCVGKFIAMVMMKA------RRCRVQTM 456
[Alligator]     LEFFPKPDEFSLENFEKSVPSRYFQPFGFGPRACVGKFIAMVMMKAILVTLLRRCRVHTQ 463
[Human]         LEFFPKPNEFTLENFAKNVPYRYFQPFGFGPRGCAGKYIAMVMMKAILVTLLRRFHVKTL 463
[Dog]           LEFFPKPNEFTLENFAKNVPYRYFQPFGFGPRSCAGKYIAMVMMKVVLVTLLRRFHVQTL 463
[Pig]           LEFFPKPNEFTLENFAKNVPYRYFQPFGFGPRACAGKYIAMVMMKVTLVILLRRFQVQTP 463
[Horse]         LEFFPKPNEFTLENFEKNVPYRYFQPFGFGPRSCAGKFIAMVMMKVMLVSLLRRFHVKTL 463
[Mouse]         LEYFPKPNEFTLENFEKNVPYRYFQPFGFGPRGCAGKYIAMVMMKVVLVTLLRRFQVKTL 463
[Rat]           LEYFPKPNEFTLENFEKNVPYRYFQPFGFGPRSCAGKYIAMVMMKVVLVTLLKRFHVKTL 463
[Frog]          IVYFPKPNEFTLENFEKTVPYRYFQPFGSGPRACAGKYIAMVMMKVILVTLLKRYKVQTL 461
[Halibut        TEFFCKPNEFRLDNFEKTAPRRYFQPFGSGPRSCVGKHIAMAMMKSILVTLLSQYSVCPH 475
[Grouper        TEFFLKANEFSLENFEKNAPRRYFQPFGSGPRSCVGKHIAMVMMKSILVTLLSQYSVCPH 475
                  :* *.:** *:** *..* ******* ***.*.**.***.***        :  * .
                                .
[Quail          KGRALINIQKMNDLSMHPIERQ---PLLVMVFTPRRA----------- 490
[Alligator]     KGRGLNNIQKNNDLSMHPNERQ---PLLEMVFTPRSITGKCQGD---- 504
[Human]         QGQCVESIQKIHDLSLHPDETK---NMLEMIFTPRNSDRCLEH----- 503
[Dog]           QGECIENMQKKYGLSLHPDETN---NLLEMVFVPRNSEKCLER----- 503
[Pig]           QDRCVEKMQKKNDLSLHPDETS---GLLEMIFIPRNSDKCFTK----- 503
[Horse]         QGNCLENMQKTNDLALHPDESR---SLPAMIFTPRNSEKCLEH----- 503
[Mouse]         QKRCIENIPKKNDLSLHPNEDR---HLVEIIFSPRNSDKYLQQ----- 503
[Rat]           QKRCIENMPKNNDLSLHLDEDS---PIVEIIFRHIFNTPFLQCLYISL 508
[Frog]          RGRCLENIQNNNDLSMHPDESQ---PSLEMIFIPKNTAEFKL------ 500
[Halibut        EGLTLDCLPQTNNLSQQPVEHQQEAPHLNMRFLPRQRGSWQTL----- 518
[Grouper        EGLTLDCLPQTNNLSQQPVEHQQEADHLSMRFLPRQRGSWQTL----- 518
                .   :  : :  .*: :  *         : *
```

(Beausoleil, Villen et al. 2006). While aromatase is a 57.5 kDa protein, the phosphorylated HeLa cell aromatase was identified from a gel region encompassing 10-25 kDa proteins, suggesting a role for the modification in aromatase degradation. The degradation of other P450 enzymes has also been correlated with phosphorylation and may play a role in regulating P450 degradation (Wang, Medzihradszky et al. 2001). It is interesting to note that a phosphorylatable residue encoded at position 162 is conserved in several species including Human, Dog, Pig, Horse and Alligator. Phosphorylation of this site imparts a reversibly acid character to this residue. Other vertebrates (Mouse, Rat, Frog, Halibut, and Grouper) encode a strictly acidic glutamic acid residue at this position. The significance of a phosphorylation site at this position remains unclear but could conceivably play a role is disease aetiology as outlined below. Our proteomic strategy was to obtain complete aromatase sequence coverage, including modified peptides. While this strategy will work for stoichiometrically present modifications, modifications incorporated at a substoichiometric level might not be detected. For substoichiometric modifications, an enrichment strategy should improve the success of modified peptide identification. The phosphoproteomic study of HeLa cells conducted by (Beausoleil, Villen et al. 2006) involved phosphopeptide enrichment by strong cation exchange chromatography prior to MS analysis. It is conceivable that a similar approach applied to placental cell lysate may also uncover substoichiometric aromatase phosphorylation. In our placental aromatase sequencing, Thr162 was clearly identified as an unphosphorylated residue

(Figure 6.3, peptide MVTVCAESLK). Thus, phosphorylation of this residue may be involved in cellular regulation of human aromatase, possibly directing its turnover. Additional studies would be required to clearly demonstrate this hypothesis.

