Development of a Mass Spectrometry-Based Assay for Measurement of Angiotensin I and Plasma Renin Activity to Diagnose Secondary Hypertension

by

Jennifer D. Reid
B.Sc., University College of the Cariboo, 2003

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Supervisory Committee

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Abstract

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The renin-angiotensin-aldosterone system (RAAS) plays an essential role in maintaining plasma volume and arterial blood pressure by regulating angiotensin II levels. Dysregulation of the RAAS can result from an underlying disorder that results in a severe and untreatable form of hypertension, known as secondary hypertension. Measurement of plasma renin activity is a commonly employed method of diagnosing secondary hypertension. Plasma renin activity is quantified by determining the amount of angiotensin I generated through the enzymatic cleavage of angiotensinogen by renin. Radioimmunoassay is routinely used to measure plasma renin activity, however there are limitations to the method. With the prevalence of hypertension on the rise, there is need for a more accurate and rapid method of assessing the RAAS for diagnostic purposes and therapeutic monitoring.

Multiplexed measurement of angiotensin I and angiotensin II would provide comprehensive understanding of the RAAS by determining dysregulation in the production of either molecule. In this thesis, the relationship between endogenous angiotensin I concentrations and plasma renin activity are studied in order to examine the research hypothesis that measurement of angiotensin I concentration correlates with

plasma renin activity and whether this may provide a more accurate and rapid method of screening for hypertension when multiplexed with angiotensin II.

To overcome the current limitations of radioimmunoassay for measuring plasma renin activity, a mass spectrometric-based method was developed to measure angiotensin I and plasma renin activity. Evaluation of the assay against radioimmunoassay demonstrates that the assay is reproducible and provides a linear response over a diagnostically relevant concentration range. Comparison of endogenous levels of angiotensin I with normal plasma renin activity levels show a correlation in this study (R=0.74). Comparison of plasma renin activity values by radioimmunoassay and iMALDI also show correlation (R=0.98), indicating that the iMALDI assay may provide an improved method for diagnosing secondary hypertension.

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Chapter 1
Regulation of Blood Pressure

1.1 Blood pressure

The primary function of the cardiovascular system is to ensure adequate blood flow to all organ systems in the body to deliver sufficient oxygen and nutrients. The driving force for the flow of oxygenated blood throughout the body is arterial pressure. Arterial pressure is determined by two factors: cardiac output and total peripheral resistance (Vander, Sherman & Luciano, 2001). Total peripheral resistance is the resistance to blood flow through the vasculature system, and is determined by the size and flexibility of the arteries (Klabunde, 2007). Cardiac output refers to the volume of blood pumped into the arteries at any given time, which is regulated by local controls, such as oxygen and potassium, the nervous system, and hormonal controls such as aldosterone and angiotensin II (Vander et al., 2007). Dysregulation of either local controls or alterations in arterial resistance results in unregulated blood pressure leading to serious diseases.

1.2 The vascular system

The vascular system is composed of the heart and a network of blood vessels that carry oxygenated blood from the lungs through the heart and to the tissues of the body in exchange for waste or nutrients. The vascular system is composed of the arterial and venous systems.

The arterial system is made of a network of arteries that pump oxygenated blood from the lungs through heart and the tissues of the body. From the arteries, blood flows into arterioles and capillaries before reaching the tissues. The pressure of blood against the arterial walls is responsible for moving blood from high to low pressure through the body (Vander et al., 2007). It is critical that blood pressure is maintained.

The venous system is responsible for returning blood to the lungs after the exchange of nutrients, waste, and gases at the interface of the capillaries and tissues. After leaving the capillaries, blood enters venules and veins before returning to the lungs. The skeletal muscle pump moves blood through the venous system to the lungs (Miller, Pegelow, Jacques, & Dempsey, 2005).

1.3 Regulation of arterial pressure

Regulation of arterial pressure is maintained by the nervous system through the action of baroreceptors and hormonal regulation (Vander et al., 2007). Arteries accommodate increased blood volume by stretching, which is detected by baroreceptors within the arteries. Baroreceptors send a signal to the medulla oblongata of the brain stem resulting in regulation of the parasympathetic and sympathetic response of the autonomic nervous system. Baroreceptors regulate arterial pressure on a scale of seconds to minutes. In addition to detecting changes in arterial pressure, baroreceptors also regulate blood pressure by measuring the rate of change in pressure. However, baroreceptors can adapt to persistent changes in blood pressure caused by diseases such as hypertension (Klabunde, 2007; Sherwood, 2010).

Long-term methods of regulating blood pressure are also important. The kidneys are responsible for long-term regulation by balancing water and sodium excretion. As blood volume increases, the kidneys increase the amount of water excreted so that the overall effect is a decrease in blood pressure. The kidneys regulate blood volume through the renin angiotensin aldosterone system (RAAS) (Brewster & Perazella, 2004).

1.4 Renin angiotensin aldosterone system

Arterial pressure is regulated through the RAAS. The key molecules of the RAAS include renin, angiotensinogen, angiotensin I and II, and aldosterone. Baroreceptors respond to low blood pressure by stimulating secretion of renin from the kidneys (Cartledge & Lawson, 2000). Renin is synthesized in the kidney as a pro-hormone called prorenin. Cleavage of a 43 amino acid segment from the amino terminus of prorenin produces renin (Cartledge et al., 2000), which is the active version of the hormone. Renin is stored in granules of the juxtaglomerular cells of the kidney (Nguyen & Danser, 2008) until released into the blood in response to low blood volume, high chloride levels in the kidneys, hormones, and neurotransmitters (Persson, 2003). Renin initiates a cascade of reactions leading to production of angiotensin II, a powerful vasoconstrictor. The first reaction in the pathway is enzymatic cleavage of angiotensinogen by renin.

Angiotensinogen is a glycoprotein produced primarily by the liver and is released constitutively into the circulation (Corvol & Jeunemaitre, 1997). Angiotensinogen is present in blood at a concentration of approximately 1 μM (Ramaha & Patson, 2002), however levels can be affected by corticosteroids, estrogen, and thyroid hormone (Corvol

et al., 1997). Renin cleaves angiotensinogen at the amino terminus to produce angiotensin I. The conversion of angiotensinogen to angiotensin I is considered the rate-limiting step of the RAAS (Allan et al., 1997; Corvol et al., 1997).

Angiotensin I is an inactive precursor to angiotensin II. Conversion of angiotensin I to II is catalyzed by angiotensin converting enzyme (ACE). ACE is a membrane-bound metalloenzyme expressed in the epithelial cells of lungs. It converts angiotensin I into angiotensin II by cleaving two carboxyl terminal amino acid residues from angiotensin I as blood flows through the lungs (Vane, 1967). Angiotensin II is a powerful vasoconstrictor that integrates cardiovascular function with blood pressure regulation. Angiotensin II increases blood pressure by activating angiotensin II receptors on blood vessels and target tissues (Atlas, 2007). This in turn, stimulates vasoconstriction. Through stimulation of the adrenal gland, angiotensin II causes release of aldosterone resulting in sodium and water reabsorption (Sealey, 1981). Aldosterone is a mineralocorticoid hormone that increases blood pressure by activating Na^+/K^+ pumps in the kidneys, leading to increased sodium and water reabsorption into the blood (Vander et al., 2001).

As a result of water and salt retention through the actions of angiotensin II and aldosterone, circulating blood volume is increased. Increased blood volume combined with the vasoconstricting effects of angiotensin II leads to increased arterial pressure. Baroreceptors responds to increased pressure by negative-feedback regulation of the

RAAS. Figure 1 provides an overview of the tissues and hormones involved in the RAAS.

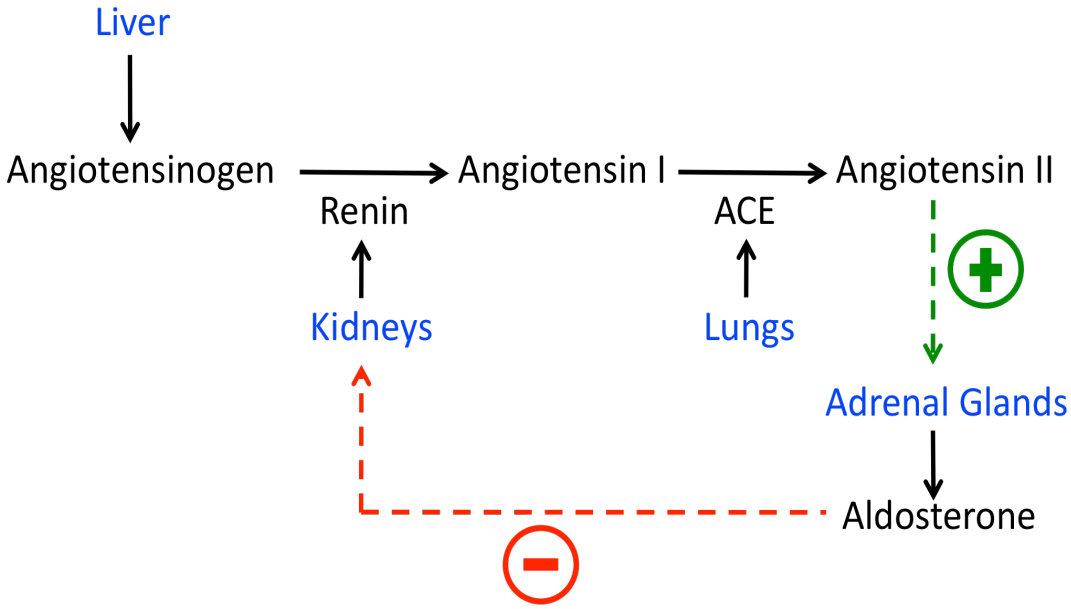


Figure 1. Renin angiotensin aldosterone system

Conversion of angiotensinogen to angiotensin I by renin leads to increased blood pressure in response to low blood volume. Cleavage of angiotensin I by ACE produces angiotensin II, a powerful vasoconstrictor. Angiotensin II regulates blood pressure by increasing sodium and water retention through the stimulation of aldosterone. Aldosterone controls renin secretion through negative feedback regulation.

1.5 Dysregulation of the renin angiotensin aldosterone system

Inadequate regulation of blood pressure can cause serious long-term health problems as a result of chronic high or low blood pressure. High blood pressure, or hypertension, is a worldwide epidemic, affecting nearly 1 billion people (Kearney et al., 2005). By 2025, the rate of occurrence is expected by another 500 million. Hypertension is a primary risk factor for serious diseases including heart attack, stroke, atherosclerosis, and renal disease

(Borzecki, Kader, & Berlowitz, 2010; Chobanian, 2009). The absence of symptoms contributes to misdiagnosis and ineffective treatment of hypertension.

Hypertension is classified into two categories: primary hypertension, which has no identifiable cause, and secondary hypertension, which results from an identifiable underlying cause and is often treatable. There are currently 90 million people diagnosed with secondary hypertension. This number may even be higher because appropriate tests to distinguish primary and secondary hypertension may not have been performed (Gordon et al., 1992; Montori et al., 2001; Sarafadis & Bakris, 2008; Young 1999). This is further exacerbated by lack of high-quality testing procedures. The ability to effectively treat secondary forms of hypertension is dependent on accurate diagnosis and identification of the underlying cause of the disease (Calhoun et al., 2008).

1.6 Causes of secondary hypertension

The underlying causes of secondary hypertension are often related to kidney and arterial disorders. Renal artery stenosis is characterized by a narrowing of the renal arteries that results in reduced blood flow to the kidneys (McLaughlin, Jardine & Moss, 2000). Renal artery stenosis most commonly arises as a result of atherosclerosis (thickening of the arterial walls) (Safian & Textor, 2001). The kidneys respond to reduced blood pressure by releasing renin in an effort to activate the RAAS (Safian et al., 2001). However, the resulting increase in angiotensin II and aldosterone levels do not effectively increase the flow of blood to the kidneys because of stenosis. The resulting stimulation of chronic activation of the RAAS leads to cardiac and vascular hypertrophy. Narrowing of the

renal arteries of both kidneys can result in renal failure. Fibromuscular dysplasia, a genetic disorder causing thickening of the vessel walls, inflammation of the vessels, or division of the vessel walls can also lead to renal artery stenosis (Safian et al., 2001).

Chronic renal disease resulting from diabetes, glomerulonephritis (inflammation of the kidney vessels), and hypertension often leads to permanent damage that prevents the kidneys from secreting normal amounts of sodium to maintain homeostatic blood levels (The Kidney Foundation of Canada). As a result, blood volume and blood pressure increase even in the presence of a normally functioning RAAS.

Primary aldosteronism is caused by adrenal adenoma (benign tumour) or hyperplasia (cellular proliferation) resulting in autonomous secretion of aldosterone. The RAAS responds to the increased blood pressure by decreasing renin secretion but with no effect. Primary aldosteronism is the most frequent form of secondary hypertension (Young, 2007). It is resistant to drug therapies for hypertension, but if diagnosed correctly and treated, can have an excellent prognosis.

Genetic mutations have also been linked to diseases that effect the RAAS and lead to secondary hypertension. Liddle's syndrome is an autosomal dominant disorder that causes hyperactivity of sodium channels resulting in increased sodium reabsorption in the kidneys followed by water retention (Carretero & Oparil, 2000). Metabolic alkalosis can result due to excessive loss of potassium through the kidneys. Patients exhibit low to normal levels of renin and aldosterone despite high blood pressure. In glucocorticoid-

remediable aldosteronism, aldosterone is regulated by adrenocorticotropic hormone leading to persistant hypertension (Carretero et al., 2000).

1.7 Diagnosis of secondary hypertension

A primary indicator of secondary hypertension is a lack of response to anti-hypertension drugs, or a sudden onset of hypertension. In such cases diagnosis is typically confirmed by determining the ratio of plasma aldosterone concentration to renin (aldosterone renin ratio, ARR) (Dunn & Espiner, 1976). Many investigators consider the ARR a better diagnostic indicator than either aldosterone or renin (Bravo, 1994; Funder et al., 2008; Ganguly, 1998; Hiramatsu et al., 1981; Rossi et al., 2006; Tiu et al., 2005; Young, 2002). A major advantage of the ARR is that it takes into account both aldosterone and renin. Consequently, this a more comprehensive method for screening (Tiu et al., 2005). Because the ARR is dependent on renin as the denominator (Montori et al., 2001), it is very important that renin assays are accurate and precise.

1.8 Disease management and prognosis

Secondary hypertension is treated by medication or surgery. In cases of autonomous aldosterone production by an adrenal adenoma or hyperplasia, surgical removal of the aldosterone producing cells leads to recovery (Jeck et al., 1994; Shenker, 1989; Weinberger et al., 1979; Young et al., 1990). In cases that do not involve autonomous aldosterone production, surgery may not be a suitable treatment. Depending on the underlying cause of the hypertension, the disease may be managed by sodium restriction, potassium supplementation, and anti-hypertensive medications (Ganguly, 1998; Young,

2007). Targeted therapeutic strategies can be developed, but are highly dependent on an accurate diagnosis (Furberg, 2010; Sarafidis et al., 2008).

