Title: Multi-site method comparison of a high-throughput immuno-MALDI (iMALDI) plasma renin activity (PRA) assay with two current methods in clinical laboratory

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List of Abbreviations

Abbreviation	
2D	Two-dimensional
Ab	Antibody
ACE	Angiotensin-converting enzyme
ACN	Acetonitrile
ACTH	Adrenocorticotropic hormone
ADH	Antidiuretic hormone
AmBic	Ammonium bicarbonate
AngI	Angiotensin I
AngII	Angiotensin II
ARR	Aldosterone-renin ratio
AQUQ	Absolute quantification of proteins
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CT	Computed tomography
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionization
FA	Formic acid
FST	Fludrocortisone suppression test
HCCA	α-Cyano-4-hydroxycinnamic acid
HILIC	Hydrophilic-interaction chromatography (HILIC)
ICAT	isotope-coded affinity tags
iMALDI	immuno-MALDI
iTRAQ	Isobaric tags for relative and absolute quantification
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
m/z	Mass-to-charge ratio
MALDI	Matrix-assisted laser desorption/ionization
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NAT	Natural form
PA	Primary aldosteronism
PAGE	Polyacrylamide gel electrophoresis
1xPBS	Phosphate buffered saline
1xPBSC	1xPBS with 0.015% CHAPS
PMSF	Phenylmethanesulfonyl fluoride
PRA	Plasma renin activity
RAAS	Renin angiotensin aldosterone system
RIA	Radioimmunoassay
RP	Reversed-phase
S/N	Signal-to-noise
SDS	Sodium dodecyl sulfate
SD	Standard deviation
SILAC	Stable isotope labeling in cell cultures

SIS	Stable isotope-labeled internal standard
SISCAPA	Stable isotope capture by anti-peptide antibodies
SIT	Saline infusion test
SPE	Solid phase extraction
TFA	Trifluoroacetic acid
TOF	Time of flight

Abstract

Primary aldosteronism (PA) is by far the most common form of secondary hypertension, accounting for approximately 10% of all hypertensive patients. Clinical laboratories play a critical role in the screening and diagnosis of primary aldosteronism, as early detection is associated with a reduction in cardiovascular risk and improved patient outcomes.

The goal of this project was to optimize and validate a novel iMALDI PRA assay against two current clinical methods using clinical samples obtained for secondary hypertension workup.

The high-throughput iMALDI PRA assay showed excellent correlation with two currently utilized clinical methods for PRA determination. Regression analyses showed R^2 value > 0.92 and > 0.95, when the iMALDI PRA assay was compared to the clinical DBC ELISA and LC-MS/MS assays respectively. The iMALDI PRA method also demonstrated excellent robustness on two MALDI-TOF instruments from different manufactures. Ninety-Six (96) clinical samples were quantitated by high-throughput iMALDI PRA assay using both the Bruker Microflex and the AB Sciex 4800, yielding an R^2 value > 0.99.

Clinical implementation of the high-throughput iMALDI PRA assay will also require accuracy assessment and cost-effective study. Through optimization of reagents, protocols, robotic systems, and software, the iMALDI platform for PRA determination has been automated into a robust, and user-friendly diagnostic platform applicable for use in clinical laboratories.

Résumé

L'hyperaldostéronisme primaire (PA) est de loin la forme la plus fréquente de l'hypertension secondaire. Ceci représente environ 10% de tous les patients souffrant d'hypertension. Les laboratoires cliniques jouent un rôle essentiel dans le dépistage et le diagnostique d'hyperaldostéronisme primaire. La détection précoce d'hyperaldostéronisme primaire est associée à une réduction du risque cardio-vasculaire et à la diminution des conséquences néfastes pour les patients.

Ce projet a pour objectif d'optimiser et de valider un nouveau test nommé iMALDI PRA, en comparaison de deux méthodes utilisées présentement en clinique. Le projet a été réalisé avec des échantillons cliniques obtenus pour l'évaluation de l'hypertension secondaire.

Le dosage à haut débit iMALDI PRA démontre une excellente corrélation avec deux méthodes utilisées présentement en clinique pour l'analyse PRA. Les analyses de régression ont démontrées la valeur $R^2 > 0.92$ et > 0.95, lorsque le dosage iMALDI PRA a été comparée aux méthodes DBC ELISA et LC-MS / MS respectivement. La méthode iMALDI PRA a également démontré une excellente reproductibilité sur deux instruments MALDI-TOF de différents fabricants. Quatre-vingt-seize (96) échantillons cliniques ont été quantifiés par dosage à haut débit iMALDI PRA en utilisant à la fois l'appareil Bruker Microflex et l'appareil AB Sciex 4800, ceci a donné une valeur $R^2 > 0.99$.

La mise en œuvre clinique du dosage à haut débit iMALDI PRA exigera également une évaluation de l'exactitude ainsi qu'une évaluation du coût-efficacité. Grâce à l'optimisation des réactifs, des protocoles, des systèmes robotisés et des logiciels, la plate-forme iMALDI pour l'analyse PRA est maintenant applicable pour une utilisation dans les laboratoires cliniques car elle est facile à utiliser, reproductible et automatisée.

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Preface & Contribution of Authors

This dissertation is original, unpublished work by the author, Michael Chen.

1. Introduction

Clinical proteomics, a field that has been exponentially growing (1), encompasses all areas of translational proteomics that involve the application of proteomic technologies on clinical specimens. The ability to accurately quantitate variations in protein expression in response to both physiological and pathological changes can play a pivotal role in clinical diagnostics (2).

Most disease-specific protein markers that are currently being measured in clinical laboratories are developed based on the principles of immunoassays with colorimetric, fluorescent, or radioactive detection methods (3).

Conventional sandwich immunoassays uses two specific, independent antibody binding sites on the respective antigen for binding and detection. Such technique only allows for detection and quantitation of single proteins. These assays always bear the risk of cross-reactivity with heterophile antibodies, or auto-antibodies, both of which can be very difficult to detect and negatively affect clinical decision making.

More importantly, proteins can exist in isoforms due to, for example, mutations or post-translational modifications, which might lead to functional differences. Nedelkov et al has previously analyzed 25 serum proteins of 96 healthy donors, and revealed a number of 76 different structural variants (4). In case of such structural changes, recognition by antibodies may or may not be complete. Discrimination and quantification of different isoforms cannot be achieved. Therefore, the method of choice should employ technology to detect and discriminate peptide and small protein isoforms.

The development of such methods for accurate protein detection and quantitation for clinical diagnostics is currently a challenging area of clinical proteomics. Mass spectrometry (MS)-

based proteomics has made significant improvements in performance by offering high sensitivity, specificity and reproducibility.

As an analytic technique in laboratory medicine, MS can achieve absolute specificity through accurate molecular weight determination and peptide sequencing in particular in the lower molecular weight range. It successfully resolves the concern of antibody cross-reactivity, hence offering a direct benefit to patient care by preventing misdiagnosis. In the aspects of laboratory safety, radioactive isotopes are not required, making MS a safer option.

In recent years, enormous amount of time and resources has been invested in clinical laboratories to setup mass spectrometers, mainly Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) in areas such as toxicology, endocrinology, and biochemical genetics (5). Although this approach is now considered as the “reference method” for many clinical assays, the utility of LC-MS/MS requires considerable amount of expertise in chromatograph and MS. Many clinical laboratories are still hesitate from implementing this technique because of its technical demands (6).

Matrix-assisted Laser Desorption/Ionization (MALDI) Time of Flight (TOF) mass spectrometer is a technically simple method that has been widely implemented in clinical microbiology laboratories for the rapid, inexpensive and accurate speciation of microorganisms (7-9).

In comparison to immunoassays such as an enzyme-linked immunosorbant technique (ELISA), the main drawback of MALDI-TOF MS is its detection limit which ranges in the lower femtomole and higher attomole range. Currently, ELISA still offers higher assay sensitivity (in lower attomole range) which is often required in biomarker quantification, especially when used as a screening tool in early stages of disease. But most importantly, in the field of clinical

routine diagnostics, all clinical assays must meet regulatory requirements (eg. CLIA) for specific and sensitive detection of the analyte of interest. Therefore, MALDI-TOF MS is not yet an established technique for clinical routine diagnostics, and still requires further assay modification and optimization.

An immune-MALDI (iMALDI) assay for the quantification of plasma renin activity (PRA), a biomarker used for screening and diagnosis of primary aldosteronism (PA), has been developed at the University of Victoria (UVic)-Genome BC Proteomics Centre. The principle behind iMALDI is immuno-enrichment of target analyte by using specific auto-antibodies to increase assay sensitivity. This novel assay has automated sample preparation with a liquid handling system to increase throughput as the basis for clinical validation and implementation.

Primary aldosteronism (PA) is a form of secondary hypertension, characterized by a dysregulation of renin-angiotensin-aldosterone system (RAAS) causing overproduction of aldosterone in a low renin state. Primary aldosteronism accounts for an estimated 10% of all hypertensive subjects (10). Clinical laboratory plays a critical role in the screening and diagnosis of primary aldosteronism by quantification of PRA, as early detection is associated with cardiovascular risk reduction and better patient outcome. While Enzyme-linked Immunosorbent Assays (ELISAs) are widely available to measure PRA, the reproducibility of PRA among different laboratories is far from being optimal (11). As mass spectrometry based assays are becoming more prevalent, the high-throughput iMALDI-based approach for PRA determination has drawn attention of clinical laboratorians.

This thesis summarizes the efforts toward clinical implementation of the high-throughput iMALDI PRA assay for secondary hypertension workup. The project therefore had the following objectives:

1. Optimization of the existing automated sample preparation protocol to meet requirements in clinical laboratories.
2. Validation of iMALDI PRA assay robustness with two MALDI TOF instruments form different manufacturers.
3. Validation of iMALDI PRA assay against two current clinical methods using clinical samples obtained for secondary hypertension workup.

2. Theoretical Background

2.1 Proteome and proteomics

The term ‘proteome’ refers to the entire complement of PROTEins that is expressed by the genOME (Wasinger et al. 1995). While the genome remains relatively static over the lifespan of a living organism, the proteome, in response to internal and external stimuli, is highly dynamic. Therefore, the proteome represents both genomic expression and post-translational modifications (PTMs) of proteins.

Based on the central dogma of molecular biology (12), that the sequential information is transferred from deoxyribonucleic acid (DNA) by transcription via messenger ribonucleic acid (mRNA) and then by translation to proteins.

The study of proteomics involves the systematic identification and quantitative study of proteins and protein variations on a large scale that includes protein modifications, interactions, activities, and functions (13).

2.2 Selection of sample type

Proteomics experiments can be carried out in biological samples of different origin, and complexity. However, the analysis of proteins from blood, urine, body fluids, or tissues can vary widely in nature and concentration.

2.3 Analytical methods for sample fractionation

Separation and isolation of proteins from a complex biological sample is often a very difficult task, and one of the challenges in proteomics is to find the most efficient protocols for different sample types. The range of protein concentration in human plasma covers over 10 orders of magnitude, with the 22 most abundant proteins accounting for approximately 99% of total protein mass (14). Detection and quantification of low abundance analytes require simplification of the biological mixture by sample fractionation to achieve sufficient sensitivity.

Analytical methods for sample fractionation can be characterized into the following categories: chromatography, gel electrophoresis, and particle-based immuno-enrichment.

2.3.1 Chromatography

Chromatographic methods are often used in a multidimensional fashion for the separation of proteins. Depending on the approach, dimensions are based on different separation mechanisms. Commonly the choice falls on affinity, ion exchange, partition, adsorption, or size exclusion, as they may offer higher selectivity and resolution of complex protein mixture. Immuno-affinity chromatography uses antibody-containing columns, usually against the 14 most abundant proteins present in plasma, to deplete high abundant proteins from the protein mixture.