Protein glycosylation is an important modification serving various functions. Glycosylated proteins may be protected against proteolytic degradation, retained in the endoplasmic reticulum until properly folded, or directed to the proper destination, serving as a transport signal. We observed that placental aromatase is an N-linked glycoprotein with a heterogeneous oligosaccharide moiety of average mass 1.65 kDa (Figure 6.7). This result is consistent with the mass of a typical N-linked oligosaccharide. For comparison, chicken ovalbumin has one N-linked glycosylation site with an average mass of 1.65 kDa as measured on the same equipment (Ge, Gibbs et al. 2005). The mass accuracy provided by the PBS2c is approximately 2000 ppm for proteins larger than 10 kDa. This corresponds to an error of ± 115 Da for a 57.5 kDa protein such as aromatase. A mass difference of 1.65 kDa was measured for deglycosylated aromatase which can be confidently measured with the PBS2c instrument. In terms of site localization, the unsequenced N-terminal segment (residues 11-30) encodes a canonical N-X-S/T site where the N-glycosylation almost certainly resides. Treatment of aromatase with N-glycosidase F cleaves the N-linked carbohydrate with concomitant conversion of the carbohydrate-linked asparagine to aspartic-acid. Chemical hydrolysis of deglycosylated aromatase with cyanogens bromide (cleavage

Figure 6.7

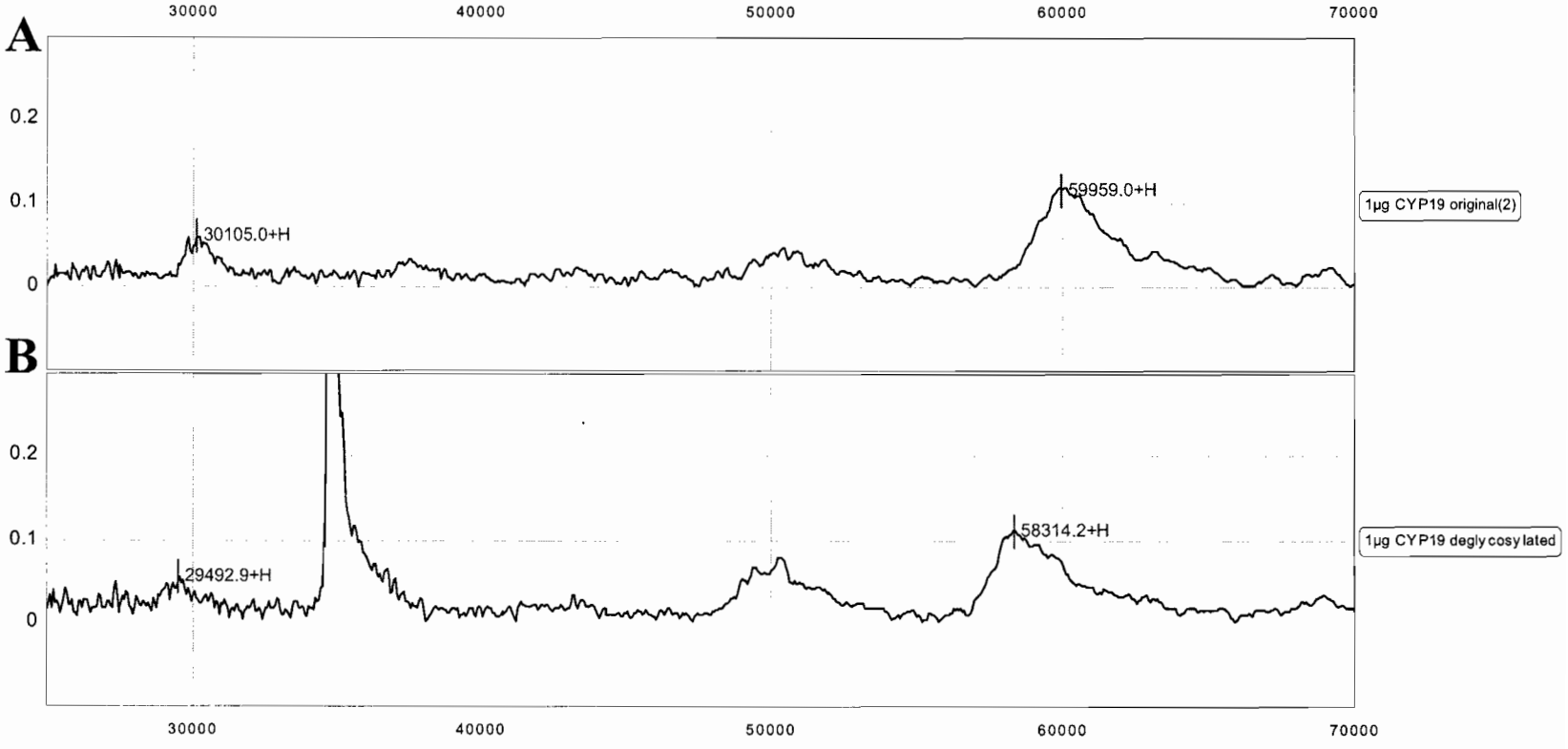

A
B
30000
40000
50000
60000
70000
0.2
0.1
0
30105.0+H
59959.0+H
29492.9+H
58314.2+H
1µg CYP19 original(2)
1µg CYP19 deglycosylated

Figure 6.7 Native (A) and N-deglycosylated (B) human placental aromatase. Deglycosylation of aromatase results in a mass shift of 1.65 kDa, corresponding to cleavage of a typical N-linked oligosaccharide. Other reports of aromatase glycosylation indicate that the N-linked glycosylation site resides at a concensus Asn-X-Thr/Ser site at residue 12, consistent with core glycosylation within the lumen of the endoplasmic reticulum. The physiological significance of aromatase glycosylation remains unclear.

specificity for methionine) would yield a peptide spanning residues 5-20 with aspartic acid at residue 11 in the event that this is the site of N-glycosylation. A targeted MS analysis could be used to confirm if this is indeed the site of N-glycosylation. The physiological significance of the N-linked oligosaccharide moiety remains unclear.

On-chip dephosphorylation of aromatase with SELDI analysis on a high accuracy, high resolution MS could be used for monitoring phosphorylation of intact aromatase. Such an approach has not yet been reported.

MS techniques under development such as ECD and ETD may also be useful for sequencing intact proteins and identifying modification sites without requiring enzymatic digestion.

While this chapter has emphasized the application of MS techniques as a fundamental discovery tool, the same technology may be implemented in a clinical setting once diagnostic modifications are identified. For example, if human aromatase phosphorylation is found to regulate enzyme activity, as in the case of Japanese quail aromatase, the modification may be directly monitored as a clinical marker using the approach outlined in Chapter 5.

Thus far, several drugs have been designed and implemented to specifically inhibit protein kinases involved in cancer (glyvec). The development of future kinase inhibitors can be guided by monitoring the phosphorylation state of relevant kinase substrates (possibly aromatase) whose activity state might be linked to disease progression and prognosis. Although aromatase phosphorylation

has not been clearly linked to estrogen dependent cancer it is conceivable that this enzyme may play such a role in specific cancer aetiology.

6.5 Conclusion

Even with sophisticated instrumentation and bioinformatics, obtaining complete sequence coverage of aromatase was not a trivial undertaking. Our best efforts using multiple complementary techniques provided extensive (90.2%) but incomplete sequence coverage. N-glycosylation of aromatase was confirmed using on-chip protein deglycosylation and MS analysis of native and deglycosylated aromatase. The unsequenced segment spanning residues 11-30 probably harbours the N-linked glycosylation site. With additional material, time and effort, experiments to obtain complete sequence coverage and clearly demonstrate the site of N-linked glycosylation could be completed. A novel amino acid substitution was observed in the N-terminal peptide, providing a new sequence variant. No evidence for placental aromatase phosphorylation was identified although this modification may have been present at a substoichiometric level. Enrichment strategies may be useful to uncover this modification in target tissues.