1.9 Aldosterone measurements

Most aldosterone tests use antibody-based approaches, such as ELISA, chemiluminescence (Hubl et al., 1990; Stabler & Siegel, 1991), or radioimmunoassay (RIA) (Lun et al., 1983). Direct measurement of aldosterone can be performed using extraction methods, such as solid phase extraction, liquid-liquid extraction or chromatographic methods (Brochu et al., 1989; Hanquez et al., 1988; Kubasik, Warren & Sine, 1979). MS assays have also been developed (De Vries & De Jong, 1980; Stockl et al., 1991; Taylor et al., 2009).

1.10 Renin measurements

The presence of two forms of renin in plasma, inactive prorenin and active renin, has led to the development of two different types of renin assays: renin concentration assays and plasma renin activity (PRA) assays. Renin concentration assays can measure both inactive and active renin, known as total renin concentration. Measurement of active renin is possible through the use of specific monoclonal antibodies (Galen et al., 1984; Menard et al., 1985). However, high concentrations of prorenin in the plasma can lead to overestimation of active renin plasma concentration as a result of antibody cross reactivity (Derkx & Schaelnkamp, 1988; Montori et al., 2001), especially in patients with low levels of active renin (Dessi-Fulgheri et al., 1987; Morganti et al., 1987).

To address the problems associated with high concentrations of inactive prorenin in plasma, the PRA assay was developed. The PRA measures the amount of angiotensin I produced through enzymatic activity of renin during an incubation period or the "angiotensin I generation period". There are several major advantages to the PRA approach, including:

1. Prevention of misdiagnosis as a result of prorenin interference
2. Potential for unlimited sensitivity as the angiotensin I generation period can be extended for up to 18 hr (Cartledge et al., 2000; Sealey, 1991; Sealey, Gordon & Mantero, 2005)
3. PRA assays have been shown to be more precise than renin concentration assays at low renin concentrations (Sealey & Laragh, 1996; Morganti et al., 1995; Sealey, 1995)

The generation period is followed by measurement of angiotensin I. Radioimmunoassay (RIA) is commonly used to measure PRA using ^{125}I as a tracer (Cartledge et al., 2000). Separation of bound and free-labelled angiotensin I is usually performed by using activated charcoal (Fyhrquist et al., 1976). Fluoregenic PRA assays have also been developed (Cartledge et al., 2000). However, the use of proprietary fluorescent reagents makes these assays too costly for clinical laboratories running numerous samples.

While the PRA assay has many advantages over renin concentration assays, it is considered a specialist assay (Cartledge et al., 2000; Morganti et al., 2010). PRA assays exhibit a high degree of imprecision, as shown in some cases (Cartledge et al, 2000; Mulatero et al., 2006), including studies in which participating laboratories performed

PRA by RIA using standard commercial kits and protocols (Morganti et al., 2010; Morganti et al., 1995). As with all immunoassays, the possibility of antibody cross reactivity in RIA can lead to misdiagnosis. In low PRA samples, even a small overestimation could lead to misdiagnosis (Cartledge, 2000; Holmes & Buhr, 2007).

1.11 Angiotensin I and II

Immunoassays for measuring angiotensin I and II are commercially available. However, due to their low concentration and instability, measurement of these analytes is not typically performed for the purpose of diagnosing secondary hypertension (Cartledge, 2000; Sealey, 1981).

1.12 Summary of current diagnostic techniques

ARR is the most commonly used method for diagnosing secondary hypertension (Gordon, 2004; Stowasser & Gordon, 2004). It considers both aldosterone and renin to provide a more comprehensive understanding of the RAAS (Tiu et al., 2005). Variability in diagnostic values has been reported, which may be a result of the low precision of the PRA test (Schwartz & Turner, 2005; Tiu et al., 2005).

The majority of commercially-available kits for measuring the RAAS are based on immunoassay, with RIAs being the most commonly used approach (Cartledge, 2000). While antibody-based approaches are highly sensitive, they are subject to false-positive results due to antibody cross reactivity. Additionally, endogenous circulating antibodies or molecules with biochemical properties similar to the target molecule are capable of

binding to the immunoassay kit reagents thereby interfering with assay results. The presence of auto-antibodies and heterophilic antibodies (antibodies that recognize self antigens) is now recognized as a major source of variability in immunoassays (Marks, 2002). Broad-spectrum blocking agents are often included in the kits to prevent antibody interference, but they are unable to eliminate all interference (Marks, 2002). Furthermore, the variation in plasma or serum between patients makes it very difficult to account for antibody interference.

As current understanding of the RAAS and its role in the development of many pathological states expands, PRA assays will be increasingly in demand (Cartledge, 2000; Dorrian et al., 2010; Stowasser, 2006) as a screening test for secondary hypertension as well as for monitoring therapeutic programs (Morganti, 2010). Recommendations have been made to screen all hypertensive patients for secondary hypertension to avoid serious cardiovascular side effects associated with the disease (Kaplan, 2007; Rossi, 2008; Rossi, 2008). There is a clear need for more robust PRA assays that can meet the accuracy and sensitivity requirements as well as the high throughput demands of clinical laboratories.

1.13 Mass spectrometry as a clinical tool

Mass spectrometric analysis of proteins and peptides allows for identification of individual analytes within a complex mixture (Trauger, 2002). Peptides are identified through molecular weight and sequence information which is made available by tandem mass spectrometry (MS/MS). Mass spectrometry is based on ionization of analytes

which generates charged molecules. These molecules are accelerated by an electric field. As charged molecules move through a vacuum they are separated by their mass (m) to charge (z) ratio (m/z) before reaching the detector. Ion signals are presented as a mass spectrum which plots the intensity at which each ion was detected against its m/z value.

With recent advancements in mass spectrometry technologies, detection of analytes at femtomole concentrations is routinely performed. Even attomole concentrations are now possible with the most sensitive instruments (Trauger, 2002; Yates, Ruse & Nakorchevsky, 2009). In addition to the sensitivity achievable with mass spectrometry, one of the greatest advantages is the ability to identify each peptide detected in a sample by peptide sequencing. This addresses the current limitations associated with non-specific antibody binding in immunoassays.

1.14 Quantitation by mass spectrometry

Mass spectrometry generates both qualitative and quantitative data. Relative quantitation is commonly achieved using label-free or differential isotope labelling methods (Lill, 2003). This allows changes in protein expression to be compared among multiple samples. Relative quantitation is useful for biomarker discovery studies. However, many applications, including clinical diagnostics, require knowledge of a target analyte's absolute concentration. Absolute quantitation is performed using the stable isotope dilution approach (Bantscheff et al., 2007). In this approach, internal standards are used that are chemically identical to the analyte of interest except for the incorporation of a stable isotope label that increases the molecular weight of the internal standard. The

mass difference is detectable by mass spectrometry. The advantage of this technique is that the standard and the target analyte behave identically during ionization and mass spectrometric analysis because of their compositional similarity. Discrepancies that normally arise from differences in analyte size, charge, and hydrophobicity are avoided.

1.15 Instrument hardware

Mass spectrometers are comprised of three basic components: the ionization source; the mass analyzer, and the detector. Technical variations of these components provide unique advantages for analyzing different sample types.

1.16 Ionization Sources

Matrix associated laser desorption/ionization (MALDI) and electrospray ionization (ESI) are the most commonly-used ionization methods for biomolecule analysis (Lill, 2003). Their soft ionization technologies prevent extensive fragmentation thereby permitting analysis of intact molecules.

In ESI liquid sample is injected into the MS through a fine-tipped emitter needle to which an electric field is applied. A fine spray of highly-charged droplets forms at the emitter tip. The droplets are then evaporated by heat and charged ions are released into the gas phase and are guided electronically into the analyzer region of the MS. ESI is also easily coupled with liquid chromatography (LC) as an on-line separation system for complex mixtures. Because ESI produces both singly and multiply charged ions, the spectra can be difficult to interpret. This is especially true when complex mixtures are being

analyzed. One of the major drawbacks of ESI is the potential for sample carry-over between analyses leading to contamination (Sparbier et al., 2009). Another disadvantage is clogging of the sample inlet.

For MALDI, a laser is used to excite analytes and transfer them to the gas phase. Sample preparation is very important in MALDI, as the sample must be mixed with a matrix that facilitates energy transfer from the laser to the sample to produce charged molecules. A UV-absorbing, weak organic acid matrix is co-crystallized with the sample. The matrix absorbs energy from the laser, permitting co-vaporization of the analyte and matrix crystals. This prevents decomposition of the sample. Although this process is not completely understood (Chang 2007; Karas 2003; Knochenmuss 2006), it is generally agreed that ionization of the analyte occurs through protonation or cationization during desorption. MALDI-generated ions are predominantly singly charged, making the spectra easy to interpret. MALDI is slightly more compatible with salts thereby permitting analysis of impure samples (Trauger, 2002). Samples are spotted directly on a MALDI stainless steel plate for analysis, which eliminates problems associated with sample carry-over and clogged tips that typify ESI; and also permits high throughput analysis of up to 384 samples per plate. MALDI is a more robust ionization technology than ESI, and its high throughput capabilities make it well suited to clinical laboratories.

1.17 Mass analyzer

After ionization, molecules enter the mass analyzer, which separates molecules based on their mass to charge ratio. Electric or magnetic fields are commonly used to separate

molecules in the mass analyzer. There are four main types of mass analyzers interfaced with ESI and MALDI sources for protein/peptide analysis: quadrupole, ion trap, time-of-flight (TOF), and Fourier transform ion cyclotron resonance (FT-ICR).

Quadrupole mass analyzers consist of four parallel rods that separate ions based on their m/z value in an electrical field. Scanning of different frequencies through the rods permits ions of different m/z to pass through the quadrupole at specific times. MS/MS is possible when three quadrupoles are aligned in the mass analyzer in a configuration known as a triple quadrupole. The first quadrupole (Q1) scans a pre-set m/z range to select ions of interest. As ions enter the second quadrupole (Q2) a gas is introduced which collides with the ions to produce fragments. In the third quadrupole (Q3) these fragment ions are selected for detection. Quadrupoles are often coupled with ESI as they can tolerate the high pressure required for ionization.

Ion traps are based on similar principles to the quadrupole. Similarly, radio frequency is applied to a quadrupole field. In ion trap instruments, ions are trapped in the electrical field instead of passing through as in the quadrupole analyzers. When frequency is applied the ions either remain trapped or are ejected based on their m/z value.

TOF analyzers measure the time it takes for ions to fly through a flight tube to the detector. Before ions begin to drift towards the detector, they are accelerated by a fixed electrical potential, which gives all ions the same starting energy. Therefore flight time is related to mass. The lower velocity of larger molecules results in increased time to reach

the detector. TOF analyzers have a high m/z range making them very compatible with MALDI.

In FT-ICR, ions orbit in a cell surrounded by a high field magnet. A pulsed radio frequency is applied to excite the ions. This excites the ions to a larger orbit radius where they induce an alternating current between detector plates.

1.18 Detector

Once the ions pass through the analyzer they reach the detector. The detector records either the charge induced or current produced when an ion reaches the surface of the detector. The most commonly-used detector is the electron multiplier which consists of a vacuum-tube and a multi-channel plate system with an electric potential. As ions strike the surface of the detector electrons are emitted and further accelerated through the detector to release secondary ions. Secondary electrons are generated as the electrons are attracted to a higher potential within the detector. Ions are collected by a metal anode.

1.19 Summary and research hypothesis

Dyregulation of the RAAS leads to secondary hypertension, which causes many serious problems, such as kidney and cardiovascular disease leading to heart attack, stroke, and diabetes. Diagnosis of secondary hypertension relies on accurate measurement of renin. PRA assay is commonly used to measure renin. Because of the prevalence and ineffective management of hypertension, it has been recommended that all hypertensive

patients be screened for secondary disorders as a means of early diagnosis and personalized therapeutic strategies.

PRA assays are most commonly performed using immunoassay technologies. Limitations to these assays include non-specific antibody binding and poor reproducibility. Highly technical procedures make the assay unsuitable for patient screening. The PRA assay also only provides information on the production of angiotensin I by enzymatic action of renin. The test does not provide information on other important molecules of the RAAS, such as angiotensin II or ACE. We hypothesize that simultaneous measurement of both angiotensin I and II would provide a comprehensive overview of the RAAS, as well as insight on the use of renin and ACE inhibitors in the treatment of hypertensive diseases. In this thesis, we study the hypothesis that endogenous concentrations of angiotensin I can be correlated with PRA and that measurement of endogenous angiotensin I will provide an improved method for diagnosing and screening hypertensive patients. To test this hypothesis, mass spectrometry methods will be developed for measuring angiotensin I and PRA.

Chapter 2
Development of methods for detection and quantitation of angiotensin I

2.1 Introduction

Plasma is routinely used in biological and biochemical research, as well as medical research, diagnostics, and treatment monitoring. Plasma is a highly desirable sample for these purposes due to easy, standardized collection protocols. It also provides a comprehensive representation of the human proteome, including tissue-secreted proteins and immunoglobulins. Plasma contains many medically-relevant molecules making measurement of multiple analytes in a single sample possible. Plasma is inherently complex because of the many different types of analytes spanning a wide dynamic range. This contributes to sensitivity problems because highly-abundant molecules can mask detection of lower concentration molecules (Lill, 2003). In mass spectrometry, this phenomenon is known as ion suppression, which results from differences in ionization efficiencies of analytes. Ion suppression can reduce sensitivity, accuracy, and precision. Sample preparation and protein separation are important in minimizing ion suppression. Numerous methods have been developed to address this issue.

2.2 Sample preparation and protein separation for mass spectrometry analysis

Enzymatic digestion of proteins is routinely employed in "bottom-up" proteomics. The primary function of protein digestion is to fragment the large intact protein into lengths that fall within the optimum detection range of MS. In this research project, enzymatic

digestion was not required since the peptide of interest, angiotensin I, is a 10-amino acid peptide, and is therefore already compatible with MALDI MS analysis.

Chromatographic techniques reduce sample complexity by separating proteins based on their size, affinities, or charge (Trauger, 2002). The resulting sample fractions are then analyzed by MS. Liquid chromatography is routinely coupled with electrospray MS instrumentation (LC-MS). LC separation prior to MALDI MS analysis requires spotting of LC fractions on to the MALDI plate. Numerous aliquots are analyzed from each sample. Although commonly used in research laboratories, LC and other chromatographic techniques can require costly separation columns and result in an increased time before MS analysis, as well as reducing sample throughput.

2.3 Enrichment methods

Immuno-affinity techniques are also commonly employed in proteomics studies as a method of concentrating, or enriching low abundance molecules to separate them from the complex background in plasma. Immuno-affinity enrichment combined with MS is a very powerful approach because, in contrast to ELISA, the MS itself is the detection system (Sparbier, 2009). This provides specificity through peptide sequence information and eliminates misdiagnosis as a result of antibody cross reactivity. Another advantage is that only one antibody (the primary antibody) is required in MS assays. This is in contrast to ELISA, which requires two antibodies (the primary antibody and a detecting antibody), or even three antibodies for a sandwich ELISA.