Two-dimensional (2D) chromatography is a type of chromatographic technique in which the sample mixture is separated by passing through two different separation stages. The two stages

employ columns that have a different separation mechanism, so that elute that are poorly resolved from the first column may be further separated in the second column. Hydrophilic-interaction chromatography (HILIC) and affinity chromatography, as well as Strong cation-exchange (SCX) and reversed-phase (RP) chromatography are routinely used 2D fractionation methods (15), which can be applied in conjunction with immune-depletion (16).

Solid phase extraction (SPE) is a quick method for concentrating and purifying target analytes without the need of columns, and is typically coupled with liquid LC-MS analysis (17).

2.3.2 Gel electrophoresis

The one-dimensional (1D) technique, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), is widely used in the separation of proteins by gel electrophoresis (18).

The solubilizing agent sodium dodecyl sulphate (SDS) has high affinity for proteins, and promote denaturation. Protein mixture separated by SDS-PAGE migrate according to their molecular weight, irrespective of charge. Two dimensional (2D) SDS-PAGE was developed to increase the resolution of protein separation (19, 20) for large-scale proteome research. The combination of two separation methods, isoelectric focusing (IEF) and SDS-PAGE allow protein separation by size and charge.

The resolution of SDS-PAGE is insufficient in terms of separation for large-scale proteome research. Other separation technique used to be carried out before the SDS electrophoresis. However, the protein identification is still limited by analysis of their co-migration proteins (21). Recent advancement in technology allows coupling of SDS-PAGE with LC-MS by in-gel digestion of protein bands, fractionation by LC and mass analysis by MS (22).

2.3.3 Immuno-enrichment

Selective enrichment of target peptides or proteins from complex sample mixture can be achieved by antibody capture (23). Two current methodologies that use anti-peptide antibodies are stable isotope capture by anti-peptide antibodies (SISCAPA) (24) and immuno-MALDI (iMALDI) (15, 25). In both cases, specific anti-peptide-antibodies have to be generated, which are anchored to magnetic beads for immuno-capture.

One of the major challenge of antibody-based method is cross-reactivity in the presence of heterophile antibody (26). This interference can lead to falsely elevated or depressed results depending on the nature of the interference involved and the assay format employed (27).

The coupling of immune-enrichment with MS-based method has effectively overcome this challenge by allowing identification and quantitation of analytes based on their specific mass-to-charge (m/z) ratio.

The use of magnetic bead-based immuno-enrichment followed by MALDI-MS analysis can be multiplexed to quantitate more than one analyte in the same run with the use of various types of specific antibodies (28).

Recently, protocol for sample preparation and MS analysis was developed with automated liquid handling system to allow robust, high-throughput assay (29) with the potential for clinical implementation.

2.4 Mass spectrometry in proteomics

Mass spectrometry (MS) is an analytical technique that separates ions in gas phase according to their mass-to-charge (m/z) ratio in a mass analyzer and records the number of ions at each m/z value by detector. Prior to mass analysis, molecules are ionized by an ion source, and

converted into gas. The use of MS-based methods, at its early stages, was mainly limited to chemistry as the technology lacked efficacy in analysis of large biomolecules such as peptides and proteins.

Each mass spectrometer is composed of three main components: an ion source ionize and transfer sample molecules ions into a gas phase, a mass analyser that separate molecules depending to their mass, and finally a detector to record separated ions.

The challenge was to ionize the biomolecules that are thermally labile and non-volatile without any fragmentation. The first ionization techniques that allowed MS detection of biomolecules were fast atom bombardment (30), plasma desorption (31) and thermospray (32). Although these techniques translated into successful proteomic studies (33-35), large quantities of samples were required for such MS analysis.

In the late 1980s, two ionization methods MALDI (36) and ESI (37) were developed that led to an important breakthrough in the field of proteomics. Both ion sources when connected to adequate mass analyser achieved significant sensitivity increase in the analysis of peptides and proteins (to femtomole level). Furthermore, polypeptide analysis with masses up to 100 kDa was proven possible with these new techniques (38, 39).

Mass analyzers, such as quadrupole, ion trap, Fourier-transform ion cyclotron resonance (FT-ICR), Orbitrap and TOF, have become very prevalent in proteomic research. Mass spectrometers may be equipped with more than one mass analysers in tandem, for example triple quadrupole, quadrupole-ion trap and tandem TOF to allow better mass resolution and specificity (40).

In tandem-MS, fragmentation spectra of the analyte of interest are produced by tandem mass spectrometer which contains two mass analysers. The tandem-in-space MS is performed by triple quadrupole, quadrupole-quadrupole-TOF and tandem TOF instruments. The first mass analyser selects and separates a precursor ion of interest which then enters a collision cell for fragmentation. The products of fragmentation are transferred to a second analyser for mass analysis.

The second type of tandem-MS is tandem-in-time MS, where precursor ion selection, fragmentation, and product ion analysis all occur in one mass analyser (quadrupole ion trap, ion trap, Fourier transform ion cyclotron resonance mass spectrometer) but sequentially in time.

Analyte fragments are generated by low energy collision-induced dissociation (CID), a method involving collisions of precursor ions with atoms of an inert gas such as helium or argon in a collision cell. In the case of peptide analysis, cleavage at amide bond occurs with CID to generate specific peptide fragments.

2.5 Quantitation strategies in Proteomics

Expression proteomics, in response to internal or external stimuli, relies upon accurate quantitation methods. Quantitation strategies for proteomics can be categorized into two major categories: gel-based and MS-based quantitation methods.

2.5.1 Gel-based quantitation

In gel-based quantitation, commonly 2D-PAGE, visualization or protein migration using various staining procedures (e.g. silver or Coomassie blue dyes) allow relative protein quantitation by gel-to-gel comparison (14).

2.5.2 Mass Spectrometry-based quantitation

In mass spectrometry, the approaches to protein quantitation can be either relative or absolute. Measurement of analyte is performed by examining the relative signal intensity between mass-shifted peptides originating from the specimen and control samples. They can be categorized as label-free quantitation and quantitation with stable isotope labels.

Label-free MS-based quantitation approaches are less costly, but these methods usually result in less accurate quantitative results and low reproducibility (41). MS-based quantitation can also be achieved using stable isotope labels that can be incorporated metabolically, chemically or enzymatically (42).

Stable isotope labeling with amino acids in cell culture (SILAC) is a metabolic labeling approach using growth medium with isotope enriched amino acids (e.g., lysine with enriched 13C) (43).

Alternatively, common chemical labeling techniques such as isotopic or isobaric labeling can be performed following protein extraction. Two most common strategies are isotope-coded affinity tags (ICAT) and isobaric tag for relative and absolute quantitation (iTRAQ).

ICAT allows comparison of samples with differential labeling of cysteine residues using heavy and light isotopic carbon atoms (22). Therefore, ICAT is only applicable for proteins or peptides with cysteine-residues.

In iTRAQ, samples undergo labeling of free amines with an isobaric reagent. Quantitation is performed by MS/MS analysis comparing reporter ions released from the isobaric reagent in the fragmentation spectra (42). iTRAQ requires instrument with MS/MS capability and appropriate software support.

A targeted approach called "absolute quantification" (AQUA) of proteins or peptides uses the addition of known amounts of stable-isotope containing analogues of the peptides of interest to the sample. These peptides, often called AQUA peptides or stable-isotope labeled internal standard peptides (SIS peptides), have identical physical properties as the endogenous peptide. The comparison of signal intensities between endogenous and SIS peptides by MS analysis allows absolute quantitation. SIS peptides are commonly used in MRM (44) experiments and in SISCAPA (24) and iMALDI (25) techniques.

2.5.3 Principle of MALDI-TOF MS and Immuno-MALDI (iMALDI) TOF MS

In Matrix Assisted Laser Desorption/Ionization (MALDI) MS, the sample is uniformly mixed and embedded in a matrix that will crystallize on a metal plate. A laser pulse is applied in the matrix, and the molecules are then desorbed and ionized by charge transfer by absorbing the laser energy (figure 1).

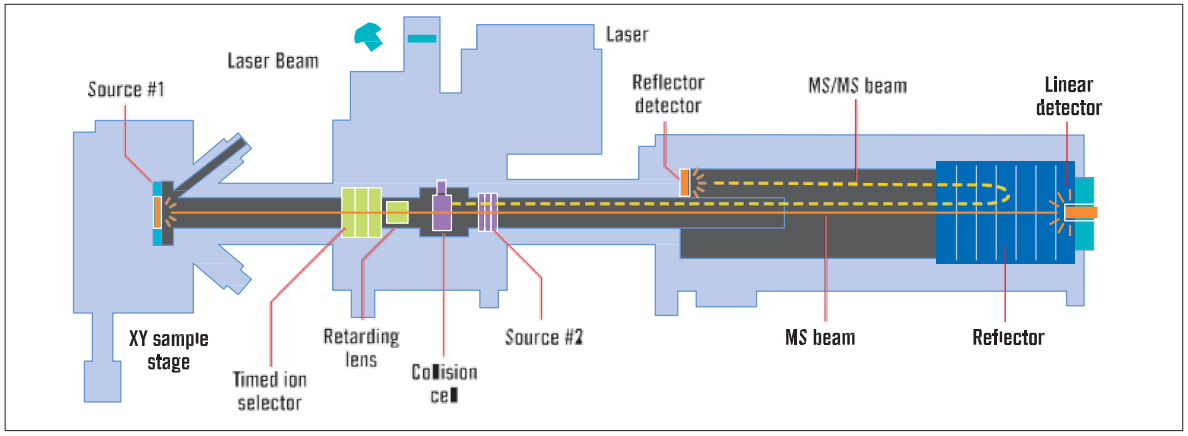


Figure 1. The MALDI-TOF/TOF mass spectrometer (Applied Biosystems 4700 Proteomics Analyzer)

The matrix is composed of chemicals that have a strong optical absorption in the range of the laser wavelength used for ionization. The choice of matrix highly depends on type of laser used and the target biomolecule (figure 2). The molecules are then both desorbed and ionized by charge transfer by absorbing the energy of a short laser pulse. Finally, the desorbed and ionized molecules are accelerated in an electrical field through a metal flight tube and collide with a

detector at the end of the flight tube. Lighter ions and more highly charged ions move faster through the flight tube to reach the detector. Consequently, biomolecules are separated according to their mass-to-charge ratio (m/z ratio), and a mass spectrum is created by both the mass and the intensity of the ions (45, 46).