7.0 Thesis Summary and Conclusion

Steady and quick improvements in MS ionization techniques, MS instrumental design, computer systems and bioinformatics have blossomed over the last two decades. Without further improvements, MS related technologies can already be applied to an impressive array of clinical chemistry and biomedical research applications. More widespread implementation of the technology may one day replace most of the traditional clinical chemistry techniques and the technology is becoming an indispensable toolbox in the basic biomedical research laboratory. The commercialization of tandem MS instrumentation (that can conveniently be coupled to LC separations through ESI) has had a profound impact in several areas of clinical chemistry including newborn screening for congenital metabolic diseases, therapeutic drug monitoring, clinical research and pharmacological and toxicological evaluation. For example, current newborn screening for congenital metabolic diseases using tandem MS is an automated, high-throughput, multiplexed technology that costs less than two dollars to test for each disease (Sechi 2007). While some progress is being made towards developing automated immunoassays, these are not multiplexed and can not perform an analysis in less than 10 minutes. In fact, no other technique currently exists that can provide a similar comprehensive metabolic evaluation as efficiently and cost effectively. As an example of the technology applied to therapeutic drug monitoring, chapter 3 of this thesis describes an efficient and cost effective MS based application for multiplexed analysis of eleven antiretroviral drugs in human plasma. The method was validated against external quality control samples and subsequently used for

analysis of over 450 patient samples (processed in triplicate in small batches of 30-40 samples requiring just several hours of analysis time per batch) spanning a period of nine months. Necessary reagents involved with sample preparation and chromatography are inexpensive (HPLC grade water, methanol, acetonitrile, trifluoroacetic acid, ammonium acetate) and tandem MS instrumentation such as the apparatus used in this study can be obtained on the aftermarket for a fraction of the cost of the latest generation instrumentation (< $10,000 US). While clinical chemistry and hospital laboratories in resource rich countries will generally invest in brand-new latest-generation equipment, research laboratories and institutes in developing countries might consider investing in aftermarket instrumentation in good working order as this technology is suitable for many different applications including qualitative and quantitative analysis of drugs, peptides and proteins. The utility of current MS technology can be optimised through the use of microfluidic devices such capillary liquid chromatography for analysis of trace quantities of analytes present at the fmol to sub-fmol level in microliter volumes of sample. Chapter 4 of this thesis outlines the quantitative analysis of fmol quantities of a peptide hormone (salmon calcitonin) in human urine and plasma. While this chapter would have been improved by direct comparison of our approach against established methods such as the immunoassay, we do illustrate that our assay provides unequivocal specificity and is a useful alternative which could be readily adapted to monitor many more peptide analytes in the same assay. The apparatus described in chapter 4 was subsequently applied to the quantitative analysis of a target protein (C-reactive protein) in rat urine, in a model of drug

induced nephrotoxicity. The approach involved trypsinization of the target protein in the presence of ^{13}C-labelled analog peptide internal standards, all of which were monitored with high sensitivity and specificity using capillary LC-TOF MS. While only two proteolytic peptides and their corresponding labelled analogues were monitored in our assay, dozens of additional peptides from other proteins could also have been monitored within the same analysis. A number of alternative MS based protein quantitation approaches have also been described in the literature for absolute and relative protein quantitation. These methods may one day become widely implemented for multiplexed analysis of diagnostic proteins in the clinical chemistry laboratory.

In the final chapter, application of MS and related technologies for identifying protein posttranslational modifications is discussed. We attempted an exhaustive analysis of an important pharmacological target protein in breast cancer (human estrogen synthase) in an effort to identify novel posttranslational modifications. Once identified, any novel aromatase posttranslational modifications could be investigated, using a targeted approach as outlined in chapter 5, for monitoring the relationship between disease progression and aromatase biochemical state. Should a clear relationship be established, this could be used as a target in pharmaceutical development. For example, identification of aromatase phosphorylation associated with regulation of enzyme activity could spawn a search for relevant kinases and the development of appropriate kinase inhibitors such as the gleevec used in the treatment of leukemia. Alternatively, tumor tissue biopsies could be interrogated using a quantitative proteomic approach in order to characterise a

neoplasm in terms of its protein complement and posttranslational state of diagnostic proteins.

Overall, our efforts to biochemically characterize human aromatase isolated from placenta yielded 90.2% sequence coverage. With additional material and time, complete sequence coverage could be achieved using targeted strategies directed towards those portions of the protein that have evaded sequence analysis. Similar studies should be performed with aromatase isolated from other tissues such as estrogen dependent neoplasms. A novel amino acid substitution was identified and the enzyme was confirmed as an N-linked glycoprotein. In particular, the unsequenced N-terminal segment spanning residues 11-30 almost certainly harbours the N-linked glycosylation site at Asn11. Current and future developments in MS and related technologies are uniquely suited for the analysis of protein posttranslational modifications. While this work is challenging at the moment, if MS based techniques continue to develop at the current pace, we will inevitably gain a complete understanding of the proteome with all of its posttranslational modifications.

References

Ablij, H. and A. Meinders (2002). "C-reactive protein: history and revival." Eur J Intern Med **13**(7): 412.

Aguiar, M., B. F. Gibbs, et al. (2005). "Sensitive capillary chromatography mass spectrometric methods for the determination of salcatonin in human biological matrices." Journal of Chromatography B **818**(2): 301-308.

Aguiar, M., R. Masse, et al. (2005). "Regulation of cytochrome p450 by posttranslational modification." Drug Metabolism Reviews **37**(2): 379-404.

Marshall, A. G., CL Hendrickson, et al.(1998). "Fourier transform ion cyclotron resonance mass spectrometry: A primer." Mass Spectrometry Reviews **17**(1): 1-35.

Allen, K. R., R. Azad, et al. (2005). "Replacement of immunoassay by LC tandem mass spectrometry for the routine measurement of drugs of abuse in oral fluid." Ann Clin Biochem **42**(Pt 4): 277-84.

Amarneh, B., C. J. Corbin, et al. (1993). "Functional domains of human aromatase cytochrome P450 characterized by linear alignment and site-directed mutagenesis." Mol Endocrinol **7**(12): 1617-1624.

Badley, A. D., A. A. Pilon, et al. (2000). "Mechanisms of HIV-associated lymphocyte apoptosis." Blood **96**(9): 2951-2964.