Stable isotope standards and capture by anti-peptide antibodies (SISCAPA) (Anderson 2004a; Anderson 2004b) is a method for specific antibody-based capture of target peptides from enzymatically digested samples. Anti-peptide antibodies are coupled to magnetic Protein G beads and incubated with the sample of interest. Capture of stable isotopically labelled peptide standards at the same time as the target peptides permits absolute quantitation. Peptides are eluted from the beads prior to LC separation and MS analysis. SISCAPA is usually performed with electrospray (LC/ESI-MS/MS) and provides excellent sensitivity in a very targeted and specific manner.

Another enrichment method is immuno-affinity MALDI (iMALDI) (Jiang, 2007a; Jiang, 2007b; Reid, 2007; Reid, 2010; Warren, 2005). It was developed for highly sensitive analysis of target peptides on the MALDI MS platform. Similar to SISCAPA, capture of target peptides and internal standards by anti-peptide antibodies bound to beads permits absolute quantitation and high sensitivity. However, the beads are placed directly on the MALDI plate which eliminates the need for peptide elution. MALDI MS is a robust platform with high throughput and automated capabilities, and is therefore ideal for clinical laboratories (Reid, 2007; Sparbier, 2009).

2.4 Materials and methods

Internal Standards

Angiotensin I was purchased from Sigma Aldrich and re-suspended in 30% acetonitrile, 0.1% formic acid. Tandem MS was performed on an Applied Biosystems 4800 MALDI TOF/TOF instrument. Angiotensin I was synthesized with a stable-isotopically coded

arginine residue (+10 Da) (D**R**VYIHPFHL) at the University of Victoria Genome BC Proteomics Centre, Victoria, Canada, according to a previously described protocol (Bordeerat, 2009).

Sample Preparation

Human plasma with unknown diagnosis of arterial pressure related disease was provided by our collaborator, Dr. Dan Holmes at St. Paul's Hospital (Vancouver, BC). Blood was collected in BD Lavender EDTA tubes. Plasma was separated, stored at -80°C, and thawed at room temperature prior to analysis. Incubation at 37°C for 1 hour enabled generation of angiotensin I through the cleavage of angiotensinogen by renin (the "angiotensin I generation period"). MALDI TOF/TOF analysis was performed on whole plasma after desalting and concentrating with Millipore ZipTipC18 pipette tips, according to the manufacturer's protocol. ZipTips were primed with 100 % acetonitrile (10 μL) by aspirating solution into the tip and dispensing. This was repeated twice. A 50 % acetonitrile solution (10 μL) was then aspirated into the tip twice, followed by 10 μL of 0.1 % TFA three times. Plasma (10 μL) was then aspirated and dispensed for 5 cycles. A wash solution of 0.1 % TFA was then aspirated three times. Trapped peptides were eluted with 10 μL of MALDI matrix solution, and 1 μL was spotted onto the MALDI target for analysis.

Antibody-Bead Conjugation

Polyclonal antibodies to angiotensin I were provided by Dr. Dan Holmes at St. Paul's Hospital. The polyclonal antibody (concentration unknown) was obtained by

immunizing rabbits with angiotensin-I/albumin complexes in Freund's adjuvant (Goodfriend, 1964). The antibody was subsequently purified by gel filtration. Monoclonal antibodies to angiotensin I (1 mg/mL) were purchased from AbCam (Cambridge, MA). Magnetic Protein G Dynabeads (30 mg/mL) (Invitrogen) were precipitated with a magnet in the manufacturer's storage buffer (PBS, pH 7.4, 0.01% Tween®-20, 0.09% sodium azide). The binding capacity of the Protein G Dynabeads was determined according to the manufacturer's specifications, and an excess of beads was used to ensure maximum antibody binding. A 5 μL volume of bead slurry was used in each reaction. The storage buffer was removed from the beads using a magnet to precipitate the beads, followed by three washes in 50 μL aliquots of 1X PBS. Antibodies (2 μL polyclonal antibody per reaction, or 1 μg monoclonal antibody) were immobilized on Protein G Dynabeads in 1X PBS, pH 7.4 for 1 hour at room temperature or 4°C. After antibody-bead conjugation, the beads were washed in three, 50 μL aliquots of 1X PBS, and then distributed into samples for immuno-affinity capture.

Immuno-affinity MALDI

Antibody beads were incubated with whole plasma (20 μL, non-digested plasma) and internal standard for 12-18 hours at 4°C ("angiotensin I capture period"). Endogenous angiotensin I and internal standard were affinity-captured from the plasma solution by the antibody-bound beads. Plasma was removed from beads prior to MS analysis in three, 50 μL aliquots of 25 mM ammonium bicarbonate. The washed beads were then re-suspended in 5 μL 25 mM ammonium bicarbonate and 1 μL of beads placed directly onto

a MALDI 384-well plate for mass spectrometry analysis. The iMALDI workflow is depicted in Figure 2.

MALDI-TOF/TOF Analysis

Captured peptide was eluted from the antibody-conjugated beads on the MALDI target with the application of 10 mM α-cyano-4-hydroxycinnamic acid (CHCA) in 0.1% trifluoroacetic acid. MS and MS/MS analysis were performed on an Applied Biosystems 4800 MALDI TOF/TOF mass spectrometer.

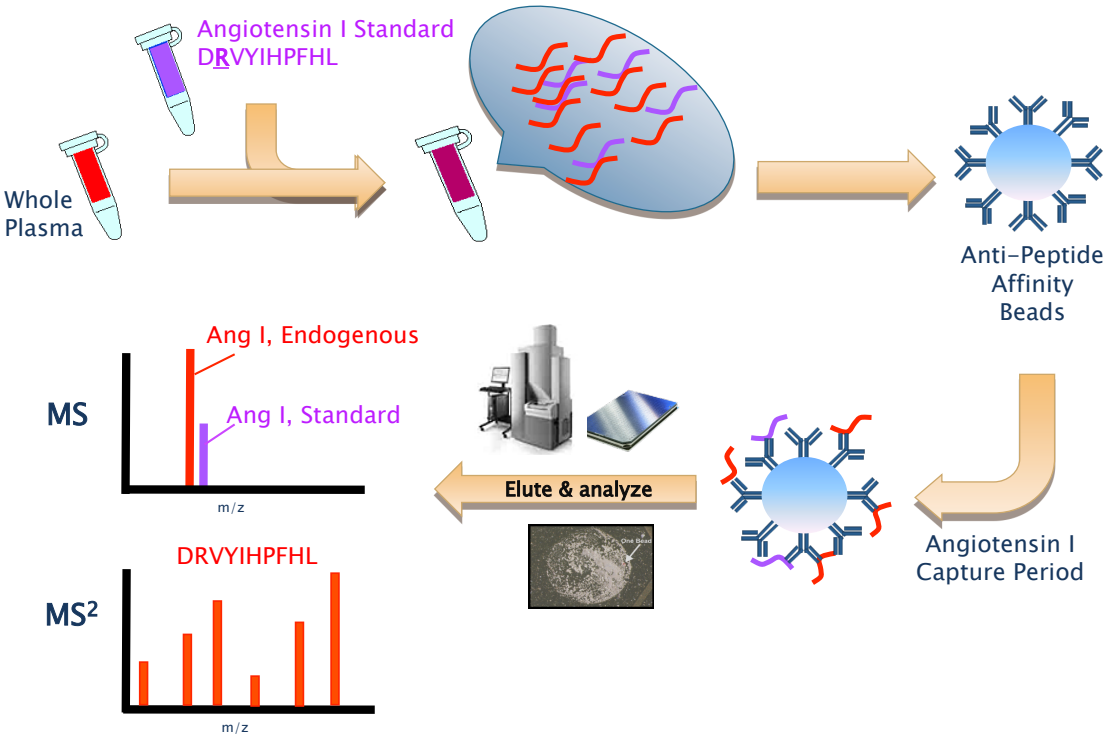


Figure 2. iMALDI assay for plasma renin activity

Whole human plasma is incubated at 37 °C to allow cleavage of angiotensinogen by renin. The internal standard is then added to the plasma, which is then incubated with anti-angiotensin I antibody conjugated beads. The endogenous angiotensin I and the internal standard are immuno-precipitated, and the beads are placed directly on a MALDI target plate. CHCA matrix is applied to elute the peptide from the antibody permitting MALDI MS analysis. The relative abundances of the molecular ion signals corresponding to endogenous peptide and internal standard are used for quantification. Absolute specificity is achieved by MS sequencing of the detected peptide.

2.5 Results

2.5.1 Internal standards

An internal peptide standard was synthesized in order to quantitate the amount of angiotensin I in samples using a calibration curve. Isotopically labelled peptide with the same sequence as endogenous angiotensin I (DRVYIHPFHL) was selected as the internal standard because of its chemically identical behaviour to the target analyte. In considering the location of a stable isotope labelled amino acid within the angiotensin I peptide, it was important to ensure that the strongest product ions observed upon fragmentation of the internal standard would show an increase in m/z respective to the mass of the isotopically labelled amino acid. This will permit peptide sequence confirmation of the internal standard even at low concentrations when weaker product ions might not be observed. Fragmentation of synthetic angiotensin I (Figure 3) was performed by MS/MS analysis, resulting in a series of b- and y- product ions detected at different intensities. Theoretical product ions for angiotensin I are listed in Appendix I. As shown in Figure 3, the strongest product ion produced upon fragmentation of angiotensin I was the y_9 ion as indicated at m/z 1181, therefore replacing arginine with a stable isotopically labelled version would increase the mass of the observed y_9 ion, as well as $b_2 – b_9$ ions.

Incorporation of a stable isotope labelled arginine ([$^{13}C_6$]) resulted in an increased mass of 10 Da (Figure 4A). MS/MS analysis of the stable isotope labelled internal standard (SIS) confirmed a similar fragmentation pattern as observed to the unlabelled angiotensin I peptide (Figure 4B).

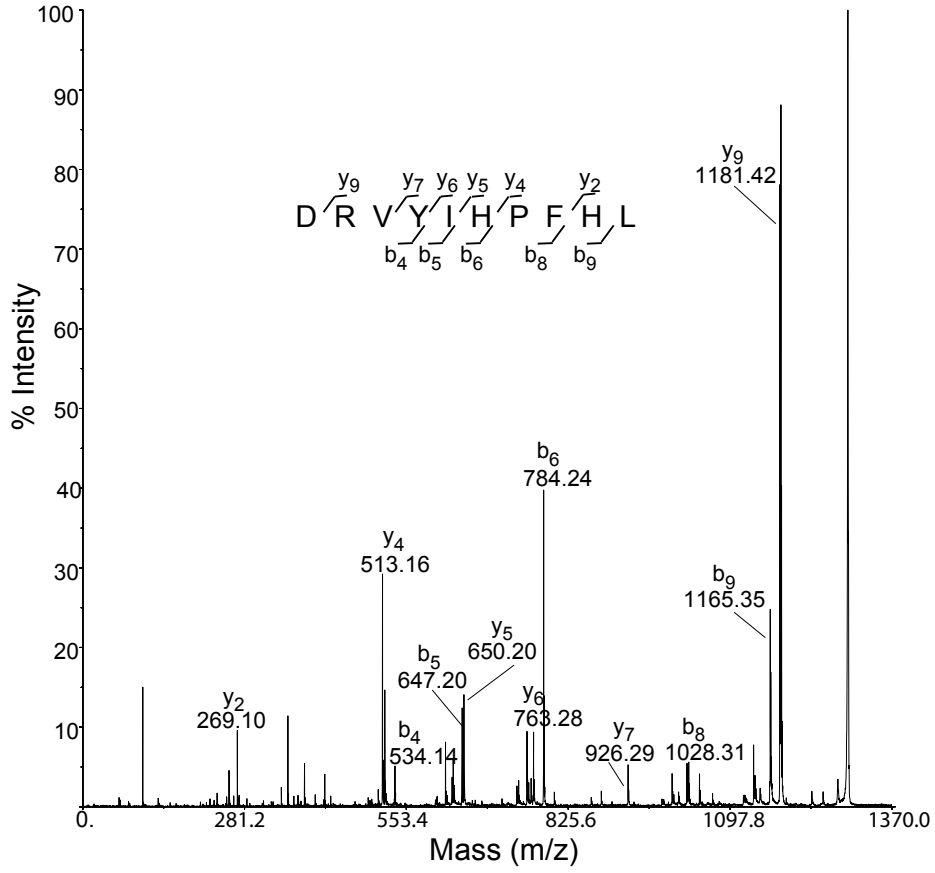
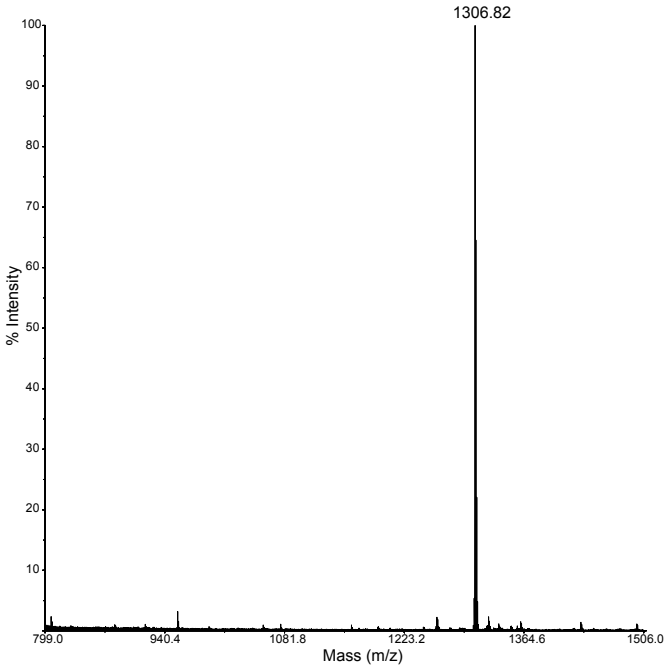


Figure 3. MS/MS fragmentation of synthetic angiotensin I.

Fragmentation of synthetic angiotensin I results in a strong y_9 ion observed at m/z 1181. Synthetic angiotensin I (1 pmol/µL) was spotted directly on MALDI target plate.

(A)



(B)

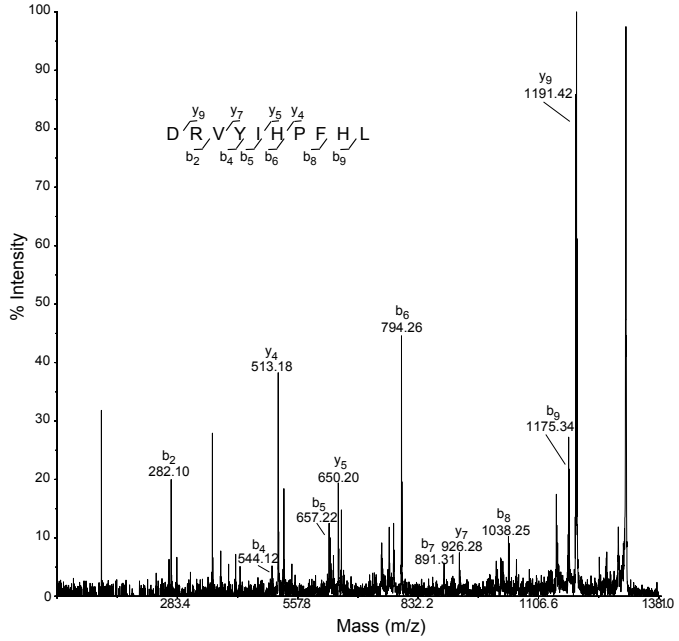


Figure 4. MS and MS/MS analysis of stable isotope labelled angiotensin I confirm presence of isotopic label in fragment ions.