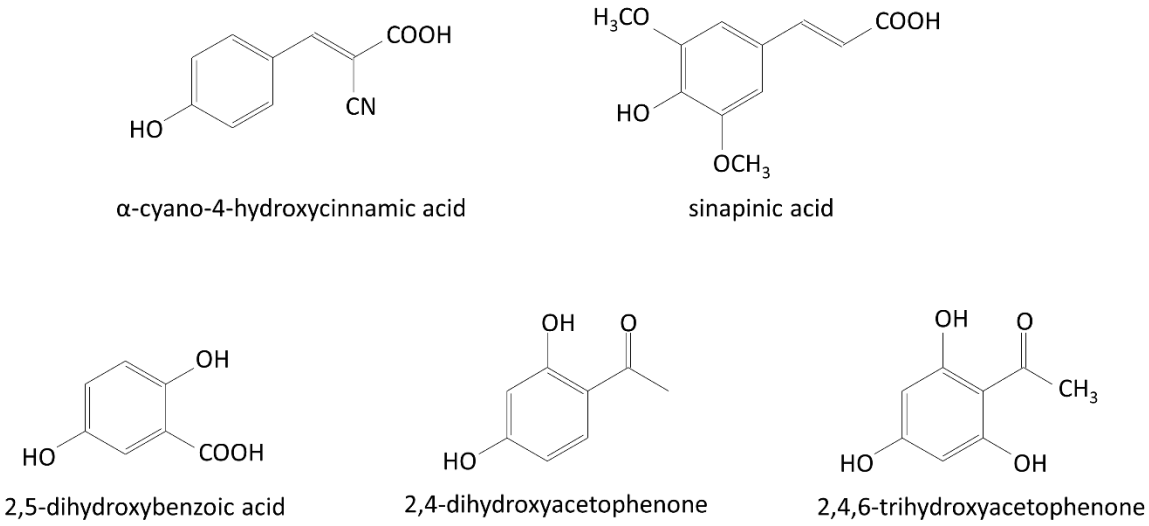


Figure 2. Structural formulas of MALDI matrices

The immuno-MALDI (iMALDI) technique couples antibody affinity capture of target analyte with MALDI MS analysis, hence, combing their unique technical advantages (47). The immune-enrichment of the analyte of interest increases the overall sensitivity and specificity of the assay (48). Very small sample volume (in this case, 1 µl) is required. Absolute quantitation is accomplished by the addition of Stable-isotope labelled Internal Standard (SIS), which are synthesized to have identical chemical properties as the analyte of interest but only slightly differ in m/z ratio. This ensures antibody (bound to beads) co-capture of both SIS standards and target analyte, whose concentration can then be quantified from its relative peak heights/areas.

The overall workflow of an iMALDI assay for the immune-capture of peptides can be summarized into the following steps (Figure 3):

1. Bead-antibody conjugation: Anti-target antibodies are conjugated with affinity beads.
2. Affinity capture of target peptides and stable isotope-labeled analogues: Incubation of digested or non-digested sample with SIS peptides of the target peptides, and bead-antibody conjugates.
3. Washing and spotting of beads onto MALDI target: Following the incubation period, bead-antibody-peptide conjugates are washed with wash buffer to remove excess peptides and plasma residues. After re-suspending the beads in a wash buffer, the beads are directly spotted onto a MALDI target.
4. Elution of peptides by acidic matrix application: After the spots are dry, an acidic matrix solution is added which elutes the peptides from the bead-antibody conjugates.
5. MALDI analysis

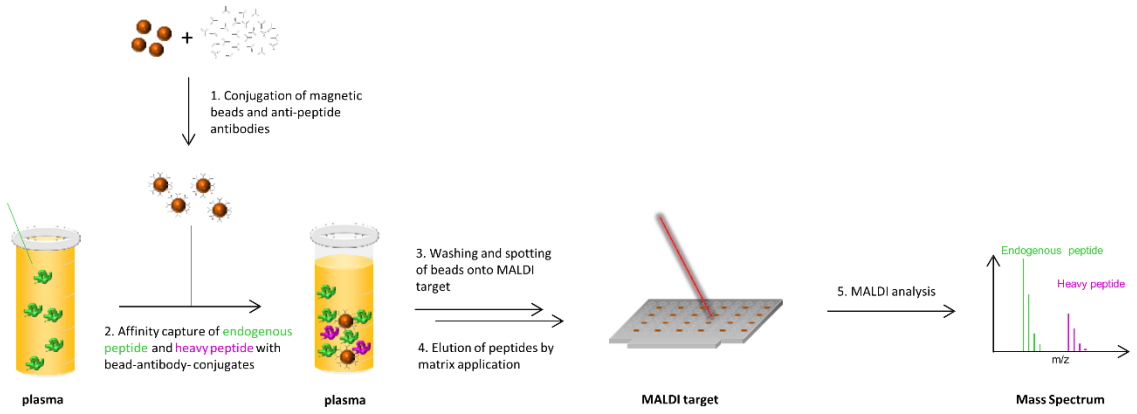


Figure 3. iMALDI workflow

3. Primary Aldosteronism

3.1 Overview

Hypertension has become a world-wide epidemic affecting more than 1 billion people and causing 7.1 million deaths per year (49). Untreated hypertension significantly increases one's risk of developing cardiovascular diseases, stroke, and chronic renal impairment. Early diagnosis of hypertension and identification of secondary causes are therefore critical to the prevention and treatment of its complications. Primary aldosteronism (PA), first described by Dr Jerome Conn in 1950s (50), is the most common type of secondary hypertension, characterized by a dysregulation of renin-angiotensin-aldosterone system (RAAS). Primary aldosteronism accounts for an estimated 10% of all hypertensive subjects, which comes to approximately 100 million people worldwide (10).

3.2 The renin-angiotensin-aldosterone-system

The renin-angiotensin-aldosterone-system (RAAS) is a coordinated enzymatic cascade in the regulation of blood pressure, and sodium-potassium homeostasis (51, 52). Stimulated mainly by low blood pressure, the kidneys release the proteolytic enzyme, renin. This triggers a signal transduction pathway whereas renin cleaves its substrate angiotensinogen, a protein synthesized by the liver, to produce angiotensin I (AngI). AngI is then converted to angiotensin II (AngII) by angiotensin-converting enzyme (ACE). An overview of the key components in the RAAS is shown in Figure 4.

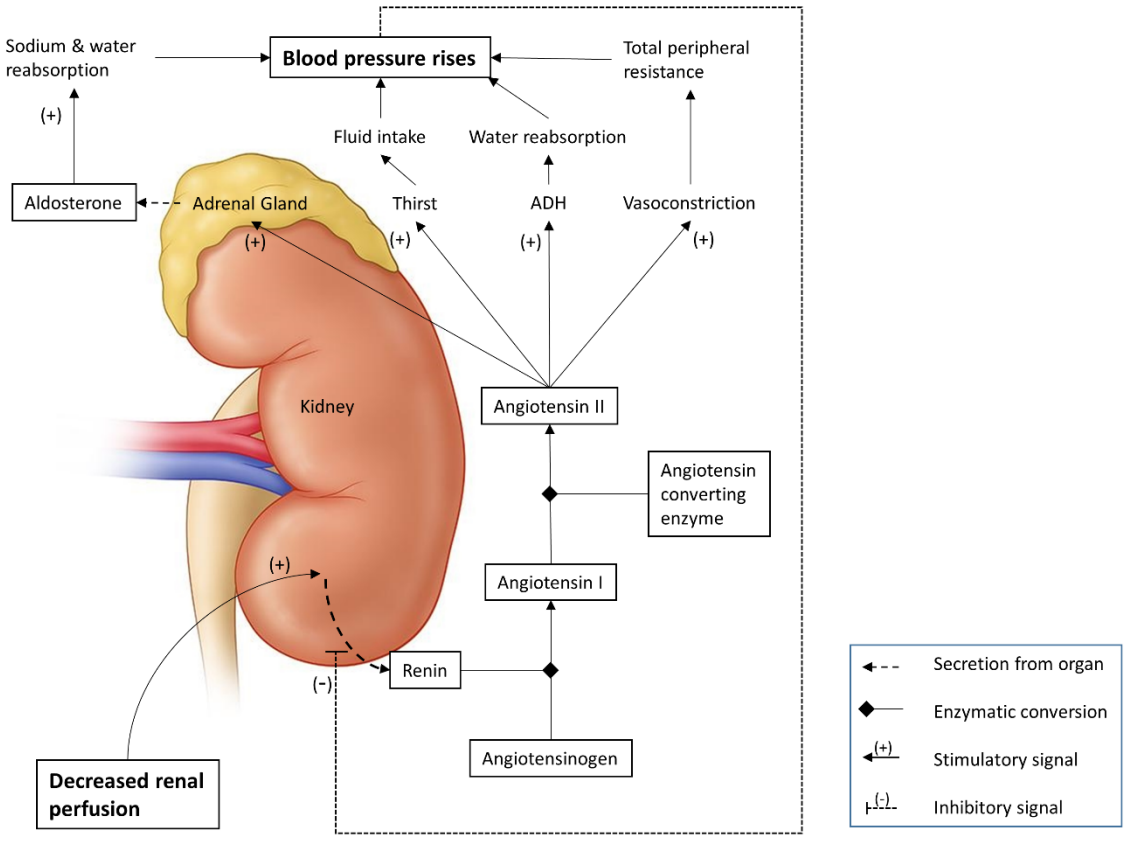


Figure 4. Overview of Renin-Angiotensin-Aldosterone System

Angiotensinogen (AGT)

Angiotensinogen belongs to the superfamily of Serpin A8 proteins, which are protease inhibitors and related proteins. The signature structural elements of serpins consist of 3 β sheets and 8 to 9 α helices (53). It is the only known substrate of renin which cleaves a 10 amino acid peptide from its N-terminus, and converts it to AngI (54). Renin has a fourfold higher catalytic activity when Agt is in its oxidized form because such conformation allows better access for cleavage (55).

Agt is a heterogeneous plasma glycoprotein mainly produced in the liver. The heterogeneity of this 452 amino-acid protein is primarily due to variable glycosylation (56). The half-life of Agt in the circulation has been estimated to be approximately 5 hours from iodinated-tracer studies in animal models (57, 58). While most Agt is mostly synthesized in the liver, other tissues have been reported to produce Agt including adipose tissue, brain, spinal cord, heart, kidney, lung, adrenal gland, large intestine, stomach, spleen, ovaries, and blood vessels (59-62). Additionally, it has been suggested that individual tissue compartments regulate local Agt levels independently of the circulatory RAAS (63-66).

Changes in plasma concentration could have significant influence on the rate of AngI generation at any given level of renin (67). Estrogens induce AGT gene transcription in the liver (68). Plasma Agt levels elevate during pregnancy and during administration of synthetic estrogens such as oral contraceptive pills (69). Genetic studies also showed the association of molecular variant (M235T) of the AGT gene and preeclampsia (70). One clinical study was able to show that patients with preeclampsia had higher level of oxidized Agt, which might contribute to hypertension (55).

Renin

Renin is an aspartyl protease mainly synthesized by the juxtaglomerular cells of the kidney, where its expression and secretion are tightly regulated by renal baroreceptor (71, 72) and sodium chloride (NaCl) delivery to the macula densa (73-75). These sensing mechanisms allow incremental titration of circulatory renin levels in response to changes in blood pressure and salt balance. Renin expression and release at the cellular level is controlled by three main intracellular mediators: cAMP, cGMP, and Ca2+.

Renin in circulation exist in two forms, as renin and prorenin, its precursor which is biologically inactive. The renin gene is highly conserved, and homologs have been identified species other than human such as mice, rats, dogs, and zebra fish. Humans have a single renin gene that spans 12 kb of DNA on chromosome 1 and contains 10 exons separated by 9 introns (76). The transcript encodes the protein pre-pro-renin with 460 amino acids (77). Removal of the 23 amino acid pre-segment from the C-terminus of pre-pro-renin generates pro-renin. Active renin is generated by removal of pro-segment N-terminal peptide by proteases in the juxtaglomerular cells of the kidney (78). The physiological role of pro-renin remains to be clarified. Current evidence suggests that pro-renin can activate pro-renin receptor and contribute to both normal and disease states (79, 80).

Renin is rate limiting for the generation of angiotensin II (AngII) in the RAAS cascade. Active renin specifically cleaves the 10 amino acids from the N-terminus of Agt to form AngI. In humans, there is an excess of Agt in serum.

Renin can be secreted in response to several stimuli such as reduced renal perfusion, increased sympathetic activity, and use of β- adrenoreceptor agonists. The source of plasma renin is not prorenin in vivo. Under steady-state conditions, the concentration of renin in blood is linearly related to its renal secretion rate. The kidney is known to secrete both renin and prorenin. However, other organs such as adrenal, ovary, placenta, testis, and retina are able to secrete prorenin as well (46). The secretion of prorenin is not regulated, therefore, the peptide exists in the circulation at concentrations that are approximately 5 to 10 times higher than those of renin (81, 82).