Balthazart, J., M. Baillien, et al. (2001). "Phosphorylation processes mediate rapid changes of brain aromatase activity." J Steroid Biochem Mol Biol **79**(1-5): 261-77.

Balthazart, J., M. Baillien, et al. (2001). "Rapid and reversible inhibition of brain aromatase activity." J Neuroendocrinol **13**(1): 63-73.

Balthazart, J., M. Baillien, et al. (2003). "Calcium-dependent phosphorylation processes control brain aromatase in quail." Eur J Neurosci **17**(8): 1591-606.

Bar-Nun, S., G. Kreibich, et al. (1980). "Synthesis and Insertion of Cytochrome P-450 into Endoplasmic Reticulum Membranes." PNAS **77**(2): 965-969.

Barber, M., R. S. Bordoli, et al. (1981). "Fast atom bombardment of solids as an ion source in mass spectrometry." Nature **293**(5830): 270-275.

Beausoleil, S. A., J. Villen, et al. (2006). "A probability-based approach for high-throughput protein phosphorylation analysis and site localization." Nat Biotech **24**(10): 1285-1292.

Becher, F., A. Pruvost, et al. (2006). "Quantification of small therapeutic proteins in plasma by liquid chromatography-tandem mass spectrometry: Application to an elastase inhibitor EPI-hNE4." Anal Chem **78**(7): 2306-13.

Bellino, F. L. and L. Holben (1989). "Placental estrogen synthetase (aromatase): evidence for phosphatase-dependent inactivation." Biochem Biophys Res Commun **162**(1): 498-504.

Biason-Lauber, A., M. Zachmann, et al. (2000). "Effect of Leptin on CYP17 Enzymatic Activities in Human Adrenal Cells: New Insight in the Onset of Adrenarche." Endocrinology **141**(4): 1446-1454.

Biswas, D. K., A. P. Cruz, et al. (2000). "Epidermal growth factor-induced nuclear factor kappa B activation: A major pathway of cell-cycle progression in estrogen-receptor negative breast cancer cells." PNAS **97**(15): 8542-8547.

Black, S., A. Agrawal, et al. (2003). "The phosphocholine and the polycation-binding sites on rabbit C-reactive protein are structurally and functionally distinct." Mol Immunol **39**(16): 1045-54.

Black, S., I. Kushner, et al. (2004). "C-reactive Protein." J. Biol. Chem. **279**(47): 48487-48490.

Blair, I. A. and A. Tilve (2002). "Analysis of anticancer drugs and their metabolites by mass spectrometry." Curr Drug Metab **3**(5): 463-80.

Boyle, M. D., J. L. Hess, et al. (2006). "Application of immunoproteomics to rapid cytokine detection." Methods **38**(4): 342-50.

Buck, R. H. and F. Maxl (1990). "A validated HPLC assay for salmon calcitonin analysis. Comparison of HPLC and biological assay." J Pharm Biomed Anal **8**(8-12): 761-9.

Burman, K. D., R. Bongiovanni, et al. (1981). "Measurement of serum T4 concentration by high performance liquid chromatography." J Clin Endocrinol Metab **53**(5): 909-912.

Cagney, G. and A. Emili (2002). "De novo peptide sequencing and quantitative profiling of complex protein mixtures using mass-coded abundance tagging." Nat Biotechnol **20**(2): 163-70.

Caron, M. G., S. Goldstein, et al. (1975). "Protein kinase stimulation of a reconstituted cholesterol side chain cleavage enzyme system in the bovine corpus luteum." J. Biol. Chem. **250**(13): 5137-5143.

Chace, D. H., J. C. DiPerna, et al. (1999). "Laboratory integration and utilization of tandem mass spectrometry in neonatal screening: a model for clinical mass spectrometry in the next millennium." Acta Paediatr Suppl **88**(432): 45-7.

Chace, D. H., T. A. Kalas, et al. (2003). "Use of Tandem Mass Spectrometry for Multianalyte Screening of Dried Blood Specimens from Newborns." Clin Chem **49**(11): 1797-1817.

Chi, J., A. L. Jayewardene, et al. (2002). "Simultaneous determination of five HIV protease inhibitors nelfinavir, indinavir, ritonavir, saquinavir and amprenavir in human plasma by LC/MS/MS." J Pharm Biomed Anal **30**(3): 675-84.

Chung, J. W., C. Ng-Thow-Hing, et al. (2007). "Outer membrane proteome of Actinobacillus pleuropneumoniae: LC-MS/MS analyses validate in silico predictions." Proteomics **7**(11): 1854-65.

Cohen, P. (2002). "Protein kinases--the major drug targets of the twenty-first century?" Nat Rev Drug Discov **1**(4): 309-15.

Comisarow, M. B. and A. G. Marshall (1974). "Fourier transform ion cyclotron resonance spectroscopy." Chemical Physics Letters **25**(2): 282-283.

Crommentuyn, K. M., H. Rosing, et al. (2003). "Rapid quantification of HIV protease inhibitors in human plasma by high-performance liquid

chromatography coupled with electrospray ionization tandem mass spectrometry." J Mass Spectrom **38**(2): 157-66.

Cross, E. and C. E. Roselli (1999). "17beta -Estradiol rapidly facilitates chemoinvestigation and mounting in castrated male rats." Am J Physiol Regul Integr Comp Physiol **276**(5): R1346-1350.

Dasgupta, A., A. Wells, et al. (1999). "Effect of heating human sera at a temperature necessary to deactivate human immunodeficiency virus on measurement of free phenytoin, free valproic acid, and free carbamazepine concentrations." Ther Drug Monit **21**(4): 421-5.

De Brabandere, V. I., P. Hou, et al. (1998). "Isotope dilution-liquid chromatography/electrospray ionization-tandem mass spectrometry for the determination of serum thyroxine as a potential reference method." Rapid Commun Mass Spectrom **12**(16): 1099-103.

Defaye, G., N. Monnier, et al. (1982). "Phosphorylation of purified mitochondrial cytochromes P-450 (cholesterol desmolase and 11 beta-hydroxylase) from bovine adrenal cortex." Mol Cell Endocrinol **27**(2): 157-68.

Ding, L., L. Yang, et al. (2006). "A sensitive LC-ESI-MS method for the determination of indapamide in human plasma: Method and clinical applications." Journal of Pharmaceutical and Biomedical Analysis **42**(2): 213-217.

Domon, B. and R. Aebersold (2006). "Mass Spectrometry and Protein Analysis." Science **312**(5771): 212-217.