A) Angiotensin I (50 fmol/µL) is observed at m/z 1306, indicating incorporation of an isotopically labeled amino acid resulting in a mass shift of 10 Da. B) MS/MS analysis of angiotensin I confirms sequence of peptide.

2.5.2 iMALDI capture of angiotensin I in plasma

Detection of Angiotensin I in Plasma: A 1 hour generation period was used to increase the endogenous levels of angiotensin I in whole (*i.e.,* non-digested) human plasma from a donor patient. However, angiotensin I was not detectable by direct MALDI TOF analysis (i.e. no enrichment) of the plasma as indicated by the absence of a corresponding peak at m/z 1296.68, the theoretical m/z value of angiotensin I (Figure 5).

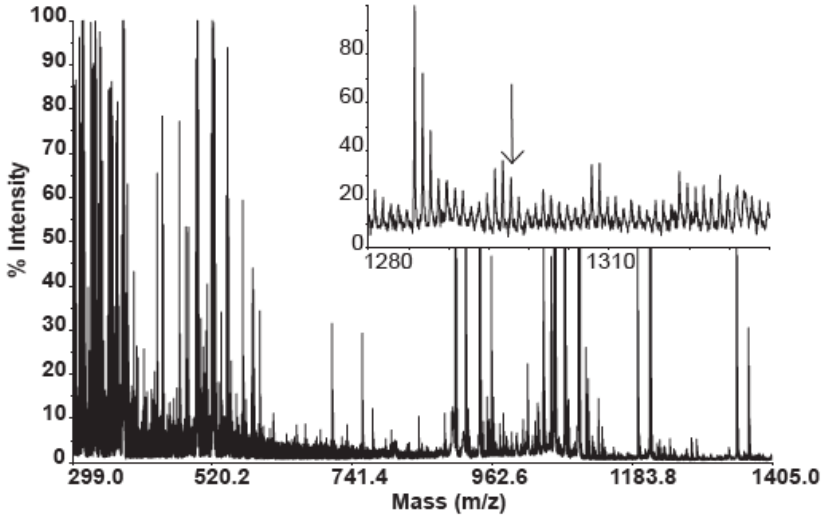


Figure 5. MALDI MS analysis of whole human plasma.

Whole human plasma was incubated at 37 °C for one hour to allow enzymatic cleavage of angiotesinogen. Angiotensin I was not detectable by MALDI TOF/TOF analysis as indicated by the arrow at 1296 (no enrichment performed).

Employing the iMALDI approach, however, angiotensin I was successfully detected in non-digested plasma (20 μL) from the same patient without an angiotensin I generation period, as shown in Figure 6A. A stronger signal at m/z 1296 was observed after the sample underwent a 1 hour angiotensin I generation period (Figure 6B). Specific fragment ions of angiotensin I were obtained by performing MS/MS of the m/z 1296 ion

from this spectrum (Figure 6C). Non-specific binding of angiotensin I was not observed when blank Protein G Dynabeads (i.e., beads with no bound antibody) were incubated with plasma (Figure 6D).

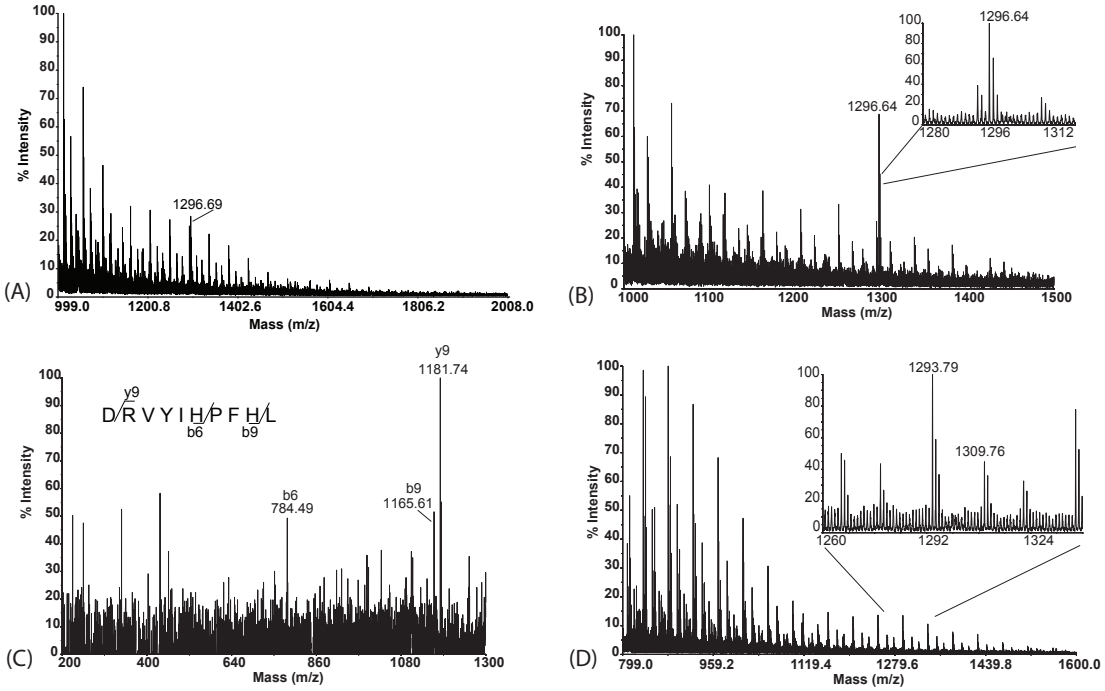


Figure 6. Detection and identification of angiotensin I in whole human plasma by iMALDI.

(A) Endogenous levels of angiotensin I (m/z 1296.69) were detectable by iMALDI. (B) An increase in signal strength of angiotensin I (m/z 1296.64) was observed after a 1 hour generation period. (C) Sequence of angiotensin I detected in whole plasma was confirmed by MS/MS. (D) Non-specific binding of angiotensin I to Protein G beads was not observed.

Sensitivity: The detection limit of angiotensin I by direct MALDI analysis was determined by analyzing the peptide spotted in solution directly on the MALDI plate. The limit of detection was determined to be 4 fmol/μL angiotensin I, equivalent to 4 fmol angiotensin I on the MALDI plate (Figure 7A). The limit of detection was improved employing the iMALDI approach. Isotopically-labeled angiotensin I spiked into 50 μL of human plasma was detectable at a concentration of 10 amol/μL (13 pg/mL) with a signal to noise ratio of 3:1, which is equivalent to 100 amol of the peptide on each spot of the MALDI plate. At a concentration of 25 amol/uL (32 pg/mL) in plasma, angiotensin I was detected with a signal to noise ratio of 20:1 (Figure 7B). This represents a 160-fold (4 fmol/μL vs. 25 amol/μL) to 400-fold (4 fmol/μL vs 10 amol/μL) improvement in sensitivity of the assay for angiotensin I by using iMALDI. The peptide sequence of angiotensin I was confirmed by MS/MS analysis of the analyte captured from a 25 amol/μL solution (Figure 7C). Although the ion signals in the MS/MS spectrum are not very intense compared to background noise, the strongest fragment ions (b_2, y_2, and y_9) are observed in a pattern similar to that seen with MS/MS analysis of higher concentrations of angiotensin I.

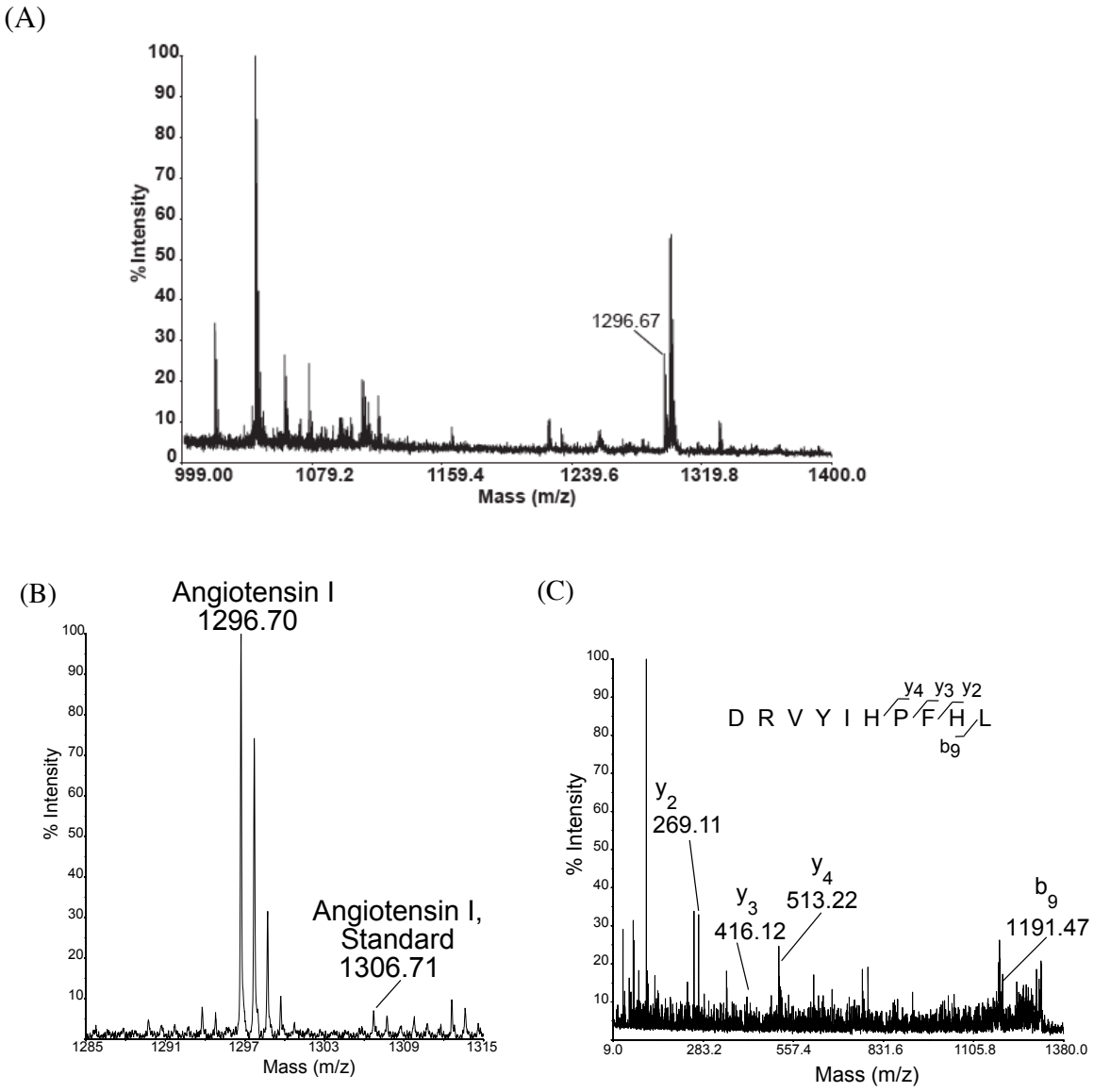


Figure 7. Sensitivity of MALDI analysis of angiotensin I improved with iMALDI.

(A) The limit of sensitivity of direct MALDI analysis of angiotensin I (no iMALDI) was 4 fmol/uL. (B) Sensitivity is improved with iMALDI methods. Angiotensin I was captured from plasma containing 25 amol/μL isotopically labeled peptide by iMALDI. (C) MS/MS spectrum (inset) of angiotensin I confirmed the identity of angiotensin I detected in this 25 amol/ μL solution.

Reproducibility of MS Analysis: In order to determine the number of data acquisitions required to provide consistent peptide ratios, light and heavy versions of the synthetic angiotensin I (100 fmol L : 300 fmol H per spot) were repeatedly analyzed by MALDI. Automatic and manual acquisition modes on the AB 4800 MALDI TOF/TOF instrument were also compared. Peak intensity ratios were calculated for 3, 5, 7, and 10 repeated measurements of the same spot on the MALDI plate. While the coefficient of variation (CV) was found to be less than 20% in both modes, the CV was 5% or less for spots measured by automatic acquisition. As shown in Table 1, the CV was between 4 % and 5 % for repeat analysis in automatic mode. Therefore, an increased number of acquisitions per spot does not appear to have a significant impact on reproducibility. In the following studies, 3 or 5 acquisitions were used to calculate average peak intensity ratios.

Table 1. Reproducibility of MALDI MS analysis

Number of Acquisitions	% CV Automatic Mode	% CV Manual Mode
10	4.9	9.2
7	4.5	11.2
5	4.1	12.9
3	5.2	16.2

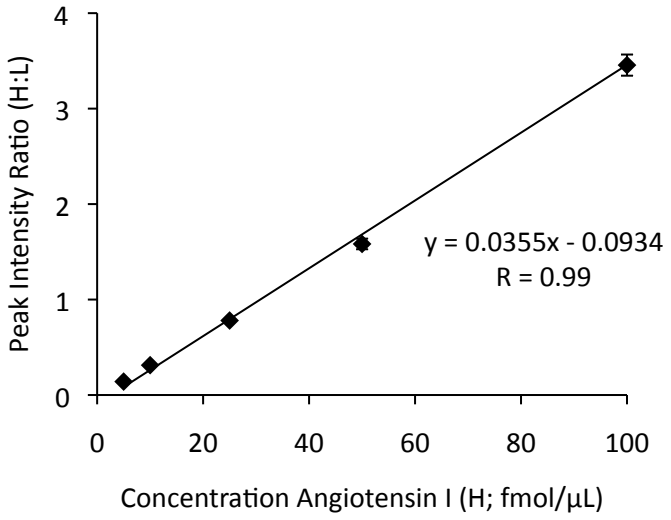
2.5.3 Linearity and Dynamic Range

Linear Response: To assess the linearity of the developed iMALDI method, standard curves were generated by placing synthetic peptides in buffer directly on the MALDI plate or by iMALDI capture of synthetic peptides in buffer. A strong correlation (R = 0.99) was obtained when varying ratios of angiotensin I (Light peptide, or L) and isotopically labeled angiotensin I (Heavy peptide, or H) were analyzed by MALDI and iMALDI (Figure 8). The heavy peptide was spiked into buffer at concentrations of 5 fmol/μL to 100 fmol/μL while the concentration of the light peptide was kept constant at 50 fmol/μL. The concentration range of heavy peptide used in these experiments corresponded to low to normal plasma renin activity values (Table 2). The reproducibility of iMALDI analysis is compared to direct MALDI analysis in Table 3. A CV of less than 10% for all iMALDI data points was calculated.