Angiotensin I (AngI)

AngI is the product of the enzymatic reaction catalyzed by renin. It is a decapeptide (DRVYIHPFHL) that is not biologically active. Particularly in the lung, AngI is converted to the octapeptide AngII (DRVYIHPF) by the cleavage of two C-terminal amino acids. This hydrolysis reaction is catalyzed by the enzyme ACE (83).

Angiotensin-converting enzyme (ACE)

ACE is a membrane-bound exopeptidase on the surface of various cell types predominantly abundant in the lung. However, it is also present in other vascular endothelial tissues such as in the heart, brain or kidney. ACE is an effector molecule in the RAAS because it generates the vasoactive peptide AngII by cleaving 2 amino acids from the c-terminus of the inactive precursor AngI (84). The enzymatic activity of ACE does not limit to the cleavage of AngI, as other biological substrates for ACE have been identified such as bradykinin (85). ACE was previously referred to as kininase II because in vivo studies showed that ACE degrades bradykinin into an inactive peptide (86). Therefore, since bradykinin has vasodilator and natriuretic properties, clinical studies have shown that blockade of this kininase activity by ACE inhibitors plays a role in the overall reduction blood pressure (85, 87-89).

Angiotensin II (AngII)

ANGII, the octapeptide [Ang-(1-8)], is the primary active product of the RAAS cascade. AngIi induces its physiological and pathophysiological actions by binding to specific angiotensin receptors. At least 4 angiotensin receptor subtypes haven been described (90). The type 1 (AT1) receptor mediates most of the classically recognized functions of AngII. These include actions on the cardiovascular system (vasoconstriction, increased cardiac contractility), pituitary gland

(release of anti-diuretic hormone), kidney (regulation of tubuloglomerular feedback (91), renal tubular sodium reabsorption, inhibition of renin release), sympathetic nervous system (release of norepinephrine), and adrenal cortex (stimulation of aldosterone synthesis) (92). In addition to circulatory homeostasis, AT1 receptor also mediates effects of AngII to promote cell growth and proliferation, inflammatory responses, and oxidative stress (93), which can be related to physiological and pathological deterioration of organ function (94).

Aldosterone

Aldosterone is a steroid hormone synthesized by the zona glomerulosa cells of the adrenal cortex. It is a major regulator of sodium and potassium balance, and thus plays a major role in regulating circulating volume and blood pressure. Aldosterone enhances the reabsorption of sodium and water in the distal tubules and collecting ducts. Consequently, a negative electrochemical gradient develops in the tubules that promotes potassium and hydrogen ion excretion (95).

AngII, together with extracellular potassium levels, are the major regulators of aldosterone. Adrenocorticotrophic hormone (ACTH) also participates in the secretion of aldosterone, but mainly in acute situations (96, 97).

3.3 Definition and causes of primary aldosteronism

PA is characterized by production of aldosterone independent of or out of proportion to angiotensin II (AngII) stimulation. PA occurs in familial and sporadic forms.

Familial forms of PA are very rare and occur in less than 1% of hypertensive patients. Three types of familial hyperaldosteronism have been identified.

Familial hyperaldosteronism type I, glucocorticoid-remediable aldosteronism (GRA), is an autosomal dominant condition caused by a fusion gene (98) between aldosterone synthase (CYP11B2) and 11-β-hydroxylase (CYP11B1). This genetic defect causes excessive aldosterone production in response to adrenocorticotropin (ACTH) (99). Familial hyperaldosteronism type II is described as familial occurrence of aldosterone-producing adenoma or bilateral idiopathic hyperplasia or both. The cause is yet to be determined (100). Familial hyperaldosteronism type III is a non-glucocorticoid-remediable hyperaldosteronism with an autosomal dominant pattern of inheritance (101). The genetic defect has been recently described as germ-line mutations in the G protein-activated inward rectifier potassium channel 4 (GIRK4) encoded by the KCNJ5 gene (102).

Sporadic forms of PA are more common with idiopathic adrenal hyperplasia (IAH) accounting for about 60% of all PA cases. Aldosterone producing adenomas (APA) and unilateral adrenal hyperplasia account for another 30% and 2%, respectively (103, 104). Aldosterone producing carcinoma causes approximately 1% of PA with very high levels of various adrenal steroids, which can also lead to Cushing syndrome, virilisation and feminization (105).

3.4 Clinical and biochemical features

Diagnosis of PA is often made in patients in their 3rd to 6th decade of life and are usually due to resistance to antihypertensive therapy (70). Patients suffering from PA have inappropriate levels of circulating aldosterone as well as suppressed plasma renin activity (PRA) independent of the AngII plasma concentration (106).

The clinical features of PA is a direct result of inappropriate production of aldosterone and its downstream activation of mineralocorticoid receptor (MR), which are expressed in tissues such

as renal tubular epithelium, cardiac myocytes, smooth muscle cells, endothelial progenitor cells, and neutrophils (107).

The physiological response of unregulated aldosterone on the distal renal tubule is excessive sodium and water retention, as well as potassium excretion (108). The overall outcome include volume expansion, hypertension, and variable hypokalemia. PRA is characteristically suppressed due to feedback inhibition from sodium excess and hypertension (109).

In the literature, 37% of patients with PA are found to have hypokalemia, mostly in later stage of the disease (110). Half of patients with APA and 17% of those with IAH were found to have serum potassium concentrations less than 3.5 mmol/L (111). Normokalemia occurs commonly in GRA. Clinically, severe hypokalemia may result in muscle weakness, cramps, palpitations and, rarely, hypokalemic paralysis.

PA leads to all of the expected sequelae of chronic hypertension. However, chronic aldosterone excess has also been shown to cause a number of cardiac (112-114) and renal (115, 116) pathologies. PA is also associated with bone demineralization (117) and may even be seen concomitantly with primary hyperparathyroidism (118).

3.5 Clinical practice guidelines

The Endocrine Society published clinical practice guidelines for diagnosis and treatment of patients with PA in 2008. Four patient groups were identified with relatively high prevalence of PA, and recommended for screening (119).

These include patients with Joint National Commission (JNC) stage 2 (>160–179/ 100–109 mm Hg), stage 3 (>180/110 mm Hg), or drug resistant hypertension; hypertension and spontaneous or diuretic-induced hypokalemia; hypertension with adrenal incidentaloma; or

hypertension and a family history of early-onset hypertension or cerebrovascular accident at a young age (<40 years). All hypertensive first-degree relatives of patients with PA were also recommended for screening.

3.6 Biochemical screening and confirmatory testing

The biochemical screening for PA in at-risk population was firstly introduced in 1981 (120). AngII is difficult to analyze in the clinical laboratory because of its short half-life (approximately 1 to 2 min). Therefore, plasma renin has been used as a surrogate for AngII. This widely accepted method measures serum (or plasma) aldosterone concentration (SAC) and plasma renin to calculate the aldosterone-renin ratio (ARR) (119). The quantification of renin can be done either be measuring plasma renin activity (PRA) or direct renin concentration (DRC). The ARR has superior test characteristics to either SAC or PRA alone (121). Elevated ARR could suggest inappropriate aldosterone release in the context of PA.

Aldosterone elevation, as a response to causes other than PA such as electrolyte imbalance (hyponatremia and hyperkalemia) or ACTH, will lead to false positive ARR results (94). Therefore, confirmatory testing is usually required to confirm the diagnosis of PA. Two common methods are saline infusion tests (SIT) or fludrocortisone suppression tests (FST) (122). Furthermore, methods to classify different forms of primary aldosteronism include adrenal computed tomography (CT), adrenal venous sampling, and genetic testing for glucocorticoid-remediable aldosteronism.

4. Laboratory analytical issues

4.1 Prorenin activation and assay interference

More than 90% of the total renin in plasma is prorenin (123). In its closed conformation, the pro-segment in the amino-terminal masks the active site of renin preventing the conversion of angiotensinogen to AngI. At physiological temperatures and pH, approximately 2% of plasma prorenin are in its open conformation leaving the active site exposed (46).

Activation of prorenin, which leads to elevated PRA and direct renin concentration (DRC), is a repeatedly reported phenomenon when plasma samples are stored at refrigeration and room temperature. However, the degree of prorenin cryoactivation may occasionally be excessively stated since it has been documented that it takes 12 h of incubation at 4 °C for 5% activation of recombinant prorenin (124). Clinical laboratory has adapted pre-analytical precautions including immediate freezing of specimen for storage, and rapid thawing followed by immediate analysis. This rapid freezing protocol not only prevents prorenin cryoactivation, it also inhibits the enzymatic activity of renin to consume its substrate (angiotensinogen) at ambient temperature. Poor specimen handling could lead to substrate depletion, higher AngI concentrations in the blank, and lower PRA results (46).

Detection of prorenin as an interferent to renin is of great concern because of its high plasma concentration relative to renin. In healthy individuals, plasma prorenin being constitutively secreted from the kidneys is 10-fold higher in concentration than renin (125, 126). In a low-renin state, such as in patient with PA, the difference in plasma concentration further increases up to 100-fold (125). Therefore, immunoassay cross reactivity with prorenin has the potential to be most significant in the screening for PA. The analytical overestimation is expected to be most significant in DRC assay, but PRA could be likewise affected by this same phenomenon.

4.2 PRA by immunoassay

A precise assessment of the activity of the renin–angiotensin system is essential for the diagnosis and treatment of PA. The most widely used method is the measurement of PRA (48), which was first introduced in the 1960s as RIA (127). Renin can be assessed by PRA assays that quantified the generation of AngI by antibody binding. Endogenous renin enzymatically cleaves endogenous angiotensinogen to generate AngI over a fixed incubation period at 37 °C under properly buffered conditions. Concurrently, a second aliquot of the same specimen is incubated in identical buffering conditions and for the same duration but on ice (at 4 °C), where renin has minimal enzymatic activity. The second aliquot is then used as a blank for PRA determination. Depending on the protocol, incubation can be 1 hour, 3 hours or in low PRA samples 18 hours. At the end of incubation, AngI is quantified in both the 37 °C and the 4 °C ("blank") aliquots and PRA is calculated by the following Equation:

$$PRA = \frac{[\mathrm{AngI}]_{37\,^{\circ}\mathrm{C}} - [\mathrm{AngI}]_{4\,^{\circ}\mathrm{C}}}{\Delta t}$$

(Δt is the incubation time)

This enzymatic assay offers two distinct advantages. Firstly, quantification of AngI generated by the reaction between renin and its substrate takes into account the contribution of both participants of the RAAS determining the production of AngII. Secondly, the amount of AngI generated during the reaction can be regulated by prolonging or shortening the duration of the incubation step, therefore allowing accurate measurement of renin despite its wide range in plasma concentration.

However, immunoassays always bear the risk of cross-reactivity with heterophile antibodies, or auto-antibodies, both of which can be very difficult to detect and negatively affect clinical

decision making. The reproducibility of PRA among different laboratories is also far from being optimal (11). Most importantly, unlike many enzymatic assays, there is no IFCC (International Federation of Clinical Chemistry) guideline for method standardization. Other than pre-analytical issues mentioned above, inter and intra-laboratory reproducibility can be affected by variations in assay conditions such as the pH of incubation, the choice of angiotensinase inhibition strategy (128), the analytical performance and the purity of the calibrators (129).

4.3 PRA by LC-MS/MS

MS-based approaches for PRA determination by quantification of AngI are becoming more prevalent. Several published methods have used solid phase extraction (SPE) and positive ion LC-MS/MS (10, 49, 130, 131). Such method requires considerable expertise in analyte extraction and chromatography, but it also offers distinct analytical advantages.