Droste, J. A., C. P. Verweij-Van Wisse, et al. (2003). "Simultaneous determination of the HIV drugs indinavir, amprenavir, saquinavir, ritonavir, lopinavir, nelfinavir, the nelfinavir hydroxymetabolite M8, and nevirapine in human plasma by reversed-phase high-performance liquid chromatography." Ther Drug Monit **25**(3): 393-9.

Duez, P., A. Kumps, et al. (1996). "GC-MS profiling of urinary organic acids evaluated as a quantitative method." Clin Chem **42**(10): 1609-1615.

Egge-Jacobsen, W., M. Unger, et al. (2004). "Automated, fast, and sensitive quantification of drugs in human plasma by LC/LC-MS: quantification of 6 protease inhibitors and 3 nonnucleoside transcriptase inhibitors." Ther Drug Monit **26**(5): 546-62.

Eliasson, E., S. Mkrtchian, et al. (1994). "Substrate-regulated, cAMP-dependent phosphorylation, denaturation, and degradation of glucocorticoid-inducible rat liver cytochrome P450 3A1." J. Biol. Chem. **269**(28): 18378-18383.

Epstein, P. M., M. Curti, et al. (1989). "Phosphorylation of cytochrome P450: regulation by cytochrome b5." Arch Biochem Biophys **271**(2): 424-32.

F. Dubois, R. Knochenmuss et al., (1999). "A comparison between ion-to-photon and microchannel plate detectors." Rapid Communications in Mass Spectrometry **13**(9): 786-791.

Freeman, J. E. and C. R. Wolf (1994). "Evidence against a role for serine 129 in determining murine cytochrome P450 Cyp2e-1 protein levels." Biochemistry **33**(47): 13963-6.

Frerichs, V. A., R. DiFrancesco, et al. (2003). "Determination of protease inhibitors using liquid chromatography-tandem mass spectrometry." J Chromatogr B Analyt Technol Biomed Life Sci **787**(2): 393-403.

Frohlich, T. and G. J. Arnold (2006). "Proteome research based on modern liquid chromatography--tandem mass spectrometry: separation, identification and quantification." J Neural Transm **113**(8): 973-94.

Garrod, A. E. (1923). Inborn Errors of Metabolism Second Edition. London, Henry Frowde and Hodder & Stoughton

Ge, Y., B. F. Gibbs, et al. (2005). "Complete chemical and enzymatic treatment of phosphorylated and glycosylated proteins on ProteinChip arrays." Anal Chem **77**(11): 3644-50.

Gerber, S. A., J. Rush, et al. (2003). "Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS." PNAS **100**(12): 6940-6945.

Ghazarian, J. G. and D. M. Yanda (1985). "Inhibition of 25-hydroxyvitamin D 1 alpha-hydroxylase by renal mitochondrial protein kinase-catalyzed phosphorylation." Biochem Biophys Res Commun **132**(3): 1095-102.

Ghoshal, A. K. and S. J. Soldin (2003). "Improved method for concurrent quantification of antiretrovirals by liquid chromatography-tandem mass spectrometry." Ther Drug Monit **25**(5): 541-3.

Gibbs, B. F., K. Itiaba, et al. (1973). "A rapid gas chromatographic method for the quantitation of volatile fatty acids in urine. Propionic acid excretion in Vitamin B12 deficiency." J Chromatogr **81**(1): 65-9.

Goodman, S. I. and S. P. Markey (1981). "Diagnosis of organic acidemias by gas chromatography--mass spectrometry." Lab Res Methods Biol Med **6**: 1-158.

Gu, J. and S. J. Soldin (2007). "Modification of tandem mass spectrometric method to permit simultaneous quantification of 17 anti-HIV drugs which include atazanavir and tipranavir." Clinica Chimica Acta **378**(1-2): 222-224.

Guo, T., M. Chan, et al. (2004). "Steroid profiles using liquid chromatography-tandem mass spectrometry with atmospheric pressure photoionization source." Arch Pathol Lab Med **128**(4): 469-75.

Guo, T., R. L. Taylor, et al. (2006). "Simultaneous determination of 12 steroids by isotope dilution liquid chromatography-photospray ionization tandem mass spectrometry." Clinica Chimica Acta **372**(1-2): 76-82.

Guthrie, R. and A. Susi (1963). "A simple phenylalanine method for detecting phenylketonuria in large populations of newborn infants." Pediatrics **32**(3): 338-343.

Gygi, S. P., B. Rist, et al. (1999). "Quantitative analysis of complex protein mixtures using isotope-coded affinity tags." Nat Biotechnol **17**(10): 994-9.

Han, K. K. and A. Martinage (1992). "Post-translational chemical modification(s) of proteins." Int J Biochem **24**(1): 19-28.

Haugen, D. A. and M. J. Coon (1976). "properties of electrophoretically homogeneous phenobarbital-inducible and beta-naphthoflavone-inducible

forms of liver microsomal cytochrome P-450." J. Biol. Chem. **251**(24): 7929-7939.

Herbert Budzikiewicz, R. D. G. (2006). "Mass spectrometry and isotopes: A century of research and discussion." Mass Spectrometry Reviews **25**(1): 146-157.

Hiwatashi, A. and Y. Ichikawa (1980). "Purification and organ-specific properties of cholecalciferol 25-hydroxylase system: cytochrome P-450D25-linked mixed function oxidase system." Biochem Biophys Res Commun **97**(4): 1443-9.

Hiwatashi, A. and Y. Ichikawa (1981). "Purification and reconstitution of the steroid 21-hydroxylase system (cytochrome P-450-linked mixed function oxidase system) of bovine adrenocortical microsomes." Biochim Biophys Acta **664**(1): 33-48.

Hlavica, P. and D. F. Lewis (2001). "Allosteric phenomena in cytochrome P450-catalyzed monooxygenations." Eur J Biochem **268**(18): 4817-32.

Holst, J. P., O. P. Soldin, et al. (2004). "Steroid hormones: relevance and measurement in the clinical laboratory." Clinics in Laboratory Medicine **24**(1): 105-118.

Holst, J. P., S. J. Soldin, et al. (2007). "Use of steroid profiles in determining the cause of adrenal insufficiency." Steroids **72**(1): 71-84.

Hortin, G. L. (2007). "A New Era in Protein Quantification in Clinical Laboratories: Application of Liquid Chromatography-Tandem Mass Spectrometry." Clin Chem **53**(4): 543-544.