Table 2. Plasma angiotensin I concentration in low, normal and high PRA patients (Locsei, 2009)

PRA (ng/L/s)	**Concentration Ang I after 1 hr incubation period** (fmol/μL)	**Concentration Ang I in 20 uL Plasma** (fmol/μL)
Low PRA, 0.06	.15	3
Normal PRA, 0.42	1.16	23
High PRA, 3.75	10	200

(A)



(B)

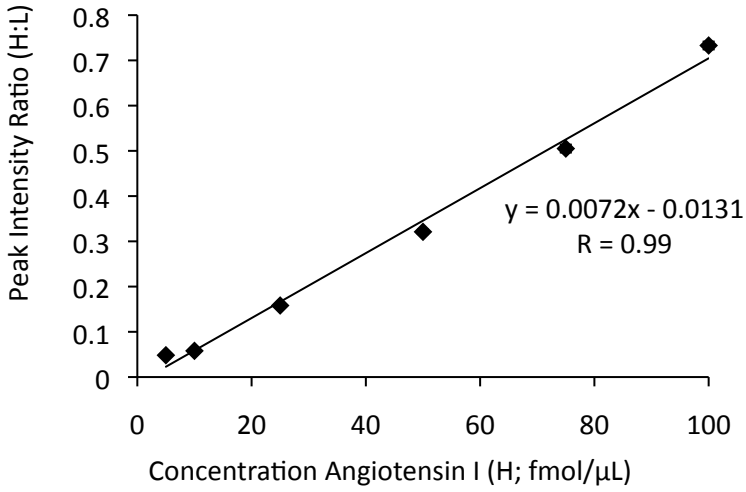


Figure 8. iMALDI assay for angiotensin I is linear over a 20-fold concentration range in buffer.

Standard curves generated by analyzing varying ratios of heavy angiotensin I (5-100 fmol/µL; L) and light angiotensin I (50fmol/µL; H) produced a linear response with an R value of 0.99 by iMALDI and MALDI analysis. Peak intensity of the heavy peptide was normalized to the peak intensity of the light peptide. (A) Analysis of peptides spotted directly on MALDI target plate. (B) iMALDI capture of peptides from solution. Error bars represent the standard error for each data point, however not all error bars are visible on the scales presented.

Table 3. Reproducibility of iMALDI assay for angiotensin I in buffer

Concentration SIS Angiotensin I (fmol/μL)	**Concentration Natural Angiotensin I** (fmol/μL)	**MALDI** (% CV)	**iMALDI in buffer** (% CV)
100	50	5.6	2.6
50	50	6.1	3.5
25	50	1.9	3.4
10	50	1.2	5.3
5	50	0.8	9.9

Linearity of iMALDI Assay in Plasma: The linear response of the assay in plasma was assessed using the standard addition method. Since plasma contains endogenous levels of angiotensin I, the concentration of isotopically labeled standard was kept constant while increasing amounts of synthetic non-labeled angiotensin I were added. Peptides were captured from 20 μL of plasma sample using iMALDI methods (Figure 9). CV's of less than 12% were calculated for each point on curve.

2.5.4 Assay optimization

Optimization of Antibody-Bead Conjugation: To determine the optimal incubation period for antibody-bead conjugation, antibody was incubated with beads over an eight-hour period. Synthetic angiotensin I was the added to the antibody beads to determine the effect of extended incubation period. Peptide capture by iMALDI was performed overnight. As shown in Figure 10, the signal produced by angiotensin I after MS analysis decreases with increased antibody-bead incubation period. The optimal incubation period was therefore determined to be one hour.

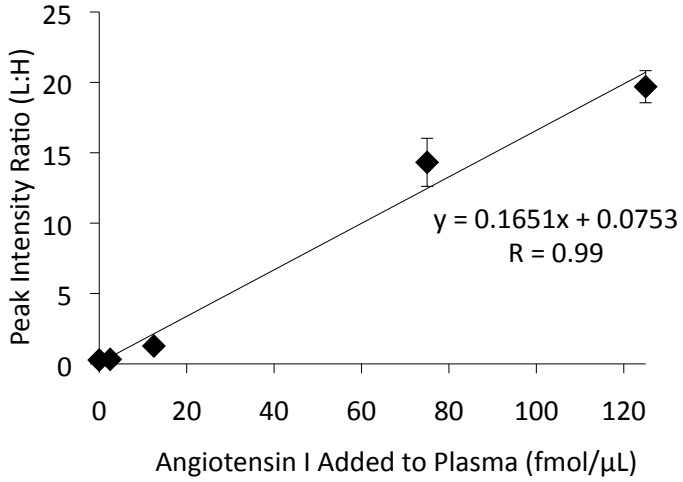


Figure 9. Linear response of iMALDI assay in plasma over 50-fold concentration range.

Linearity of iMALDI assay (R = 0.99) demonstrated by adding varying ratios of light (0-120 fmol/µL) and heavy angiotensin I (50 fmol/µL) peptides to 20 µL of human plasma. Peak intensity of the light peptide was normalized to the peak intensity generated from the heavy peptide. Error bars represent the standard error for each data point, however not all error bars are visible on the scale presented.

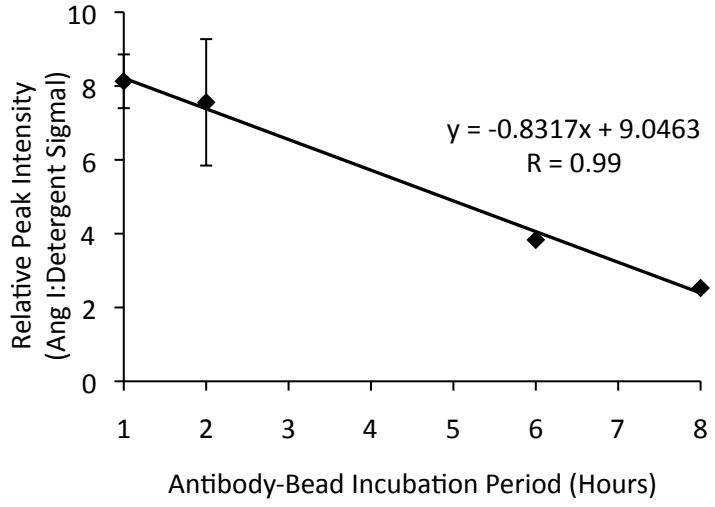


Figure 10. Optimization of antibody-bead conjugation period

Antibodies were bound to Protein G Dynabeads in PBS solution over a period of 8 hrs followed by addition of synthetic angiotensin I and iMALDI capture. Peak intensity of angiotensin I and background signal were used to determine relative changes in amount of peptide captured by antibody-conjugated beads.

Optimization of Peptide Capture Period: Synthetic angiotensin I was incubated with antibody-conjugated beads over a 6-hour period. As shown in Figure 11, an incubation period of 4 hours provides optimal peptide capture.

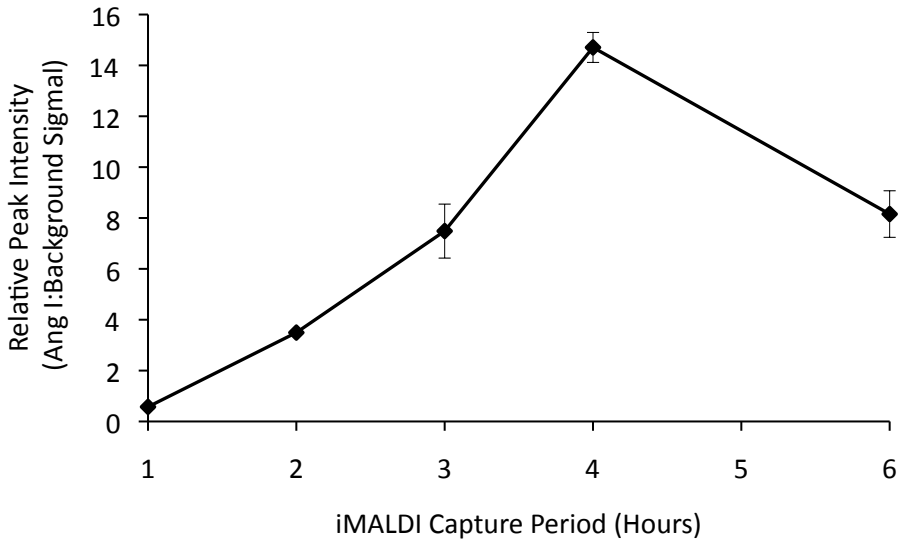


Figure 11. Optimization of antibody capture period

Antibodies were bound to Protein G Dynabeads in PBS solution followed by addition of synthetic angiotensin I. iMALDI capture was performed over a period of 6 hours. Peak intensity of angiotensin I and background signal were used to determine relative changes in peptide binding over time.

Reduction of Background Signal from Beads: As a result of the affinity beads containing Tween-20 detergent, a pattern of repeating peas was observed by MS analysis. In order to reduce background signal caused by the detergent, alternate washing solutions and detergent-free Protein G DynaBeads were examined. A citrate phosphate buffer was used to wash beads prior to antibody-bead conjugation, as described in the manufacturer's protocol. A ten-minute wash period was also tested, however, neither approach proved successful in noticeably reducing detergent signal. Furthermore,

difficulties in bead handling were experienced when detergent was removed. This may contribute to the loss of beads during the various steps of the iMALDI method.

Detergent-free prototype Protein G DynaBeads, provided by Invitrogen Dynal AS, Norway, were also tested. While these prototype beads produced a spectra with lower-abundance detergent peaks, non-specific binding of angiotensin I was observed (Figure 12). Consequently, subsequent experiments were conducted using the commercially available Protein G DynaBeads with Tween-20.

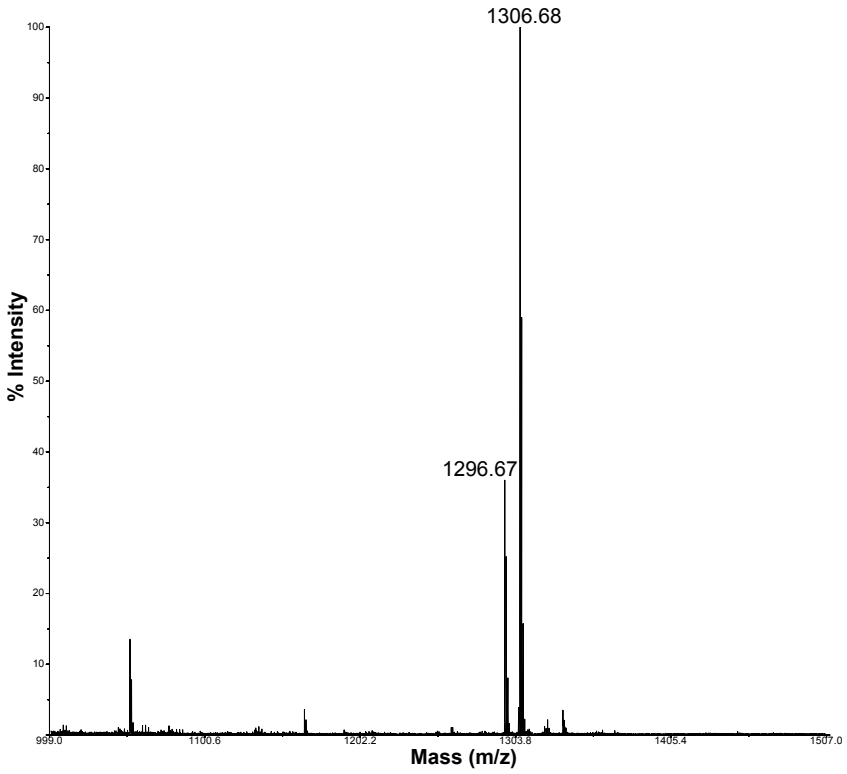


Figure 12. Non-specific binding of angiotensin I with plain prototype beads

Prototype beads containing no bound antibody were incubated synthetic angiotensin I peptide (light and heavy) in PBS buffer. Both peptides were detected, indicating non-specific binding to the antibody-free bead surface.

2.6 Discussion

A combination of immuno-affinity enrichment with MALDI MS analysis permitted detection of endogenous angiotensin I in 20 µL of whole plasma from a normo-tensive patient. The sensitivity of the assay was determined to fall between 10 and 25 amol/µL in plasma. According to the Tietz Clinical Guide to Laboratory Tests, the normal concentration of angiotensin I is <19 amol/µL (Tietz, 2006). The developed assay therefore shows a limit of detection in plasma that corresponds to normal circulating concentration of angiotensin I, indicating the potential of the assay for measuring endogenous angiotensin I concentrations in patients with normal and high concentrations as a direct means of assessing the RAAS. Applicability of the assay for measuring changes in angiotensin I concentration as a result of enzymatic activity was also demonstrated using a 1 hour angiotensin I generation period to increase analyte concentration. The advantage of incorporating the generation period is to overcome sensitivity limitations. It is not uncommon for 3 – 18 hour angiotensin I generation periods to be incorporated in PRA assays (Sealey, 1995). Low and normal PRA values determined by commercially available RIA range from 0.06 – 0.42 ng/L/s (Locsei, 2009). The iMALDI assay provided a linear response in plasma over this concentration range indicating its potential use for diagnostic purposes.

Considerable background signal was observed in the MS spectra shown in Figures 5A, 5B, and 5D, which is caused by the Tween20-containing buffer that the Protein G Dynabeads are stored in. Tween-20 is a polysorbate surfactant that is produces a series of repeating peaks in the MS spectra, which are a result of a complex mixture of oligomers

(Ayorinde, 2000). While Tween aids in sample handling by preventing the loss of beads, which might otherwise stick to the walls of the tube, the resulting background signals can suppress detection of low-abundance peptides such as angiotensin I. Detergent-free beads were assessed to determine whether improved sensitivity could be achieved. However, non-specific binding of angiotensin I peptides to detergent free beads was observed. The PRA method provides a means for unlimited sensitivity because of the ability to increase angiotensin I levels through enzymatic action of renin. Since the sensitivity of the assay was demonstrated to fall within the concentration of normal plasma renin activity, PRA should be measurable in normal and high PRA patients with the developed methods. Measurement of endogenous angiotensin I levels in patients with low PRA however may be beyond the limit of detection of the current iMALDI assay. However, larger sample volumes may address this problem. In these experiments, 20 μL of plasma was used. RIAs for PRA typically require 50 μL to 1 mL (Hartman, 2004) of plasma. It is therefore feasible to extend the sensitivity limits of the current iMALDI methods with a larger sample volume.

The concentration of angiotensin I generated in high PRA samples (3.75 ng/L/s as reported by a commercial RIA kit, (Locsei, 2009)) exceeds the linear range tested in the development of this iMALDI assay. However, the sensitivity of the assay should permit measurement of angiotensin I in normal and high PRA samples without a generation period which would provide a very fast method for screening elevated PRA levels for diagnostic and therapeutic monitoring purposes. Alternatively, dilution of high PRA

samples or smaller sample volumes would result in a final concentration fitting within the linear region shown in this curve.