First, AngI calibration curves generated by LC-MS/MS method is linear to allow larger dynamic ranges (130), rather than sigmoidal curves in the case of immunoassay. Even though in the screening of PA, the context of interest is low-renin state, the increase in dynamic range allows accurate measurement of high PRA concentrations without sample dilution (132).

Secondly, LC-MS/MS methods are free of the use of radiotracers. The mass spectrometer offers analyte detection by scanning for unique mass fragments, which improves analytical specificity, and eliminates non-specific immunoreactivity in the blank specimen. Based on these promises, published MS-based methods have reported that PRA assays do not require blanking and blank subtraction (29, 130). This change effectively removes the requirement for duplicate preparation, incubation, and analysis for each sample at 37 °C and 4 °C. Therefore, MS-based

methods are less prone to error propagation because the calculation for PRA has been simplified to the following equation:

$$PRA = \frac{[\mathrm{AngI}]_{37\,^{\circ}\mathrm{C}}}{\Delta t}$$

(Δt is the incubation time)

4.4 PRA by iMALDI-TOF MS

Because the utility of LC-MS/MS requires considerable amount of expertise in chromatography, many clinical laboratories are still hesitate from implementing this technique for routine analysis. Chromatography-free, immuno-matrix-assisted laser desorption/ionization (iMALDI) mass spectrometric assays for PRA has been developed at the University of Victoria (UVic)-Genome BC Proteomics Centre (15).

This iMALDI PRA assay has recently been automated with a liquid handling system to allow PRA determination of 29 patient samples within 7 hours (using a 3-hour Ang I generation period), a 7.5-fold faster analysis time than LC–MS/MS. The assay is performed on 350 µl of plasma, and has a linear range from 0.04 to 5.3 ng/L/s. The analytical precision has a coefficient of variation (CV) of 1.5 to 14.3% (29).

The sensitivity of the iMALDI assay promises agreement with the Endocrine Society's Clinical Practice Guidelines requirement, which recommend a minimum sensitivity of 0.6–0.08 ng/L/s for clinically suitable PRA assays (119), and is below the established PRA cut-off value of 0.18 ng/L/s for low PRA patients (125).

5. Experimentation

5.1 Introduction

The goal of this project assess the high-throughput iMALDI PRA assay developed by University of Victoria for clinical implementation.

The existing automated iMALDI PRA assay protocol had been published for high-throughput sample preparation in research laboratory. Therefore, as the first step, the protocol was optimized for implementation as a clinical assay in routine clinical laboratories.

In a second step, iMALDI PRA assay robustness was assessed with clinically relevant samples using two MALDI-TOF MS instruments from two different manufacturers (Bruker Microflex LRF and Ab Sciex 4800).

Lastly, the high-throughput iMALDI PRA assay was compared against two current clinical methods as part of a multi-site correlation study. The two clinical assays are DBC (Diagnostics Biochem Canada Inc.) ELISA PRA assay in Jewish General Hospital in Montreal, QC and Ab Sciex LC-MS/MS PRA assay in St. Paul's Hospital in Vancouver, BC.

Overall, determination of plasma renin activity was carried out on 4 different instruments for method comparison. 140 clinical samples were analyzed using DBC ELISA PRA assay for diagnostic purposes over a 10 months period at Jewish General Hospital in Montreal, QC. A separate aliquot was collected for each sample for comparison with the automated, high-throughput iMALDI PRA assay in University of Victoria, BC. PRA determination by iMALDI was done by using an Agilent Bravo automated liquid handling system.

40 of the 140 clinical samples were also analyzed using clinically employed LC-MS/MS method. DBC ELISA calibrators were used to re-calibrator iMALDI and LC-MS/MS methods. Bio-Rad EQAS external quality control results were also correlated with all methods.

The study was approved by the Jewish General Hospital Research Ethics Committee.

5.2 Materials

5.2.1 ELISA

5.2.1.1 Reagents and chemicals

The ELISA kit and its contents are listed in Table 1.

Table 1. List of required reagents for DBC ELISA PRA assay

Plasma Renin Activity (PRA) ELISA kit (DBC, Cat. # CAN-RA-4600)	
REAGENTS PROVIDED IN THE KIT: • Generation Buffer: 5mL bottle. • PMSF (Phenylmethylsulfonyl fluoride) • Rabbit Anti-Ang-l Antibody Coated Microwell Plate: two 96 well pre-coated microwell plates • Angiotensin-l-Biotin Conjugate:one bottle containing buffer,protease inhibitors, Ang-l-Biotin conjugate. • Streptavidin-Horseradish Peroxidase Conjugate Concentrate • Angiotensin-l Calibrators: 8 vials. • Controls: Two vials (High/Low). • Assay Buffer: 40mL bottle • Wash Buffer: Two bottles • TMB Substrate: 32mL bottle. • Stopping Solution: 12mL bottle.	REAGENTS AND EQUIPMENT NEEDED BUT NOT PROVIDED • Disodium EDT A (2 mg/ml blood) collection tubes • Single and multi-channel pipettes and disposal tips. • Distilled or deionized water. • Disposable test tubes (glass or polypropylene). • Plate shaker. • Microwell plate absorbance reader equipped with a 450 nm filter. • 37'C incubator. • Ice bath. • 95% Ethanol.

5.2.2 High-throughput iMALDI

5.2.2.1 Internal Standards

Internal standards, AngI NAT (DRVYIHPFHL) and SIS (D**R**VYIHPFHL) were synthesized at the University of Victoria Genome BC Proteomics Centre by using solid phase peptide

synthesis (SPPS). The lyophilized peptides were re-suspended in 30% ACN, 0.1% formic acid (FA), and stored at -80 °C until used.

The AngI SIS peptide differs from the AngI NAT peptide by 10 Da. During synthesis, a stable isotope-coded arginine residue, which contains six 13C- and four 15N-atoms, is incorporated into the peptide, resulting in a monoisotopic mass of 1305.71 Da.

5.2.2.2 Antibodies

Anti-AngI polyclonal antibodies were provided by Santa Cruz Biotechnology, (Cat.#: sc-7419).

5.2.2.3 Chemicals and Reagents

The chemicals used for iMALDI PRA assay are listed in Table 2.

Table 2. List of chemicals for iMALDI PRA assay

Chemical	Abbreviation	Manufacturer
3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate	CHAPS	Invitrogen
Acetonitrile (LC-MS CHROMASOLV®, ≥99.9%)	ACN	Fluka
Albumin from chicken egg white	CEWA	Sigma Aldrich
Ammonium bicarbonate	AmBic	Sigma Aldrich
Ammonium citrate dibasic	-	Sigma Aldrich
Ethylenediaminetetraacetic acid (EDTA)	EDTA	BDH
Maleic acid ≥99.0% (HPLC)	-	Fluka
Methanol (LC-MS CROMASOLV®)	MeOH	Fluka
Neomycin trisulfate salt hydrate	-	Sigma Aldrich
Phenylmethanesulfonyl fluoride (≥99.0%)	PMSF	Fluka
Phosphate buffered saline	PBS	Sigma Aldrich
Trifluoroacetic acid	TFA	Thermo Scientific

Water (LC-MS CHROMASOLV®)	H_2O	Fluka
α-Cyano-4-hydroxycinnamic acid (≥98%)	HCCA	Sigma Aldrich

A 1xPBS solution was prepared following the Sigma Aldrich protocol by dissolving a PBS tablet in 200 µL H2O. The HCCA matrix solution was prepared by mixing 4.5 mg HCCA, 2.7 mg ammonium citrate dibasic, 1050 µL ACN, 450 µL H2O and 1.5 µL TFA.

5.2.2.4 Analytical Equipment and Supplies

The analytical equipment and labware used are listed in Table 3.

Table 3. Instrumentation and labware for iMALDI PRA assay

Instrument/Labware	**Manufacturer**
Bravo equipped with 96-channel LT head	Agilent
Microflex LRF MALDI-TOF-MS	Bruker
4800 MALDI-TOF/TOF	AB Sciex
Labquake Tube Rotator	Thermo Scientific
Microcentrifuge 5415D	Eppendorf
Thermomixer® compact	Eppendorf
MicroScout Plate targets • MSP AnchorChip 96 • MSP BigAnchor 96 • MSP Polished steel 96	Bruker
96-well Full Skirt PCR plate, 200 µL (PCR-96-FS-C)	Axygen Scientific
96-well Deep well plate, 1.1 mL, sterilized (P-DW-11-C-S)	Axygen Scientific
Bravo disposable pipette tips 250 µL for 96LT Head (2.0– 250 µL), (Cat.-#: 19477-002)	Agilent
DynaMag™ magnet	Invitrogen
VP771RM magnet	VP Scientific

5.2.3 LC-MS/MS

5.2.3.1 Chemicals and Reagents

Table 4. List of required chemicals reagents for LC-MS/MS PRA assay

Plasma Renin Activity by LC-MS/MS (St. Paul's Hospital, Vancouver, BC)
• Bovine Serum Albumin, Fraction V, Omnipur® (EMD Millipore. Billerica, MA)
• Phenylmethanesulfonylfluoride (PMSF), >98.5% (Sigma-Aldrich St. Louis, MO)
• Buffer A: 0.1M Tris Base pH 6
• Buffer B: 1% BSA (w/v) in Stock Buffer A: Dissolve 0.1 g of BSA into 10 mL of 0.1M Tris Buffer pH 6.
• Generation Buffer: Weigh 121.1 g of Tris Base + 74 g EDTA into a 1000 mL volumetric flask. Add DI water to about 900 mL. Adjust to pH 5.45 – pH 5.50 with glacial acetic acid.
• Mobile Phase A: 0.2% formic acid in water
• Mobile Phase B: 0.2% formic acid in methanol
• Lyphochek™ Hypertension Markers Control, Trilevel (Bio-Rad, Montreal QC)

5.2.3.2 Internal Standards

- Primary standard: AngI : 3 × 10 µg (Proteochem, Loves Park IL)
- Stable isotopically labelled internal standard (SIS): AngI (DRVYIHPFHL) with isotopically labelled arginine residue (13C,15N) was synthesized by the University of Victoria Genome BC Proteomics Centre.

5.2.3.3 Calibrators and Controls

- Controls used are BioRad Hypertension controls Levels 1, 2 and 3.
- An in-house patient pool is prepared approximately once yearly by pooling discarded anonymized previously analyzed patient plasma samples.
- Calibrators are prepared in-house using the AngI stock solution in 1% BSA in Buffer A.

5.2.3.4 Analytical Equipment and Supplies

Table 5. Instrumentation and labware for LC-MS/MS PRA assay

Instrument/Labware	Manufacturer
API-5000 or API-5500 QTRAP® triple quadrupole mass spectrometer	AB SCIEX
Shimadzu 20AC LC System	Shimadzu
Anaytical column: 4u Proteo 90 Å, 50 × 2.0 mm	Phenomenex
Guard column, C12, 4 × 2.0mm	Phenomenex
Nunc® cap mats for round bottom plates	Thremo Scientific
2 mL Nunc® 96 DeepWellTM round-bottom well plates	Thremo Scientific
Strata-X 33u Polymeric Reversed Phase 96-Well Plate, 60mg/well	Phenomenex
2 mL 96 deep square well, V-bottom plates	Corning
Silicone cap mats with PTFE barrier for square well plates	Microliter Analytical Supplies

5.3 Methods

5.3.1 ELISA

This kit measures PRA and the results are expressed in terms of mass of Ang-l generated per volume of human plasma in unit time (ng/mL/h). The plasma is separated and either stored frozen or kept at room temperature for immediate use, samples should not be chilled on ice or stored at temperatures between 0 and 10°C during collection or processing before adjustment of pH, this could lead to overestimation of renin activity.

Before the start of the immunoassay the protease inhibitor (PMSF) and the Generation buffer is added to the plasma sample, which will prevent Ang-l in plasma from degradation. The pH of the plasma sample should be around 6.0 after the addition of the supplied Generation buffer.