Hurlimann, J., G. J. Thorbecke, et al. (1966). "The liver as the site of c-reactive protein formation." J. Exp. Med. **123**(2): 365-378.

Ichikawa, Y. and A. Hiwatashi (1982). "The role of the sugar regions of components of the cytochrome P-450-linked mixed-function oxidase (monooxygenase) system of bovine adrenocortical mitochondria." Biochim Biophys Acta **705**(1): 82-91.

Jansson, I., M. Curti, et al. (1990). "Relationship between phosphorylation and cytochrome P450 destruction." Arch Biochem Biophys **283**(2): 285-92.

Jellum, E., O. Stokke, et al. (1971). "Screening for metabolic disorders using gas-liquid chromatography, mass spectrometry, and computer technique." Scand J Clin Lab Invest **27**(3): 273-85.

Jellum, E., O. Stokke, et al. (1972). "Combined Use of Gas Chromatography, Mass Spectrometry, and Computer in Diagnosis and Studies of Metabolic Disorders." Clin Chem **18**(8): 800-809.

Jialal, I., S. Devaraj, et al. (2004). "C-Reactive Protein: Risk Marker or Mediator in Atherothrombosis?" Hypertension **44**(1): 6-11.

Julka, S. and F. Regnier (2004). "Quantification in proteomics through stable isotope coding: a review." J Proteome Res **3**(3): 350-63.

Karas, M. and F. Hillenkamp (1988). "Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons." Anal Chem **60**(20): 2299-301.

Keil, K., V. A. Frerichs, et al. (2003). "Reverse phase high-performance liquid chromatography method for the analysis of amprenavir, efavirenz,

indinavir, lopinavir, nelfinavir and its active metabolite (M8), ritonavir, and saquinavir in heparinized human plasma." Ther Drug Monit **25**(3): 340-6.

Kilby, J. M. and J. J. Eron (2003). "Novel Therapies Based on Mechanisms of HIV-1 Cell Entry." N. Engl. J. Med. **348**(22): 2228-2238.

Kimura, M., T. Yamamoto, et al. (1999). "Automated metabolic profiling and interpretation of GC/MS data for organic acidemia screening: a personal computer-based system." Tohoku J Exp Med **188**(4): 317-34.

Kirkpatrick, D. S., C. Denison, et al. (2005). "Weighing in on ubiquitin: the expanding role of mass-spectrometry-based proteomics." Nat Cell Biol **7**(8): 750-757.

Kobayashi, N., N. Yamamura, et al. (2000). "In vitro metabolism of human and salmon calcitonins in rat liver and kidney evaluated by liquid chromatography-tandem mass spectrometry." Xenobiotica **30**(7): 655-64.

Koch, J. A. and D. J. Waxman (1989). "Posttranslational modification of hepatic cytochrome P-450. Phosphorylation of phenobarbital-inducible P-450 forms PB-4 (IIB1) and PB-5 (IIB2) in isolated rat hepatocytes and in vivo." Biochemistry **28**(8): 3145-52.

Kuhara, T. (2001). "Diagnosis of inborn errors of metabolism using filter paper urine, urease treatment, isotope dilution and gas chromatography-mass spectrometry." J Chromatogr B Biomed Sci Appl **758**(1): 3-25.

Kuhn, E., J. Wu, et al. (2004). "Quantification of C-reactive protein in the serum of patients with rheumatoid arthritis using multiple reaction monitoring

mass spectrometry and ^{13}C-labeled peptide standards." Proteomics **4**(4): 1175-86.

Kuta, A. E. and L. L. Baum (1986). "C-reactive protein is produced by a small number of normal human peripheral blood lymphocytes." J. Exp. Med. **164**(1): 321-326.

Lannigan, R., R. Kark, et al. (1962). "The effect of a single intravenous injection of aminonucleoside of puromycin on the rat kidney: a light- and electron-microscope study." J Pathol Bacteriol **83**: 357-62.

Lappin, G., W. Kuhnz, et al. (2006). "Use of microdosing to predict pharmacokinetics at the therapeutic dose: Experience with 5 drugs[ast]." Clin Pharmacol Ther **80**(3): 203-215.

Lee, I. H., S. Pollack, et al. (1991). "Influence of the mobile phase on salmon calcitonin analysis by reversed-phase liquid chromatography." J Chromatogr Sci **29**(4): 136-40.

Lee, K. C., S. C. Moon, et al. (1999). "Isolation, characterization, and stability of positional isomers of mono-PEGylated salmon calcitonins." Pharm Res **16**(6): 813-8.

Lohr, J. B. and W. N. Kuhn-Velten (1997). "Protein phosphorylation changes ligand-binding efficiency of cytochrome P450c17 (CYP17) and accelerates its proteolytic degradation: putative relevance for hormonal regulation of CYP17 activity." Biochem Biophys Res Commun **231**(2): 403-8.

Mamer, O. A. and B. F. Gibbs (1973). "Simplified Gas Chromatography of Trimethylsilyl Esters of C1 through C5 Fatty Acids in Serum and Urine." Clin Chem **19**(9): 1006-1009.

Mangelsdorf, D. J., C. Thummel, et al. (1995). "The nuclear receptor superfamily: the second decade." Cell **83**(6): 835-9.

Mann, M. (1999). "Quantitative proteomics?" Nat Biotech **17**(10): 954-955.

March, R. E. (2000). "Quadrupole ion trap mass spectrometry: a view at the turn of the century." International Journal of Mass Spectrometry **200**(1-3): 285-312.

Martinetti, A., E. Seregni, et al. (2003). "Evaluation of circulating calcitonin: analytical aspects." Tumori **89**(5): 566-8.

Masaru Miyagi, K. C. S. R. (2007). "Proteolytic ^{18}O-labeling strategies for quantitative proteomics." Mass Spectrometry Reviews **26**(1): 121-136.

McGowan, J. P. and S. S. Shah (2000). "Prevention of perinatal HIV transmission during pregnancy." J. Antimicrob. Chemother. **46**(5): 657-668.

McLafferty, F. W. (1980). "Interpretation of Mass Spectra." University Science Books, Mill Valley, CA, **3rd edition**: 303 pp.

Melvin A. Park, J. H. C. A. V. (1994). "An inductive detector for time-of-flight mass spectrometry." Rapid Communications in Mass Spectrometry **8**(4): 317-322.