The linear response of the iMALDI assay in plasma was determined using the standard addition method (National Institute of Standards and Technology), where known amounts of internal standards are added to a sample of unknown concentration. In this method, the sample itself is used to produce the calibration curve. While this approach is less common, it takes into consideration the effect of other molecules in the sample that can contribute to instrument response thereby providing a more accurate method of quantitating molecules in complex backgrounds (Abbyad, 2001; Ito, 2001). The iMALDI assay produced a linear response in a normo-tensive patient plasma sample over a concentration range of 0 – 120 fmol/μL. This concentration range reflects the concentration of synthetic angiotensin I added to the plasma sample. Endogenous levels of angiotensin I were also present in the sample. Therefore, it is expected the y-intercept of the standard curve should be > 0 fmol/μL. A y-intercept of 0.0753 was calculated for the plasma sample analyzed here. While the angiotensin I concentration in this sample was unknown, it was calculated to be 0.456 fmol/μL through linear extrapolation (Bruce & Gill, 1999) of the calibration curve.

MALDI MS has been critiqued as a quantitative tool because of variability between shot-to-shot analyses (Silvertand, 2008; Szájli, 2008). This is thought to arise from non-homogenous crystallization of analytes during matrix application (Preston,

Murray, & Russell, 1993) or laser ablation (Lill, 2003). Reproducibility of the AB 4800 MALDI TOF/TOF platform was assessed in this study by performing repeat analyses of angiotensin I peptides in automatic and manual acquisition modes. Greater reproducibility was observed for repeat analyses in automatic mode compared to manual mode, which is an advantage for high throughput analysis. In automatic mode, user input is not required while in manual mode the user must select the region of the MALDI spot to be analyzed during each acquisition (Trauger, 2002). The CV calculated for shot-to-shot variability on the 4800 MALDI TOF/TOF platform was ≤ 5%. This instrument therefore provides a reliable method of measuring peptides spotted directly on the plate. The effect of placing the magnetic protein G beads with captured peptide on the MALDI plate was also examined. While CV's were slightly higher for iMALDI compared to direct MALDI analysis (≤10 % vs. ≤6%), analysis of beads on the plate only results in a slight increase in shot-to-shot variability.

The assay was optimized by determining the most efficient incubation periods for antibody-bead conjugation and peptide capture. A one-hour incubation period is required for optimal antibody-bead conjugation. After one hour, capture of target analytes decreases, which may be a result of dissociation of the antibody from the bead. Although long-storage of antibody-bound beads has not been considered here, this would be desirable for laboratories analyzing numerous samples. Crosslinking of the antibody to the beads for long-term storage has been described by Dr. Terry Pearson (personal communications, October 2010). The manufacturer's Dynabeads Protein G protocol suggests that increased peptide capture periods may increase assay sensitivity. When

capturing angiotensin I in buffer by iMALDI, we observed the strongest MS signal after a 4-hour incubation period.

Comparison of iMALDI and Radioimmunoassay for PRA: RIAs for PRA typically require 50 μL to 1 mL (Hartman, 2004) of plasma, while 20 μL of plasma has been shown to be sufficient for iMALDI analysis. Small sample volumes are desirable when multiple tests are requested, as commonly seen when diagnosing, monitoring, and treating hypertension. Assays requiring only small sample volumes are also important in large population studies where limited sample is available.

In RIA, angiotensin I is measured by competitive RIA. In this technique, radiolabelled angiotensin I and unlabelled angiotensin I compete for antibody binding sites (Zettner, 1973). This results in a sigmoidal calibration curve, limiting the linear quantitation range of RIA (Hennion & Barcelo, 1998). The iMALDI protocol used here enabled detection of endogenous levels of angiotensin I in normo-tensive plasma samples (Figures 5A, 5B) without the standard angiotensin I generation period. The iMALDI is also more efficient through the use of affinity beads, which were shown to bind maximally after 4 hours, a significant reduction in time from the incubation periods required for some RIA protocols.

The major advantage provided by this mass spectrometry-based approach is the ability to distinguish between detected molecules, and confirm their identity through tandem MS analysis. This added level of specificity eliminates the major pitfall of other immuno-

based assays (Hoofnagle, 2009). The ability to differentiate between analytes based on their unique mass and peptide sequences also makes the iMALDI assay well suited for multiplexed diagnostics. The use of a stable isotopically coded internal standard eliminates the need for a radioactive label, making the assay safer for technicians and the environment.

Chapter 3
Evaluation of iMALDI assay for measuring plasma renin activity

3.1 Introduction

While many different assays have been described for measuring different components of the renin angiotensin pathway, the PRA assay is considered by some investigators to be the most accurate and sensitive method for measuring renin (Sealey, 2005). Some major advantages of this particular assay are its unlimited sensitivity through the accumulation of angiotensin I in the presence of protease inhibitors (Sealey, 1991), and, when appropriate precautions are taken, a lowered risk of misdiagnosis due to false positive results that occur due to measurement of prorenin (Sealey, 2005). Radioimmunoassay is the most commonly used approach for measuring PRA. While highly sensitive, RIA is considered technically demanding. It also relies on the use of hazardous radioactive isotopes. Additionally, as with any other immunoassay, antibody cross-reactivity can lead to inaccurate quantitation. The ability to identify individual molecules by mass spectrometry prevents such misdiagnosis. In order to evaluate the developed iMALDI assay for diagnostic purposes, it was compared it to a PRA radioimmunoassay. Endogenous levels of angiotensin I were compared to PRA to determine diagnostic relevance.

3.1.1 Plasma renin activity protocols

Plasma renin activity assays measure the rate at which renin converts angiotensinogen to angiotensin I, which correlates with angiotensin II levels (Bragat, 1997; Christen, 1991).

Therefore, the PRA assay provides a measurement that can be used for diagnosing disorders related to the regulation of the renin angiotensin aldosterone system. To avoid cryoactivation of prorenin and degradation of angiotensin I by angiotensin converting enzyme (ACE) and other proteases, several precautionary measures must be taken in plasma renin activity assays (Sealey, 1991). These include precautions in collection, incubation and control samples.

Blood is collected into a tube containing ethylenediaminetetraacetic acid (EDTA) to inhibit ACE, a zinc metalloenzyme. Blood should be processed at room temperature (Ulmer & Meikle, 2000). Plasma is then separated by centrifugation, and immediately stored at -80 °C until analysis.

Angiotensin I generation is performed at 37 °C in the presence of ACE and protease inhibitors (Sealey, 1991; Cawood, 2006). EDTA is typically used as an ACE inhibitor and serine protease inhibitors, such as phenylmethylsulfonyl fluoride (PMSF), are included to prevent degradation of angiotensin I by angiotensinases. During incubation, plasma pH increases leading to inactivation and denaturation of renin (Sealey, 1991). It is therefore critical that pH is maintained between 5.5 – 6.0 (Boer, 1985; Favre, 1973), which is the optimal range of renin activity. Maleic acid buffer is commonly used to maintain plasma pH range. Incubation also requires careful control so that prorenin is not activated (Ulmer & Meikle, 2000). Prorenin is constitutively secreted into the circulation and accounts for 70-90% of the total renin in plasma (Atlas, 2007). Prorenin can be found at concentrations 10x higher than renin (Cartledge, 2000). While prorenin is

inactive it is converted to active renin through exposure to low temperatures (4-8°C) (Sealey, Moon, Laragh & Alderman, 1976). It is critical that exposure of samples to this temperature range is avoided. Rapid thawing of frozen plasma at room temperature is recommended (Cawood, 2006, Sealey, 1991). Ulmer & Meikle (2000) have shown that limited exposure of samples to 4 °C has minimal or no affect on active renin. To avoid any concerns with this issue, it is recommended that samples remain on ice through the remainder of the assay, unless undergoing incubation at 37 °C.

Control or blank samples are used to establish a baseline level of angiotensin I concentration in plasma. This reflects endogenous angiotensin I levels prior to renin conversion of angiotensinogen during the generation period. The blank value is then subtracted from the concentration of angiotensin I after generation at 37 °C to determine PRA (ng/L/s). Blank samples are defined as patient plasma incubated on ice (0 °C) to prevent conversion of angiotensinogen to angiotensin I, and are referred to as "0 °C Samples" compared to "37 °C Samples" to describe samples undergoing angiotensin I generation for 1 hour. Reference ranges for PRA will vary depending on the assay used because of differences in pH (Cartledge, 2000; Sealey, 1991), as well as different standards. The RIA that the developed iMALDI assay is compared against, has reference ranges of <0.28 ng/L/s in supine patients, and 0.05 – 0.55 ng/L/s in erect patients with normal blood pressure.

3.2 Materials and Methods

3.2.1 Sample Collection

Venous blood samples were collected at room temperature in BD Vacutainer tubes containing EDTA (1.8 mg/mL). After centrifugation, plasma was split for RIA and iMALDI analysis. Plasma was stored at -80 °C and shipped to the UVic Genome BC Proteomics Centre on dry ice for iMALDI analysis.

3.2.2 Plasma Renin Activity by iMALDI

Angiotensin I Generation: Plasma was thawed rapidly at room temperature and immediately placed on ice. PMSF (1.4 mM) (Fluka BioChemika) was added to 20 µL plasma to prevent serine protease activity and 0.0276M maleic acid (Fluka) was added to maintain a pH 6. Bacterial growth was inhibited with 1.1 mM neomycin trisulfate (Sigma-Aldrich). Angiotensin I generation was performed at 37 °C for 1 hour while blank samples remained on ice at 0 °C. Immediately following the angiotensin generation period, ice-cold Tris-acetate buffer was added to the plasma to stop the enzymatic reaction.

ACE and Angiotensinase Inhibition: Levels of angiotensin I and II were compared in samples containing EDTA (incorporated through the collection process) and samples containing both EDTA and PMSF. Two different plasma samples were incubated at 37 °C over a period of 16 hours. The PRA values of these two samples were determined by RIA (0.09 ng/L/s and 0.383 ng/L/s). Peak intensity ratios of endogenous angiotensin I

and II (L) to isotopically labelled angiotensin I and II (H) from the acquired mass spectra were plotted against the angiotensin I generation period (hours).

Antibody-Bead Conjugation: Protein G Dynabeads and polyclonal antibodies (St. Paul's Hospital) were conjugated as previously described (Chapter 2). A 5 µL volume of bead slurry was used in each reaction. The storage buffer was removed from the beads using a magnet to precipitate the beads, followed by three washes in 50 µL aliquots of 1X PBS. Beads were incubated with antibody for 1 hour at 4 °C, followed by three consecutive washes in 50 µL aliquots of 1X PBS. The beads were then distributed into blank, 37 °C generation samples, and standard tubes for immuno-affinity capture.

Calibration Curve: Calibration curves were produced by adding increasing amounts of synthetic angiotensin I (1 – 40 fmol/µL) and constant amounts of isotopically labelled angiotensin I (5 fmol/µL) as the internal standard to 20 µL volumes of plasma. Standard curves were produced for both the angiotensin I generation sample at 37 °C and the sample kept at 0 °C. Isotopically labelled angiotensin I was added to the 0 °C samples and 37 °C generation samples (5 fmol/ µL). Synthetic, endogenous, and isotopically labelled angiotensin I were co-captured with iMALDI methods.

Multiplexed iMALDI: A multiplexed assay with antibody-bound beads for angiotensin I and angiotensin II was used to determine the efficiency of enzyme inhibition during angiotensin I generation. Simultaneous capture of angiotensin I and angiotensin II was performed by incubating plasma with anti-angiotensin I and anti-angiotensin II

(BACHEM, Torrance, CA) bound to beads. These multiplexed iMALDI methods were developed by Randal D. Mason at the UVic Genome BC Proteomics Centre. Following iMALDI capture, beads were washed with 25 mM ammonium bicarbonate and spotted on MALDI plate for MS analysis. Mass spectrometry analysis was performed on an Applied Biosystems 4800 MALDI TOF/TOF instrument.

3.2.3 Plasma Renin Activity by Radioimmunoassay

Plasma renin activity assay by radioimmunoassay was performed in Dr. Dan Holmes' laboratory at St. Paul's Hospital, Vancouver, BC, following in-house standard operating procedures. Plasma was thawed rapidly at room temperature and immediately placed on ice. Polyclonal antibody against angiotensin I was added to 50 µL of plasma. Angiotensin I generation was performed at 37 °C for 1 hour while the blank samples remained on ice. Immediately following the generation period, ice-cold Tris-acetate buffer was added to the plasma to stop the enzymatic reaction. Calibration curves were created by adding increasing amounts of synthetic angiotensin I (Sigma-Aldrich) to 0.1M Tris acetate buffer with 1 % bovine serum albumin (BSA) and polyclonal antibody.

Following angiotensin I generation, radiolabelled angiotensin I (^{125}I-Angiotensin I, Amersham Biosciences) tracer was added to both plasma samples and angiotensin I standards in 1% BSA. Active competition between angiotensin I and radiolabelled angiotensin I occurred at 2-8 °C for 72 hours. Following incubation, 500 µL of cold, dextran-coated charcoal were added to the samples to separate free and bound antibody

complexes. The tubes were mixed and centrifuged, and the radioactivity in the supernatant (bound fraction) was measured using a gamma counter.

3.3 Results

3.3.1 Inhibition of PRA by EDTA and PMSF

Enzymatic action of renin resulted in increased levels of angiotensin I over time (Figure 13A and B). Angiotensin II levels were also shown to increase over an incubation period of 16 hours in EDTA plasma containing no additional inhibitors. Conversion of angiotensin I to angiotensin II was inhibited by adding PMSF to EDTA plasma. Constant baseline levels of angiotensin II indicate complete inhibition of ACE and plasma proteases over a 16 hour period. These results therefore demonstrate that iMALDI methods can be used for determining plasma renin activity when ACE and other plasma proteases are inhibited. In the presence of these inhibitors, the amount of angiotensin I measured increased indicating enzymatic conversion of angiotensinogen by renin.

3.3.2 Determination of Plasma Renin Activity

Correlation coefficients of 0.99 were obtained for calibration curves generated in triplicate for both 0 °C and 37 °C samples (Figure 14). The standard addition method was used to produce these calibration curves. Synthetic unlabelled angiotensin I was added to each sample at a concentrations ranging from 0.5 – 10 fmol/μL and the isotopically labelled version was added a constant concentration of 5 fmol/μL. The peak intensity of the light, or unlabelled peptide, was normalized to the peak intensity of the heavy, or isotopically labelled peptide. Linear equations were calculated for each curve,

with the y-intercept representing endogenous amounts of angiotensin I in the sample without any additional synthetic angiotensin I added. The same concentration ratios of light and heavy peptides were added to both 0 °C and 37 °C samples. Differences of 1 – 9 % were calculated for the slopes of the curves generated at the two different temperatures. The CV for the calculated slopes of the curves generated in triplicate at 0 °C was 6.8%, and 1.7% for the slopes calculated for the curves generated at 37°C.

PRA was measured in triplicate by iMALDI and RIA for eight different plasma samples. iMALDI calibration curves were generated for each experiment in samples incubated at 0 °C. Synthetic angiotensin I was added to each sample over a concentration range of 0 – 40 fmol/μL, while the isotopically labelled peptide was added at 5 fmol/μL. Correlation coefficients of 0.99 were calculated for the calibration curves generated for all eight samples (Figure 15). The concentration of angiotensin I was calculated in samples incubated at 0 °C and samples undergoing angiotensin I generation at 37 °C by extrapolation of the calibration curves. PRA was calculated by subtracting the mean angiotensin I concentration at 0 °C from the mean angiotensin I concentration at 37 °C. The results are summarized in Table 4. The normal range of PRA for the RIA protocol used in these experiments is 0.05 – 0.55 ng/L/s. The eight samples measured here fall within the normal range of this assay. CV's for samples incubated at 0 °C and 37 °C ranged from 2 – 34% and 4 – 19%, respectively. The PRA rates measured by iMALDI were higher than RIA for seven out of the eight samples assayed. CV's for PRA values were calculated based on triplicate values, and ranged from 5 – 35%, with CV's of $\leq$ 15% for six out of the eight samples. PRA values measured by iMALDI and RIA were

compared by linear regression. These data showed a correlation coefficient of 0.98 (Figure 16).