The plasma sample is split in two and the fractions are incubated at 37˚C for 18hrs and 3hrs. If samples with normal or high PRA are incubated for 3hrs and those with <1 ug/L/h are incubated for 18hrs, the blank amounts to <10% of the total angiotensin-l and can be ignored.

The immunoassay of Ang-l is a competitive assay that uses two incubations, with a total assay incubation time of less than two hours. During the first incubation unlabelled Ang-l competes with biotinylated Ang-l to bind to the anti-Ang-l antibody.

In the second incubation the labelled Streptavidin-HRP conjugate, binds to the immobilized Ang-l-Biotin. The washing and decanting procedures remove unbound materials. The colorimetric HRP substrate is added and after stopping the color development reaction, the light absorbance (OD) is measured. The absorbance values are inversely proportional to the concentration of Ang-l in the sample. A set of calibrators is used to plot a standard curve from which the concentrations of Ang-I in the samples and controls can be directly read.

5.3.1.1 General ELISA protocol for determination of PRA

5.3.1.1.1 Specimen

A minimum of 0.5 mL of plasma is required per duplicate determination. The in-vitro generation and degradation of angiotensin-1 can be minimized by the following recommended collection procedure:

- Collect 2 mL of blood into an EDTA venipuncture tube or syringe.
- Centrifuge blood for 15 minutes at 5000 rpm at room temperature.
- Transfer plasma sample to a test tube at room temperature.
- If samples are to not be assayed, freeze samples immediately at -20ºC or less.

5.3.1.1.2 Angiotensin I Generation

1. If a freshly drawn plasma sample is being used proceed to step 2. If frozen plasma samples are being used thaw them as follows. Quickly bring frozen plasma samples to room temperature by placing the tubes in a container with room temperature water.
2. Transfer 0.5 mL of the plasma sample into a test tube.
3. Add 5 µL of the PMSF solution to the 0.5 mL of plasma sample (1 :100 ratio). Vortex the tube to mix thoroughly.
4. Add 50 µL of the generation buffer to the treated sample from step 3 (1:10 ratio). Vortex the tube again to mix thoroughly.
5. Divide the treated sample from step 4 equally into two aliquots by transferring 0.25 mL into two test tubes. Incubate one aliquot for 90 minutes or longer (do not exceed 180 minutes), place the second aliquot on an ice bath. Be sure to record the incubation time used for the aliquots as this is used for calculations.
6. At the end of the incubation period place the 37 ºC aliquot on the ice-bath for 5 minutes to cool it down quickly.
7. Bring both aliquots to room temperature by placing in a bath with room temperature water for 5-10 minutes.

5.3.1.1.3 Standards Preparation

1. Allow kit components to reach room temperature.
2. Bring aliquots to room temperature by placing in a room temperature water bath for 5-10 minutes.
3. (do not exceed 10 minutes)
4. 8 standards : (0.0), (0.2), (0.5), (1.5), (4.0), (10.0) (25.0) (60.0) (ng/mL)
5. 2 Kit Controls: High and Low (ng/mL)

5.3.1.1.4 Reagent Preparation

1. Wash Buffer : Dilute 1:10 in distilled water (50mL of wash buffer in 450mL of water)
2. Streptavidin-Horseradish Peroxidase Conjugate: Dilute the HRP conjugate concentrate 1:100 in assay buffer before use.

5.3.1.1.5 ELISA analysis

1. Allow all kit components to reach room temperature. Remove the required number of microwell strips and assemble into the plate frame.
2. Pipette 50 µL of each calibrator, control and treated plasma sample (both 37 ºC and 0 ºC aliquots) into correspondingly labelled wells in duplicate.
3. Pipette 100 µL of the angiotensin-1-biotin conjugate into each well (the use of a multichannel pipette is recommended).
4. Incubate on a plate shaker (-200 rpm) for 60 minutes at room temperature.
5. Wash the wells 5 times each time with 300 µL/well of diluted wash buffer. After washing tap the plate firmly against absorbent paper to remove any residual liquid (the use of an automatic strip washer is strongly recommended).
6. Pipette 150 µL of the streptavidin-HRP conjugate working solution into each well (the use of a multichannel pipette is recommended).
7. Incubate on a plate shaker (-200 rpm) for 30 minutes at room temperature.
8. Wash the wells 5 times with the same procedure as in step 5.
9. Pipette 150 µL of the TMB substrate into each well (the use of a multichannel pipette is recommended). Incubate on a plate shaker (-200 rpm) for 10 to 15 minutes at room temperature.
10. Add 50 µL of stopping solution to each well and mix thoroughly by gently tapping the plate

11. Measure the absorbance at 450 nm in all wells with a microplate reader between 0-20 minutes after addition of the stopping solution.

5.3.1.1.6 PRA Calculation

1. Using immunoassay software, choose either a 4-parameter or 5-parameter curve fitting method for calculating results.

2. If a sample reads more than 60 ng/mL then dilute it with calibrator A at a dilution of no more than 1 :10 and rerun the sample. The result obtained should be multiplied by the dilution factor.

3. Calculate the plasma renin activity (PRA) in each sample using the following equation:

$$PRA = \frac{[\mathrm{AngI}]_{37\,^{\circ}\mathrm{C}} - [\mathrm{AngI}]_{4\,^{\circ}\mathrm{C}}}{\Delta t}$$

(Δt is the incubation time)

5.3.2 High-throughput iMALDI

5.3.2.1 General iMALDI protocol for determination of PRA

The procedure for the determination of PRA by iMALDI consists of the following general steps: standard preparation, bead-antibody conjugation, AngI generation, affinity-capture, washing and spotting, MALDI analysis and PRA calculation.

5.3.2.1.1 Specimen

Plasma collected in EDTA tubes is the only acceptable sample type for this assay.

5.3.2.1.2 Standard preparation

Calibration standards consisting of varying amounts of AngI NAT and constant amounts of AngI SIS are prepared by dissolving the corresponding stock solutions in 1xPBSC. The

prepared standard solutions are transferred to a pre-cooled 96-well plate (Reagent Plate) and put on ice until used. An overview of the standard concentrations used is given in Table 3.

Table 6. Standard concentrations of iMALDI PRA assay

Standard	c(AngI NAT) in fmol/µL	c(AngI SIS) in fmol/µL	Application in PRA assay
A	80	25	Calibration curve
B	40	25	Calibration curve
C	20	25	Calibration curve
D	10	25	Calibration curve
E	5	25	Calibration curve
F	-	25	Calibration curve + plasma samples

5.3.2.1.3 Bead-antibody conjugation

Magnetic Protein G Dynabeads® are washed seven times with 25% ACN/1xPBSC and three times with 1xPBSC. CHAPS in the wash buffer prevents the beads from sticking to the walls, thereby significantly improving automated liquid handling performance. After the final wash, all liquid is discarded. Subsequently, 0.2 µg of sc-7419 polyclonal antibody and 1 µL 1xPBSC per 1 µL bead slurry are added, followed by a 1-hour incubation at 4 °C while rotating on a Thermo Scientific Labquake Tube Rotator.

Following the 1-hour incubation, the bead-antibody conjugates are washed three times with 1xPBSC and are resuspended in 20 µL of 1xPBSC per µL of bead slurry. The resuspended bead-antibody conjugates are then transferred manually to different wells of the same 96-well plate as the standard solutions (Reagent Plate), and placed on ice.

5.3.2.1.4 Angiotensin I generation

To each human plasma sample, 350 µL of plasma are brought from -80 °C to room temperature in a beaker that contains water at room temperature, and the thawed samples are spun down at

room temperature for 10 min at 13,000 g in an Eppendorf Microcentrifuge 5415D, mainly to remove insoluble material from the plasma.

Stock solutions of EDTA, maleic acid, and neomycin trisulfate salt hydrate are prepared in H2O. A stock solution of PMSF is dissolved in 2-propanol because of its short half-life in aqueous solutions.

The four solutions are mixed together less than 1 minute before the end of the plasma centrifugation in order to keep PMSF degradation as low as possible.

Next, 290 µL of supernatant are added to 17.81 µL of the reagent mix. The resulting solution has a pH of 6. The solution is split into two separate 1.5-mL Eppendorf tubes (130 µL each). The final plasma-reagent solution contains EDTA at 5 mmol/L, maleic acid at 25 mmol/L, neomycin trisulfate salt hydrate at 0.0275 mmol/L, and PMSF at 1 mmol/L. One tube is put on ice for 1 hour (4 °C plasma aliquot), the second one is incubated at 37 °C for exactly 1 hour on an Eppendorf Thermomixer at 750 rpm (37 °C plasma aliquot).

EDTA, a metalloprotease inhibitor, is used to inhibit conversion of AngI to AngII by ACE. PMSF, a serine protease inhibitor, reduces degradation of AngI by serine proteases, whereas maleic acid maintains a constant plasma pH of 6. Neomycin trisulfate prevents bacterial growth during AngI generation.

After the 1-hour incubation, the 37 °C plasma aliquot is stored on ice for 10 minutes. A 110-µL aliquot of each 4 °C and 37 °C plasma sample is then transferred manually to adjacent wells of an ice-cooled 96-well plate (Plasma Plate).

Additionally, a solution of 3 mg/mL CEWA in 1xPBS is prepared and transferred to the Plasma Plate. It is used as a plasma substitute for the calibration curve.

5.3.2.1.5 Automation with liquid handling system

The solutions of the Reagent Plate and Plasma Plate are transferred to a new 96-well plate (Incubation Plate) and mixed using the Agilent Bravo liquid handling robot.

Affinity-capture

The Incubation Plate is placed on the Thermo Scientific Labquake rotator and incubated for 1 hour at 4 °C.

Washing and spotting with Agilent Bravo

After the MALDI spots are dry, 0.5 µL of HCCA-matrix are added manually. This elutes the captured peptides from the antibodies.

5.3.2.1.6 MALDI-TOF analysis

The dried spots are analyzed on a Bruker Microflex LRF MALDI-TOF instrument in both reflector and linear mode. One AutoExecute method was optimized for each mode. The "fuzzy control" feature which automatically adjusts the laser intensity to achieve a compromise of optimal signal intensity and resolution was used. For each spectrum, 1500 shots were summed, in increments of 300.

5.3.2.1.7 PRA calculation

The PRA value of a patient sample is calculated by determining the amount of AngI NAT which has been produced during the 1-hour AngI generation period. For each sample, three captures of the 4 °C and 37 °C plasma aliquots are performed. The resulting AngI NAT/SIS intensity ratios for each plasma aliquot are averaged, resulting in one average AngI NAT/SIS intensity ratio for each 4 °C and 37 °C plasma aliquot.

The slope of the CEWA standard addition curve is applied to the calculated average AngI NAT/SIS intensity ratios of the 4 °C and 37 °C plasma aliquots which allow the calculation of their corresponding x-intercepts. The difference between the two x-intercepts is the amount of endogenous AngI generated in 28.26 µL of plasma of one sample.

5.3.3 LC-MS/MS

5.3.3.1 General LC-MS/MS Protocol for determination of PRA

5.3.3.1.1 Specimen

Plasma collected in EDTA tubes is the only acceptable sample type for this assay. Specimens should be collected into EDTA plasma tubes and centrifuged within 30 min (preferably within 10 min) and rapidly frozen until analysis.

5.3.3.1.2 Standard preparation

1. AngI Standard (100 ng/mL) is thawed at room temperature.
2. Prepare Working Buffer B.
3. Prepare calibration standards by hand or on robotic liquid handler.
4. The calibration standards are serial dilutions as follows:

    - S6: concentration is 30.00 ng/mL.
    - S5: concentration is 9.000 ng/mL.
    - S4: concentration is 2.700 ng/mL.
    - S3: concentration is 1.350 ng/mL.
    - S2: concentration is 0.6750 ng/mL.
    - S1: concentration is 0.3375 ng/mL.
    - Blank: Working Buffer B with no AngI added.