Menendez-Arias, L. (2002). "Molecular basis of fidelity of DNA synthesis and nucleotide specificity of retroviral reverse transcriptases." Prog Nucleic Acid Res Mol Biol **71**: 91-147.

Menez, J. F., T. K. Machu, et al. (1993). "Phosphorylation of cytochrome P4502E1 (CYP2E1) by calmodulin dependent protein kinase protein kinase c and cAMP dependent protein kinase." Alcohol Alcohol. **28**(4): 445-451.

Merchant, M. and S. R. Weinberger (2000). "Recent advancements in surface-enhanced laser desorption/ionization-time of flight-mass spectrometry." Electrophoresis **21**(6): 1164-77.

Moslemi, S., A. Vibet, et al. (1997). "Purification and characterization of equine testicular cytochrome P-450 aromatase: comparison with the human enzyme." Comp Biochem Physiol B Biochem Mol Biol **118**(1): 217-27.

Motie, M., K. W. Schaul, et al. (1998). "Biodistribution and Clearance of 125I-Labeled C-Reactive Protein and 125I-Labeled Modified C-Reactive Protein in CD-1 Mice." Drug Metab Dispos **26**(10): 977-981.

Munson, M. S. B. and F. H. Field (1966). "Chemical Ionization Mass Spectrometry. I. General Introduction." J. Am. Chem. Soc. **88**(12): 2621-2630.

Nagant de Deuxchaisnes, C. (1983). "Calcitonin in the treatment of Paget's disease." Triangle **22**: 103-128.

Nakajin, S., P. F. Hall, et al. (1981). "Testicular microsomal cytochrome P-450 for C21 steroid side chain cleavage. Spectral and binding studies." J. Biol. Chem. **256**(12): 6134-6139.

Negishi, M., N. M. Jensen, et al. (1981). "Structural gene products of the murine Ah complex. Differences in ontogenesis and glucosamine incorporation

between liver microsomal cytochromes P1-450 and P-448 induced by polycyclic aromatic compounds." Eur J Biochem **115**(3): 585-94.

Nelson, D. R., T. Kamataki, et al. (1993). "The P450 superfamily: update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature." DNA Cell Biol **12**(1): 1-51.

Nguyen, L. B., S. Shefer, et al. (1996). "Cholesterol 7alpha-hydroxylase activities from human and rat liver are modulated in vitro posttranslationally by phosphorylation/dephosphorylation." Hepatology **24**(6): 1468-74.

Niwa, T. (1986). "Metabolic profiling with gas chromatography-mass spectrometry and its application to clinical medicine." J Chromatogr **379**: 313-45.

Oesch-Bartlomowic, B. and F. Oesch (1990). "Phosphorylation of cytochrome P450 isoenzymes in intact hepatocytes and its importance for their function in metabolic processes." Arch Toxicol **64**(4): 257-61.

Oesch-Bartlomowic, B., P. R. Padma, et al. (1998). "Differential modulation of CYP2E1 activity by cAMP-dependent protein kinase upon Ser129 replacement." Exp Cell Res **242**(1): 294-302.

Oesch-Bartlomowic, B., B. Richter, et al. (2001). "cAMP-dependent phosphorylation of CYP2B1 as a functional switch for cyclophosphamide activation and its hormonal control in vitro and in vivo." Int J Cancer **94**(5): 733-42.

Palmer, S., M. Kearney, et al. (2005). "Multiple, Linked Human Immunodeficiency Virus Type 1 Drug Resistance Mutations in Treatment-

Experienced Patients Are Missed by Standard Genotype Analysis." J. Clin. Microbiol. **43**(1): 406-413.

Patricia Hernandez, M. Muller et al., (2006). "Automated protein identification by tandem mass spectrometry: Issues and strategies." Mass Spectrometry Reviews **25**(2): 235-254.

Poirier, J. M., N. Radembino, et al. (2000). "Simultaneous determination of the five HIV-protease inhibitors: amprenavir, indinavir, nelfinavir, ritonavir, and saquinavir in human plasma by solid-phase extraction and column liquid chromatography." Ther Drug Monit **22**(4): 465-73.

Pyerin, W. and H. Taniguchi (1989). "Phosphorylation of hepatic phenobarbital-inducible cytochrome P-450." EMBO J **8**(10): 3003-10.

Pyerin, W., H. Taniguchi, et al. (1987). "Isoenzyme-specific phosphorylation of cytochromes P-450 and other drug metabolizing enzymes." Biochem Biophys Res Commun **142**(3): 885-92.

Qizhi Hu, R. J. Noll et al., (2005). "The Orbitrap: a new mass spectrometer." Journal of Mass Spectrometry **40**(4): 430-443.

Qu, J., Y. Qu, et al. (2007). "Ultra-Sensitive Quantification of Corticosteroids in Plasma Samples Using Selective Solid-Phase Extraction and Reversed-Phase Capillary High-Performance Liquid Chromatography/Tandem Mass Spectrometry." Anal. Chem. **79**(10): 3786-3793.

Rifai, N., R. P. Tracy, et al. (1999). "Clinical Efficacy of an Automated High-Sensitivity C-Reactive Protein Assay." Clin Chem **45**(12): 2136-2141.

Robb, D. B. and M. W. Blades (2006). "Atmospheric Pressure Photoionization for Ionization of Both Polar and Nonpolar Compounds in Reversed-Phase LC/MS." Anal. Chem. **78**(23): 8162-8164.

Robb, D. B., T. R. Covey, et al. (2000). "Atmospheric Pressure Photoionization: An Ionization Method for Liquid Chromatography-Mass Spectrometry." Anal. Chem. **72**(15): 3653-3659.

Roberts, B. J., B.-J. Song, et al. (1995). "Ethanol Induces CYP2E1 by Protein Stabilization." J. Biol. Chem. **270**(50): 29632-29635.

Sakaguchi, M., K. Mihara, et al. (1987). "A short amino-terminal segment of microsomal cytochrome P-450 functions both as an insertion signal and as a stop-transfer sequence." EMBO J **6**(8): 2425-31.

Sanchez, R., D. Nguyen, et al. (2002). "Diversity in the mechanisms of gene regulation by estrogen receptors." Bioessays **24**(3): 244-54.

Schneider, L. V. and M. P. Hall (2005). "Stable isotope methods for high-precision proteomics." Drug Discov Today **10**(5): 353-63.

Sechi, S. (2007). Quantitative Proteomics by Mass spectrometry. Methods in Molecular Biology. S. Sechi. Totowa, New Jersey, Humana Press. **359:** v-vi.