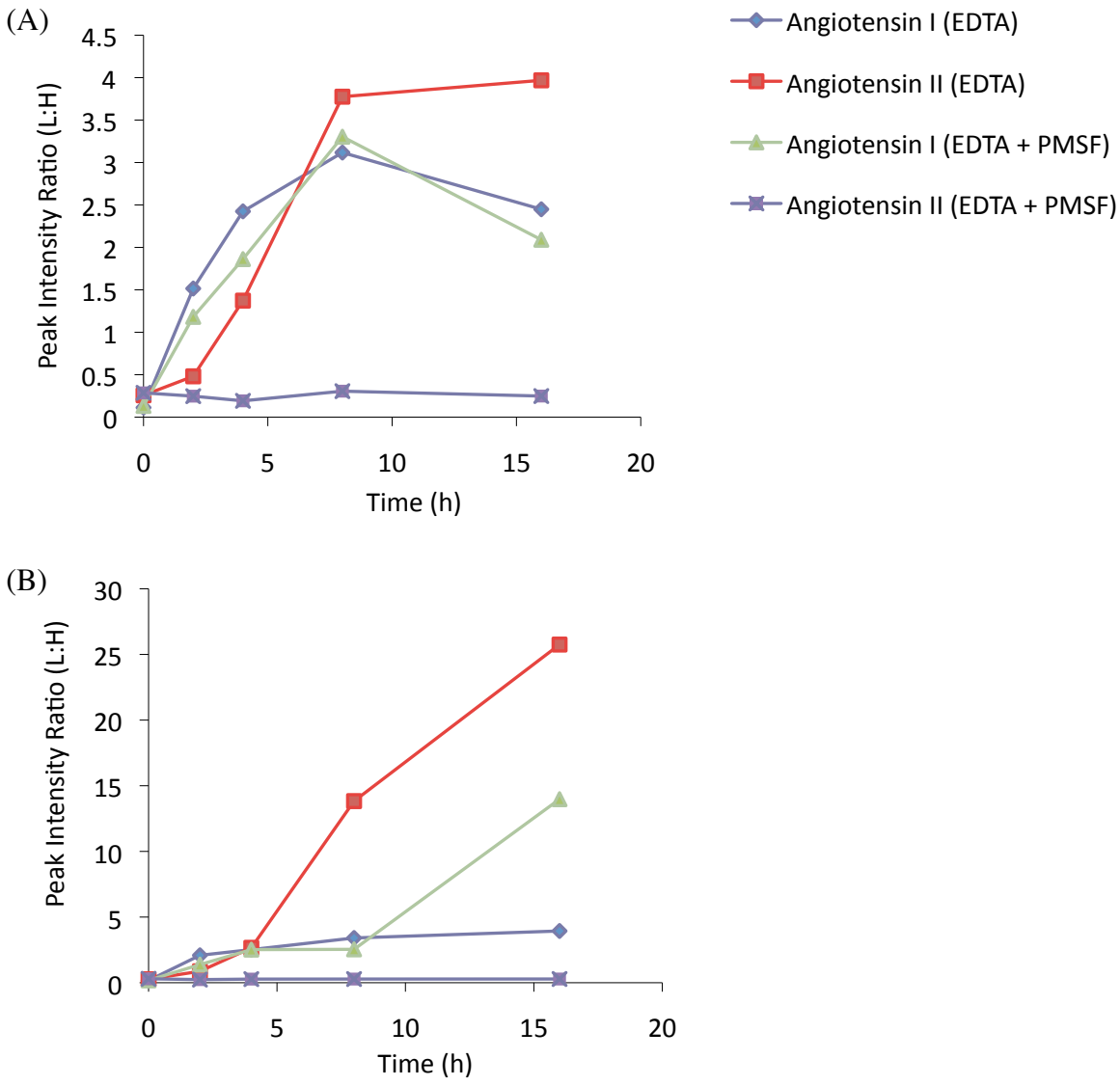


Figure 13. Inhibition of ACE and plasma proteases with EDTA and PMSF prevents angiotensin I degradation without effecting renin activity.

Angiotensin II levels increase over time as a result of degradation of angiotensin I to angiotensin II by plasma angiotensinases, as observed in EDTA plasma samples. With the addition of PMSF, degradation of angiotensin I is completely inhibited as indicated by baseline levels of angiotensin II. PRA values were determined by RIA: (A) 0. 090 ng/L/s and (B) 0.383 ng/L/s.

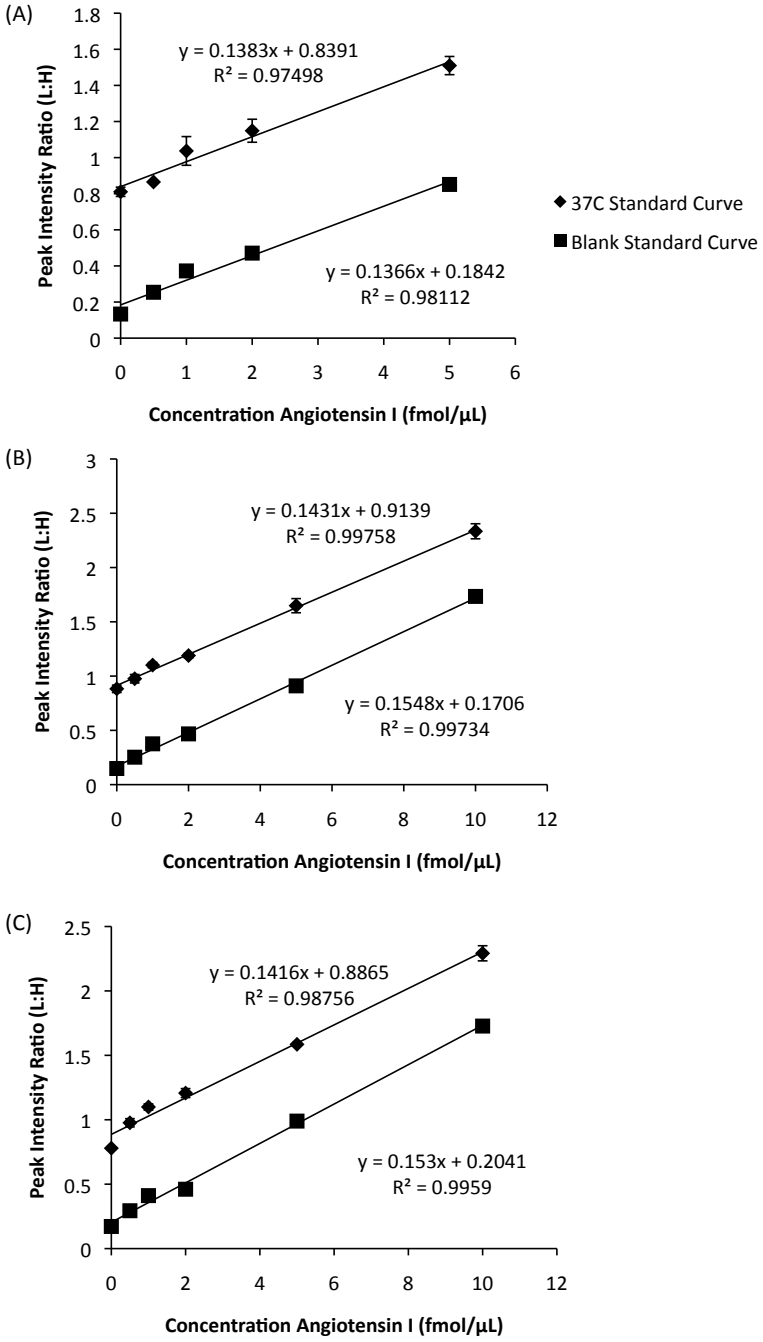


Figure 14. Triplicate analysis of calibration curves generated by iMALDI at 37 °C and 0 °C show a linear correlation.

Calibration curves were generated by adding varying ratios of light and heavy peptides to human plasma. Plasma was incubated at either 37 °C (indicated by ◆) or on ice at 0 °C (indicated by ■) followed by iMALDI capture and MS analysis. Correlation coefficients of 0.99 were achieved for all analyses; the difference between slopes at different temperatures was calculated at 1-8%; and CV <7%. Error bars represent standard error, however not all error bars are visible on the scale presented.

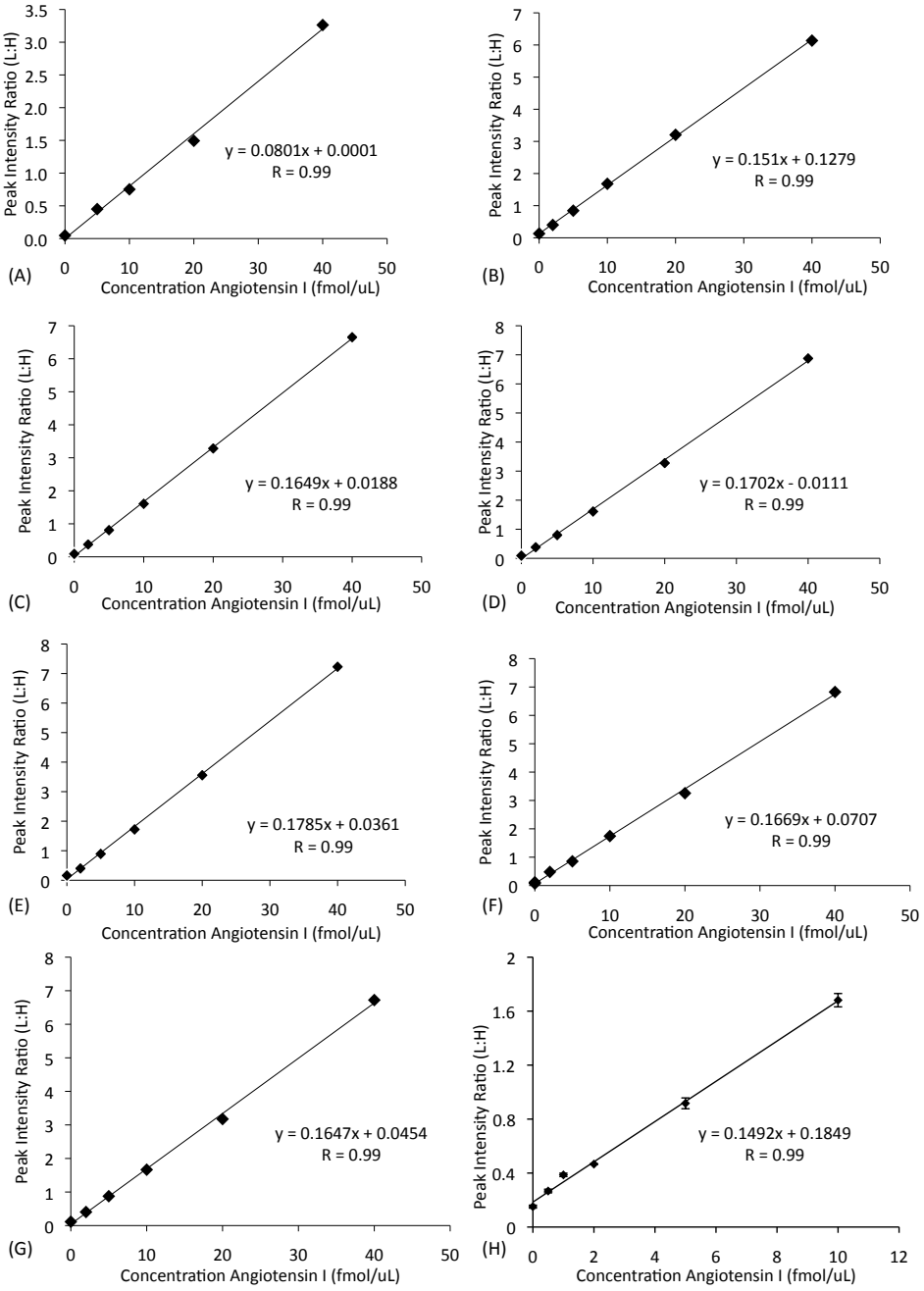


Figure 15. Measurement of PRA by iMALDI

Plasma from 8 different patients was incubated at 37 °C or on ice (0 °C) over a one hour period to determine PRA by iMALDI. Calibration curve samples were incubated on ice (0 °C), and concentrations of angiotensin I in 37 °C-incubated and 0 °C-incubated samples were used to calculate plasma renin activity. Correlation coefficients of 0.99 were calculated for each calibration curve.