5.3.3.1.3 Angiotensin I generation

1. Samples and QCs are thawed in room temperature water bath for 5 min.
2. Samples and QCs are transferred to an ice bath to complete thawing.
3. Samples and QCs mixed and centrifuged for 5 min at 2100 g at <5 ºC.
4. 50 µL of generation buffer is added to 2 mL 96 deep square well plate.

5. 250 µL of calibrators, patient samples and QCs are added to the appropriate wells of a 2 mL 96 deep square well plate.
6. The plate is sealed with a silicone cap-mat and votex-mixed briefly, then placed in 37 ºC water bath for 3 hours.
7. After 3 hours, 300µL of AngI SIS in 10% formic acid is added to the incubation plate to stop generation of AngI. The plate is sealed with a new silicone cap mat and mixed briefly.

5.3.3.1.4 Solid phase extraction (SPE) of Angiotensin I

The AngI is extracted by solid phase extraction (SPE):

- Condition step 1: 1mL of methanol is added to each well of the SPE plate and vacuum is applied for 1 min.
- Condition step 2: 1 mL of 5% formic acid is added to each well of the SPE plate and vacuum is applied for 1 min.
- 600 µL of sample is added to each well and vacuum is applied for 1 min.
- Wash 1: 1 mL of 5% formic acid is added to each well of the SPE plate and vacuum is applied for 1 min.
- Wash 2: 1 mL of 20% methanol is added to each well of the SPE plate.
- Apply 10 minutes vacuum at 100 mbar to dry the SPE plate.
- Elute the AngI from the SPE plate to a 2 mL deep well round-bottom well plate with 250 µL of methanol. Apply vacuum for 2 min.

5.3.3.1.5 LC-MS/MS analysis

Seal the 2 mL deep well round plate with the appropriate cap-mat and load on instrument. Inject 20 µL of extract into the LC-MS/MS.

5.3.3.1.6 PRA quantification

1. Data are analyzed using Analyst® Software (AB SCIEX, Concord, ON)
2. Standard curves are generated based on linear regression with 1/x2 weighting of the analyte/IS peak area ratio (y) versus analyte concentration (x) using the quantifying ions indicated in table 2.
3. Run acceptability is based on control values falling within 2 standard deviations of the target value. Target values and SD are set based on the mean of 10 runs as described in section 2.4.1.
4. An ion ratio limit of 15% of the quantitation to qualifying ion is used to positively identify AngI peaks.

6. Results

The automated, high-throughput iMALDI PRA assay has previously demonstrated clinically suitable sensitivity, precision (2.0% to 9.7% CV in the reflector mode) and a high-throughput of up to 360 samples per run over a 2 day period. This assay protocol was optimized to decrease turn-around-time to allow sample analysis within the same day. Internal quality control material and patient pool specimen were added to the existing protocol to detect drift in system performance and maintain quality.

This method comparison study used the modified automated sample preparation procedures in the determination of PRA by iMALDI.

Our clinical specimen had PRA results ranging from 0.03 ng/L/s to 5.94 ng/L/s measured by DBC ELISA PRA assay, with approximately 45% of patients in the low renin state (lower than our ELISA reference range). Eight (6%) out of the 140 sample results were removed from this comparison study. Three of them were outside of the (DBC ELISA) linear range. The other 5 were found to be statistical outliers.

Linear regression was performed with the software Chemical Pathology R (cpR).

6.1 Method comparison studies

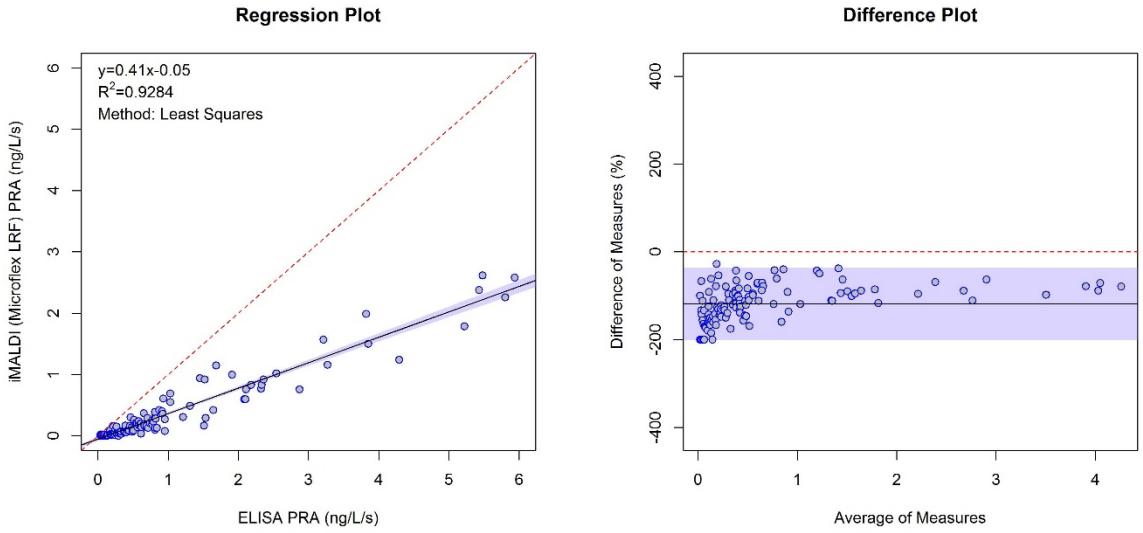
6.1.1 iMALDI PRA assay vs DBC ELISA PRA assay

6.1.1.1 iMALDI PRA assay (Bruker Microflex LRF)

The automated, high-throughput iMALDI PRA assay on the Bruker Microflex LRF instrument shows strong correlation with the DBC ELISA methods for PRA determination. Linear regression analysis (Figure 5) with 132 samples showed R-squared value ≥ 0.92 with slopes of 0.41. Results of 35 samples were found to be outside of the linear range of the iMALDI PRA assays, and therefore removed from the data set. Linear regression analysis was performed again using data within the linear range for both methods, and an R-squared value ≥ 0.91 with slopes of 0.42 were yielded.

Additionally, the two methods were compared by Bland Altman plots plotting the %-difference in PRA as determined by iMALDI and ELISA against the average PRA values for both methods. The mean difference is 118.9% lower for iMALDI PRA values compared to ELISA PRA values in all data points. The mean difference is 103.5% lower in samples within the linear range.

A) all patient samples (N=132)



B) all samples within linear range (N=97)

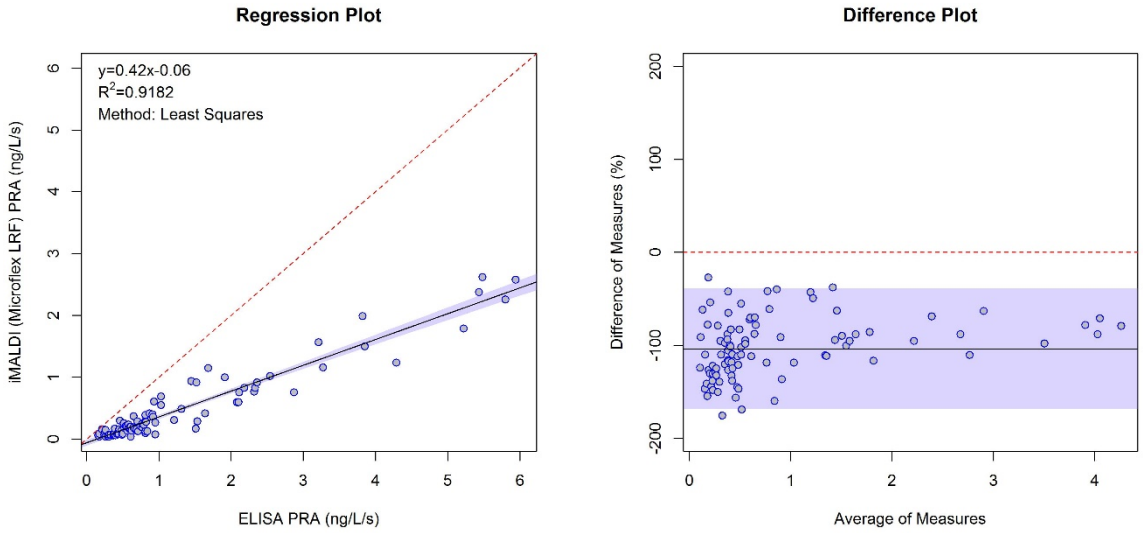


Figure 5. Regression & Bland-Altman analyses for patient samples analyzed by iMALDI (Microflex LRF) vs ELISA

6.1.1.2 iMALDI PRA assay (AbSciex 4800)

Linear regression analysis (Figure 6) with 92 samples analyzed by AbSciex MALDI-TOF/TOF showed R-squared value ≥ 0.88 with slopes of 0.4. The mean difference is 114% lower for iMALDI PRA values compared to ELISA PRA values.

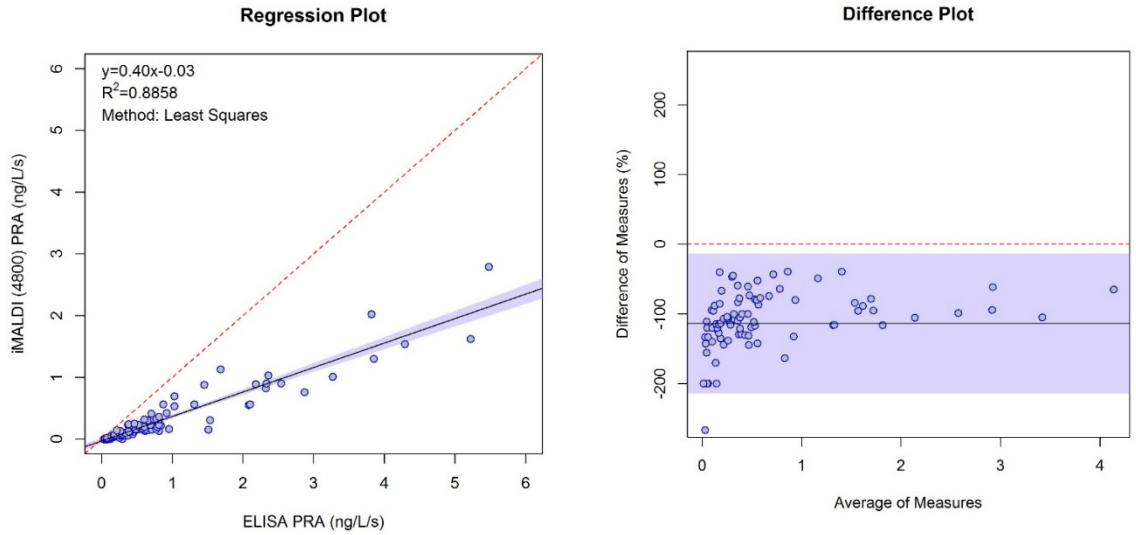


Figure 6. Regression & Bland-Altman analyses for 92 patient samples analyzed by iMALDI (AbSciex 4800) vs ELISA