Sethumadhavan, K., F. L. Bellino, et al. (1991). "Estrogen synthetase (aromatase). The cytochrome P-450 component of the human placental enzyme is a glycoprotein." Mol Cell Endocrinol **78**(1-2): 25-32.

Shevchenko, A., M. Wilm, et al. (1996). "Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels." Anal Chem **68**(5): 850-8.

Shimozawa, O., M. Sakaguchi, et al. (1993). "Core glycosylation of cytochrome P-450(arom). Evidence for localization of N terminus of microsomal cytochrome P-450 in the lumen." J. Biol. Chem. **268**(28): 21399-21402.

Silverman, S. L. (2003). "Calcitonin." Endocrinol Metab Clin North Am **32**(1): 273-84.

Simpson, E. P. D. (2004). "Aromatase: Biologic Relevance of Tissue-Specific Expression." Seminars in Reproductive Medicine(1): 11-23.

Soldin, S. J., N. Soukhova, et al. (2005). "The measurement of free thyroxine by isotope dilution tandem mass spectrometry." Clinica Chimica Acta **358**(1-2): 113-118.

Song, K. H., H. M. An, et al. (2002). "Simple liquid chromatography-electrospray ionization mass spectrometry method for the routine determination of salmon calcitonin in serum." J Chromatogr B Analyt Technol Biomed Life Sci **775**(2): 247-55.

Sonoda, Y., C. Amano, et al. (1995). "Lanosterol 14 alpha-demethylase (cytochrome P-45014DM): modulation of its enzyme activity by cholesterol feeding." Biol Pharm Bull **18**(7): 1009-11.

Soukhova, N., O. P. Soldin, et al. (2004). "Isotope dilution tandem mass spectrometric method for T4/T3." Clinica Chimica Acta **343**(1-2): 185-190.

Steel, D. M. and A. S. Whitehead (1994). "The major acute phase reactants: C-reactive protein, serum amyloid P component and serum amyloid A protein." Immunol Today **15**(2): 81-8.

Szalai, A. J., A. Agrawal, et al. (1999). "C-reactive protein: structural biology and host defense function." Clin Chem Lab Med **37**(3): 265-70.

Szanto, J., S. Jozsef, et al. (1986). "Pain killing with calcitonin in patients with malignant tumours." Oncology **43**(2): 69-72.

Tai, S. S. C., L. T. Sniegoski, et al. (2002). "Candidate Reference Method for Total Thyroxine in Human Serum: Use of Isotope-Dilution Liquid Chromatography-Mass Spectrometry with Electrospray Ionization." Clin Chem **48**(4): 637-642.

Tang, P. M. and J. Y. Chiang (1986). "Modulation of reconstituted cholesterol 7 alpha-hydroxylase by phosphatase and protein kinase." Biochem Biophys Res Commun **134**(2): 797-802.

Thienpont, L. M., V. I. De Brabandere, et al. (1994). "Development of a new method for the determination of thyroxine in serum based on isotope dilution gas chromatography mass spectrometry." Biol Mass Spectrom **23**(8): 475-82.

Thomas W. Burgoyne, G. M. H. (1996). "An introduction to ion optics for the mass spectrograph." Mass Spectrometry Reviews **15**(4): 241-259.

Titier, K., F. Lagrange, et al. (2002). "High-performance liquid chromatographic method for the simultaneous determination of the six HIV-protease inhibitors and two non-nucleoside reverse transcriptase inhibitors in human plasma." Ther Drug Monit **24**(3): 417-24.

Tribut, O., C. Arvieux, et al. (2002). "Simultaneous quantitative assay of six HIV protease inhibitors, one metabolite, and two non-nucleoside reverse

transcriptase inhibitors in human plasma by isocratic reversed-phase liquid chromatography." Ther Drug Monit **24**(4): 554-62.

Turecek, F., C. R. Scott, et al. (2007). "Tandem mass spectrometry in the detection of inborn errors of metabolism for newborn screening." Methods Mol Biol **359**: 143-57.

Verbeck, G. F., B. T. Ruotolo, et al. (2002). "A fundamental introduction to ion mobility mass spectrometry applied to the analysis of biomolecules." J Biomol Tech **13**(2): 56-61.

Vigushin DM, P. M., Hawkins PN (1993). "Metabolic and scintigraphic studies of radioiodinated human C-reactive protein in health and disease." J. Clin. Invest. **91**(4): 1351-1357.

Vilgrain, I., G. Defaye, et al. (1984). "Adrenocortical cytochrome P-450 responsible for cholesterol side chain cleavage (P-450scc) is phosphorylated by the calcium-activated, phospholipid-sensitive protein kinase (protein kinase C)." Biochem Biophys Res Commun **125**(2): 554-61.

Volosov, A., C. Alexander, et al. (2002). "Simple rapid method for quantification of antiretrovirals by liquid chromatography-tandem mass-spectrometry." Clin Biochem **35**(2): 99-103.

Walsh, G. and R. Jefferis (2006). "Post-translational modifications in the context of therapeutic proteins." Nat Biotech **24**(10): 1241-1252.

Wang, X., K. F. Medzihradszky, et al. (2001). "Phosphorylation of native and heme-modified CYP3A4 by protein kinase C: a mass spectrometric

characterization of the phosphorylated peptides." Biochemistry **40**(38): 11318-26.

Whiteaker, J. R., L. Zhao, et al. (2007). "Antibody-based enrichment of peptides on magnetic beads for mass-spectrometry-based quantification of serum biomarkers." Anal Biochem **362**(1): 44-54.

Whitehouse, C. M., R. N. Dreyer, et al. (1985). "Electrospray interface for liquid chromatographs and mass spectrometers." Anal Chem **57**(3): 675-9.

Yue, W., R. J. Santen, et al. (1999). "Aromatase within the breast." Endocr Relat Cancer **6**(2): 157-164.

Yue, W., J. P. Wang, et al. (2003). "Adaptive hypersensitivity following long-term estrogen deprivation: involvement of multiple signal pathways." J Steroid Biochem Mol Biol **86**(3-5): 265-74.

Zhang, L., H. Rodriguez, et al. (1995). "Serine Phosphorylation of Human P450c17 Increases 17,20-Lyase Activity: Implications for Adrenarche and the Polycystic Ovary Syndrome." PNAS **92**(23): 10619-10623.