Table 4. Comparison of mean PRA values determined by RIA and iMALDI

% CV for 0 °C Samples (n=3)	**% CV for 37 °C Samples** (n=3)	**PRA by RIA** (ng/L/s)	**PRA by iMALDI** (ng/L/s)	**% CV for Mean PRA by iMALDI**	**Blank Ang I Concentration** (fmol/μL)
1.8	5.4	0.16	0.1721	15.2	0.8737
2.1	4.3	0.48	0.3781	8.0	0.847
3.5	7.7	0.128	0.3985	8.5	0.114
34.1	8.3	0.488	0.8275	9.0	0.2022
12.8	6.4	0.936	1.792	5.8	1.3334
7.6	4.5	0.085	0.3955	4.7	0.0652
3.4	17.8	0.16	0.1617	34.6	0.4236
5.4	19.3	0.144	0.1908	29.3	0.2757

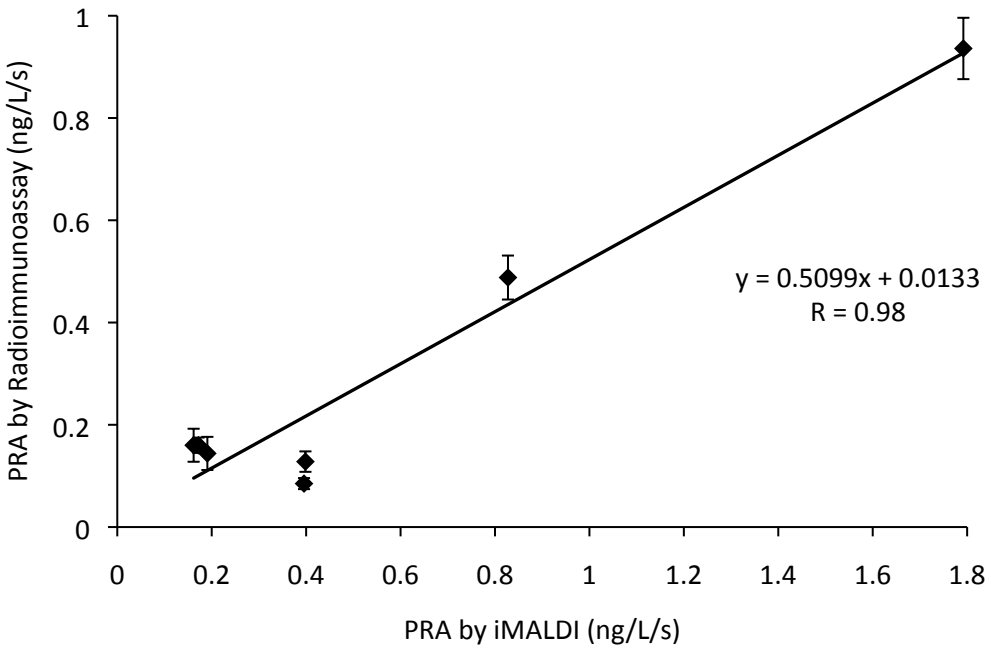
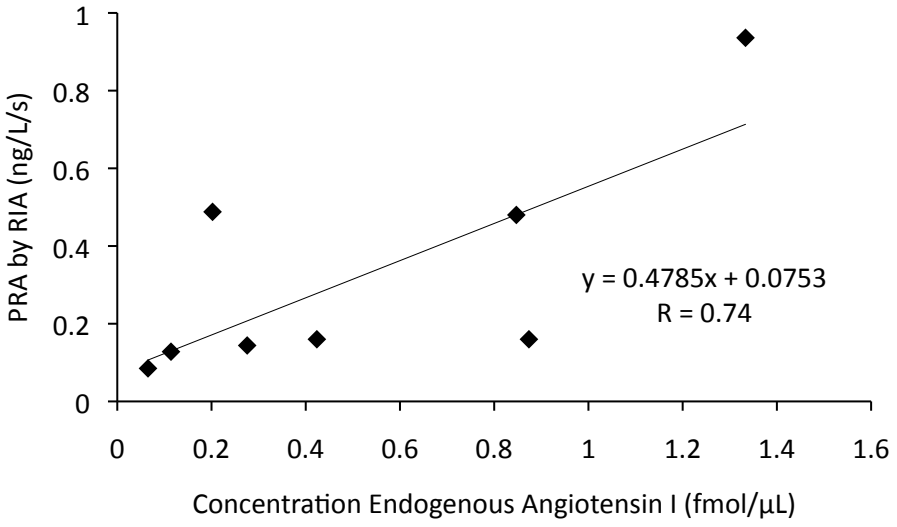


Figure 16. Comparison of PRA measurements by RIA and iMALDI

PRA was measured for 8 different plasma samples by RIA (n=2), performed at St. Paul's Hospital, and iMALDI, performed at the UVic Genome BC Proteomics Centre (n=3). One outlier was removed from the dataset, which had a lower PRA measurement by iMALDI than RIA (0.3781 ng/L/s and 0.48 ng/L/s, respectively). Error bars were calculated as standard error between triplicate PRA measurements.

To test our hypothesis, the concentration of angiotensin I in the 0 °C samples measured by iMALDI was compared to PRA values obtained by both iMALDI and RIA (Table 4, Figure 17). Correlation coefficients of 0.74 and 0.63 were calculated.

(A)



(B)

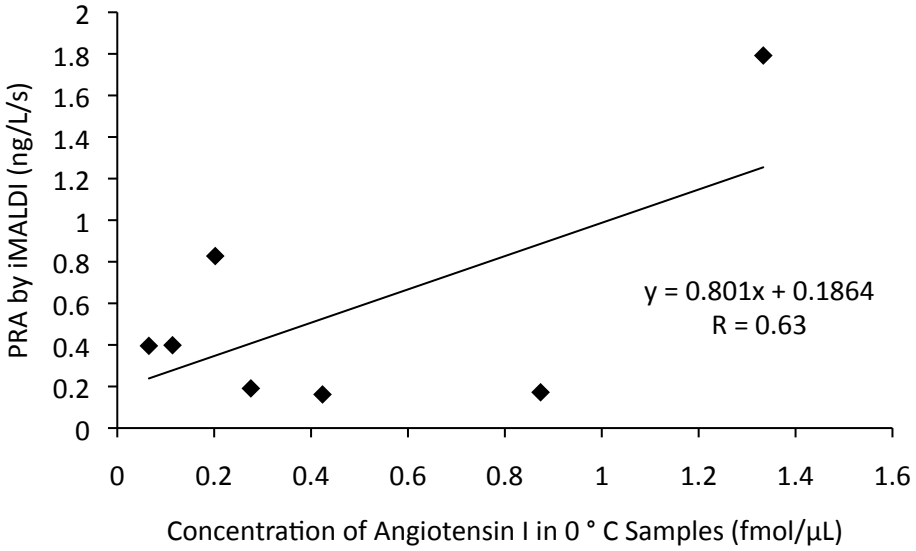


Figure 17. Comparison of endogenous angiotensin I concentration to PRA measured by RIA and iMALDI

Concentration of angiotensin I was determined in 0°C-incubated samples by iMALDI. Angiotensin I concentrations were compared to PRA values obtained by RIA (R = 0.74; A) and iMALDI (R = 0.63; B).

3.4 Discussion

Measurement of PRA by the developed iMALDI assay shows a linear correlation (R = 0.98) with PRA values obtained by RIA. PRA values calculated by iMALDI were higher in seven out of eight samples than PRA values determined by RIA, which may be a result of the use of different pH levels and enzyme inhibitors. Renin values are known to differ between methods as a result of the assay being carried out at different pH levels (Sealey, 1981). The iMALDI assay described here was developed at pH 6.0, while the PRA radioimmunoassay at St. Paul's hospital was performed at pH 7.4. The iMALDI assay was optimized at a lower pH for two reasons: 1) to maintain optimal conditions for renin and 2) to directly compare the developed assay to a commercially available radioimmunoassay kit produced by Diasorin (Vercelli, Italy), which uses a pH of 6.0 (manufacturer's protocol, Diasorin GammaCoat PRA RIA). Because of increased activity of renin at pH 6 compared to 7.4 (Sealey, 1981), higher PRA values would be expected at a lower pH. Another source of variation that may have contributed to the difference in PRA values obtained by RIA and iMALDI is the use of PMSF as an angiotensinase inhibitor in the iMALDI assay. In contrast, the PRA protocol performed by our collaborator's at St. Paul's Hospital includes the anti-angiotensin I antibody in the plasma sample during the angiotensin I generation period. Capture of angiotensin I by the antibody protects the peptide from degradation by steric hindrance (Poulsen, 1974). No additional inhibitors are added. Therefore, any angiotensin I that happens to dissociate from the antibody during the angiotensin generation I period may be degraded by proteases. In the iMALDI protocol, inhibitors are added to prevent degradation during the generation period.

By comparing the slopes calculated for calibration curves produced in plasma incubated at 0 °C and 37 °C, the reproducibility of the iMALDI assay was demonstrated. The only difference between the calibration curves was the incubation temperature. Therefore, linearity of the assay should be maintained during incubation, while the y-intercept would be expected to increase in samples incubated at 37 °C as a result of accumulation of angiotensin I. A difference ≤9 % between curve slopes at 0 °C and 37 °C demonstrates that angiotensin I can be measured in 0 °C and 37 °C samples from the same calibration curve. This is important because two separate standard curves are not required which reduces the time and reagent requirements for analyzing a single sample.

The reproducibility of iMALDI was demonstrated by generating calibration curves (R = 0.99) in plasma over concentration ranges corresponding to normal PRA patients (as determined by RIA). CVs of ≤ 15 % for 6 out of 8 samples demonstrate the assay's reproducibility. Interbatch CVs between 3.9 – 11.5 % have been reported for commercially-available radioimmunoassays for PRA (Cartledge, 2000). Lock (2006) suggests that an acceptable level of precision for radioimmunoassay is 8 – 12%. In these experiments, imprecision does not appear to be associated with low or high concentration values. Heterogeneity of MALDI matrix crystallization leading to so-called hot spot (Szájli, 2008) may have affected the reproducibility in these experiments, or other uncontrollable factors such as instrument laser intensity, bead deposition, or sample loss during the procedure. Further optimization of the current iMALDI protocol may improve

the assays reproducibility to within the suggested range. Such improvements may include the use of alternative detergents to prevent non-specific binding while minimizing suppression effects caused by the detergent and loss of beads during sample transfer.

The concentration of angiotensin I in samples incubated at 0 °C was determined by iMALDI, and compared against PRA values obtained by both RIA and iMALDI. Linear regression analysis resulted in a correlation coefficient of 0.74 for RIA and 0.63 for iMALDI. The sample size used here was limited (n=8) so it is difficult to determine whether a there is a correlation between endogenous angiotensin I and PRA, as hypothesized. A larger sample set may be useful in proving the hypothesis of this study, and determining whether endogenous angiotensin I concentrations correlate to PRA and may be useful for diagnostic purposes. In order to more accurately compare the two PRA methods, reference materials should be used. Lyophilized hypertension controls are available (Lyphochek Hypertension Markers, Bio-Rad) that include three clinically significant levels of renin which could be used to compare PRA values in both methods. An international standard for renin and angiotensin is also available (68/356) (Bangham, 1975) that could be used for method comparison.

Accurate measurement of PRA requires inhibition of proteases to prevent degradation of angiotensin I to angiotensin II by ACE (Sealey, 1991). Other proteases, such as cathepsin G, elastase, and protease can also cleave angiotensinogen to produce angiotensin I or II, as well as degrade angiotensin I and II (Ramaha, 2002). Kodish and

Katz (1974) have shown that complete inhibition of ACE and angiotensinases is achieved for up to 18 hours with EDTA and PMSF at pH 5.7. To assess the efficiency of protease inhibition, the generation of angiotensin I and its subsequent cleavage to angiotensin II by ACE and angiotensinases was measured using the developed multiplexed iMALDI methods. This multiplexed method allowed simultaneous measurement of angiotensin I and II production during a 16 hour period. Angiotensin II levels were seen to increase during the incubation period when the assay was conducted on plasma collected in EDTA tubes, without any additional enzyme inhibitors added. This confirms that conversion of angiotensin I to angiotensin II occurs in the presence of EDTA, as a result of proteases present in the plasma that are not inhibited by EDTA. By adding PMSF, angiotensin I degradation was inhibited over the 16 hour incubation period, without impacting renin activity. The developed iMALDI assay can therefore be utilized to measure PRA with a 16-hour angiotensin generation period. The significance of this is two-fold. By inhibiting angiotensin I degradation, we have demonstrated that the assay can be utilized for accurately measuring the rate of angiotensinogen conversion to angiotensin I. In addition, the sensitivity of the iMALDI assay can be greatly increased by extending the angiotensin I generation period. The work presented here incorporated a 1-hour generation period. Extension of the generation period would effectively increase the concentration of angiotensin I over time, thereby presenting the possibility of unlimited sensitivity for this assay.

Chapter 4

Future Directions

This study presents a novel method for measuring PRA by combining immuno-affinity enrichment and MALDI MS analysis. The assay was shown to provide a linear response over the concentration range for measuring low and normal rates of PRA, 0.06 – 0.42 ng/L/s (Locsei, 2009). With continued method development, it is feasible that the linearity could be extended to the range of high PRA, making the test useful for population-wide screening for primary hypertension where high levels of PRA would be expected. The sensitivity of the assay was determined to be 10-25 amol/μL in plasma. It is aso feasible that the linear range of this assay may be extended to a lower plasma renin activity range (<0.06 ng/L/s, equivalent to < 150 amol/μL in order to diagnose secondary hypertension in very small sample volumes (< 20 μL). Large-scale studies that aim to study specific diseases in blood-banked samples from several generations of subjects, such as the Framingham Heart Study (http://www.framinghamheartstudy.org/), often favour technologies that require very small amount of sample in order to maximize the research findings from these highly characterized cohorts.

This study also aimed to determine whether there is a relationship between endogenous angiotensin I concentrations prior to an angiotensin I generation period. This would eliminate the need for an incubation period during which renin enzymatically cleaves angiotensinogen. A direct assay would provide a faster method for diagnosing hypertensive diseases. The results presented here indicate that there is a correlation (R =

0.74 for RIA) between the initial angiotensin I concentration and PRA . Since a small sample size was tested here, analysis of a larger sample size may be needed to conclusively determine whether direct measurement of angiotensin I concentration in plasma might provide an alternative method for diagnosing hypertensive diseases. A larger sample size should also include patients with high and low PRA values

Additional future studies may focus on measurement of angiotensin II as a more diagnostically-relevant tool, since angiotensin II concentrations would provide a more direct measure of the RAAS (Sealey, 1981). In this study, multiplexed iMALDI methods that were developed outside of this thesis by Randal D. Mason at the UVic Genome BC Proteomics Centre, were used to simultaneous measure angiotensin I and II levels over a 16-hour incubation period. Therefore, an iMALDI assay for measuring both angiotensin I and angiotensin II concentrations is also feasible. A multiplexed assay would be beneficial for studying anti-hypertensive drugs such as renin and ACE inhibitors.

In order to advance the developed iMALDI assay to the clinical laboratory, reference ranges for low, normal, and high renin levels must be established. International standards are available for renin and angiotensin, which could be used to establish the precision of the assay. These standards would also provide reference values to which PRA values obtained by iMALDI and RIA could be compared.

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Appendix I

Product ions resulting from MS/MS fragmentation of angiotensin I and angiotensin II

	b_9	b_8	b_7	b_6	b_5	b_4	b_3	b_2
Angiotensin I	**1165.59**	**1028.53**	881.46	784.41	647.35	534.27	371.2	272.14
Angiotensin II			881.46	784.1	647.35	534.27	371.2	272.14
	y_9	y_8	y_7	y_6	y_5	y_4	y_3	y_2
Angiotensin I	**1181.66**	**1025.56**	926.49	763.43	650.34	513.28	416.23	269.16
Angiotensin II			931.51	775.41	676.35	513.28	400.2	263.14

Appendix II

List of Abbreviations

μ	micro
μL	microlitre
a	atto
ACE	angiotensin Converting Enzyme
ARR	aldosterone renin ratio
amol	attomol
CV	coefficient of variation
D	aspartic acid
Da	Dalton
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ESI	electrospray ionization
F	phenylalanine
f	femto
fmol	femtomole
FT-ICR	fourier transform ion cyclotron resonance
H	histidine

Hr	hour
I	isoleucine
iMALDI	immuno-MALDI
L	lysine
LC	liquid chromatography
M	molarity
m	mass
MALDI	matrix-assisted laser desorption/ionization
mg	milligram
mL	millilitre
mol	mole
MS	mass spectrometry
MS/MS	tandem mass spectrometry
n	nano
ng	nanogram
P	proline
p	pico
PBS	phosphate buffered saline
pg	picogram
PMSF	phenylmethylsulfonyl fluoride
ppm	part per million
PRA	plasma renin activity

Q	quadrupole
R	Arginine
r	Correlation coefficient
RAAS	renin angiotenin aldosterone system
RIA	radioimmunoassay
SELDI	surface-enhanced laser desorption/ionization
SIS	stable isotope standard
S/N	signal to noise
SISCAPA	stable isotope standards and capture by anti-peptide antibodies
TOF	time-of-flight
V	valine
Y	tyrosine
z	charge