6.1.1.3 Robustness of iMALDI PRA assay

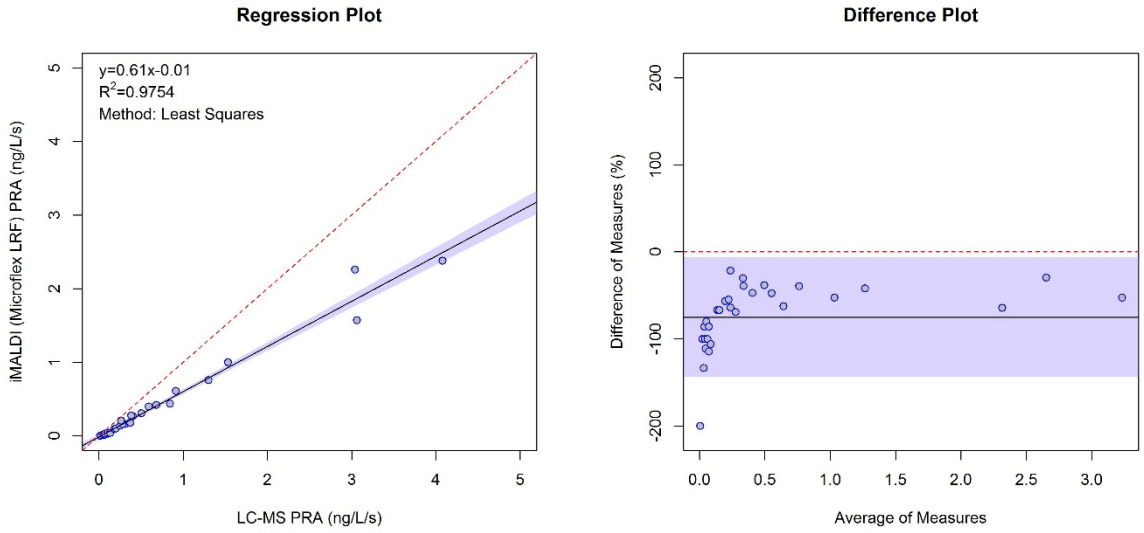
Nighty-Six (96) clinical samples were quantitated by high-throughput iMALDI PRA assay 2 different MALDI TOF instrument (Bruker Microflex and AB Sciex 4800), yielding an R-squared value ≥0.99.

6.1.2 iMALDI PRA assay vs LC-MS/MS PRA assay

The automated, high-throughput iMALDI PRA assay on the Bruker Microflex LRF instrument also showed strong correlation with the LC-MS/MS method for PRA determination. Linear regression analysis with 38 samples showed R-squared value ≥ 0.97 with a slope of 0.61. (Figure 7). Thirteen samples were outside of the linear range, and removed from the data set. The updated linear regression analysis yielded results of insignificant differences.

The mean difference is 75.08% lower for iMALDI PRA values compared to LC-MS/MS PRA values in all data points. The mean difference is 56.07% lower in samples within the linear range.

A) all patient samples (N=38)



B) all samples within linear range (N=25)

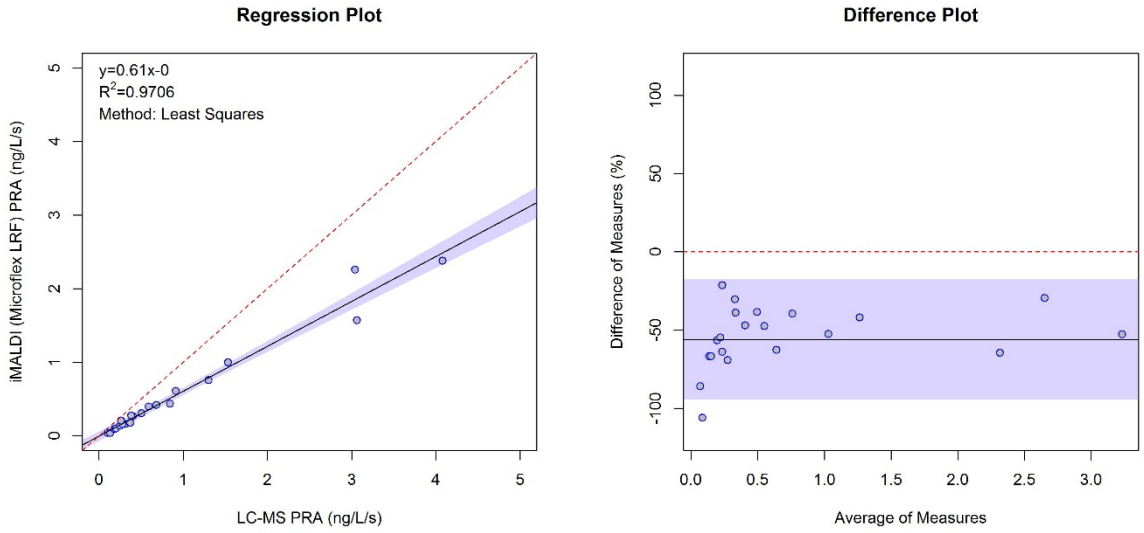


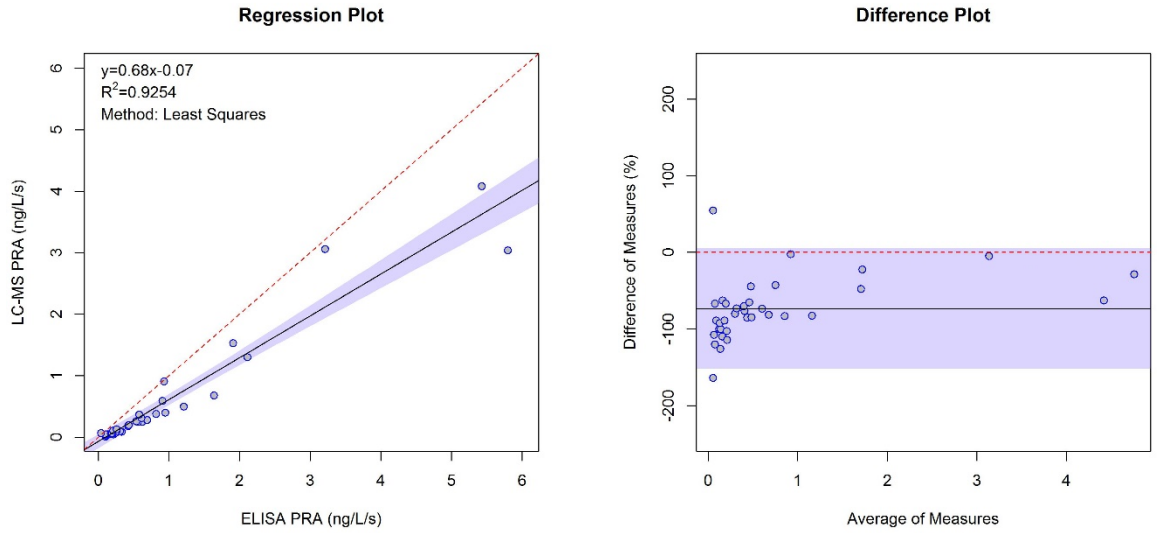
Figure 7. Regression & Bland-Altman analyses for patient samples analyzed by iMALDI (Microflex LRF) vs LC-MS/MS

6.1.3 LC-MS/MS PRA assay vs DBC ELISA PRA assay

Our current DBC ELISA PRA assay also revealed strong correlation when compared to the clinical LC-MS/MS method with an R-squared value ≥0.92 and a slope of 0.68 by linear regression analysis (Figure 8). One sample was outside of the linear range, but did not affect the overall correlation.

The mean difference is 73.27% lower for LC-MS/MS PRA values compared to ELISA PRA values in all data points. The mean difference is 70.61% lower in samples within the linear range.

A) all patient samples (N=36)



B) all samples within linear range (N=35)

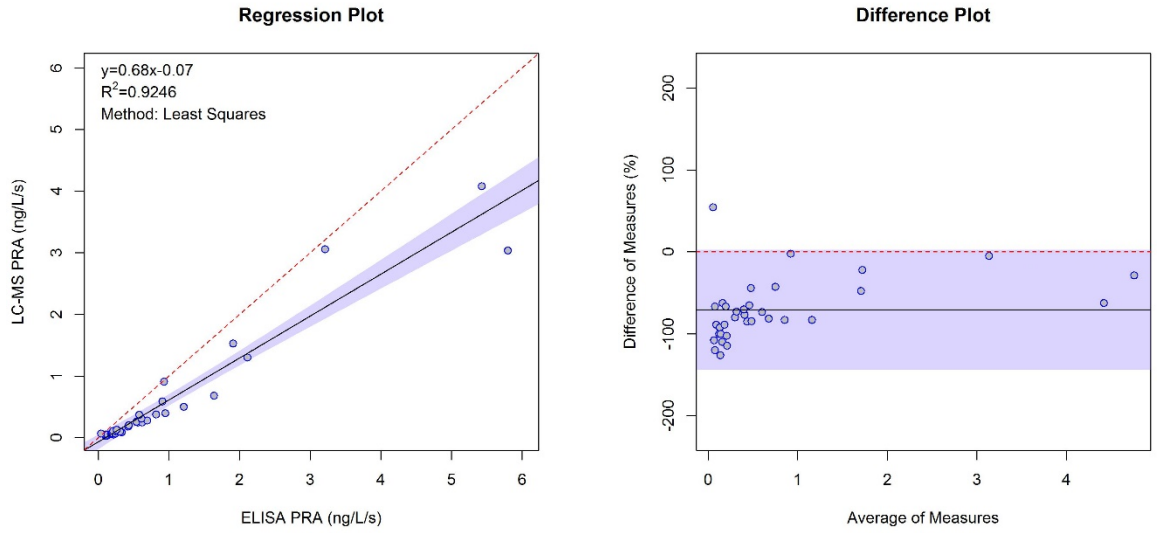
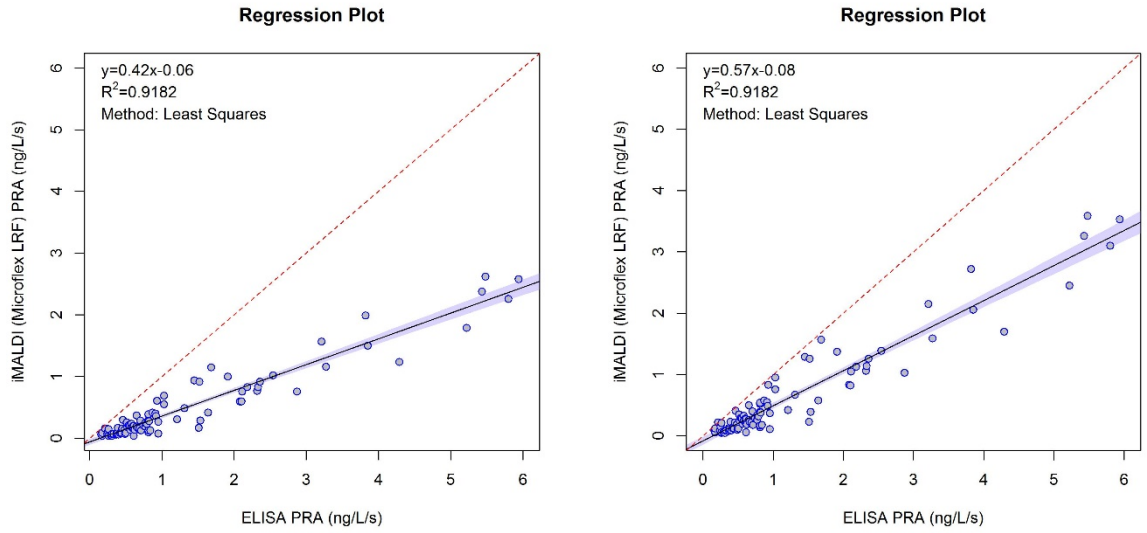


Figure 8. Regression & Bland-Altman analyses for patient samples analyzed by LC-MS/MS vs ELISA

6.1.4 Recalibration

A) Re-Calibration of iMALDI by ELISA calibrators



B) Re-Calibration of LC-MS/MS by ELISA calibrators

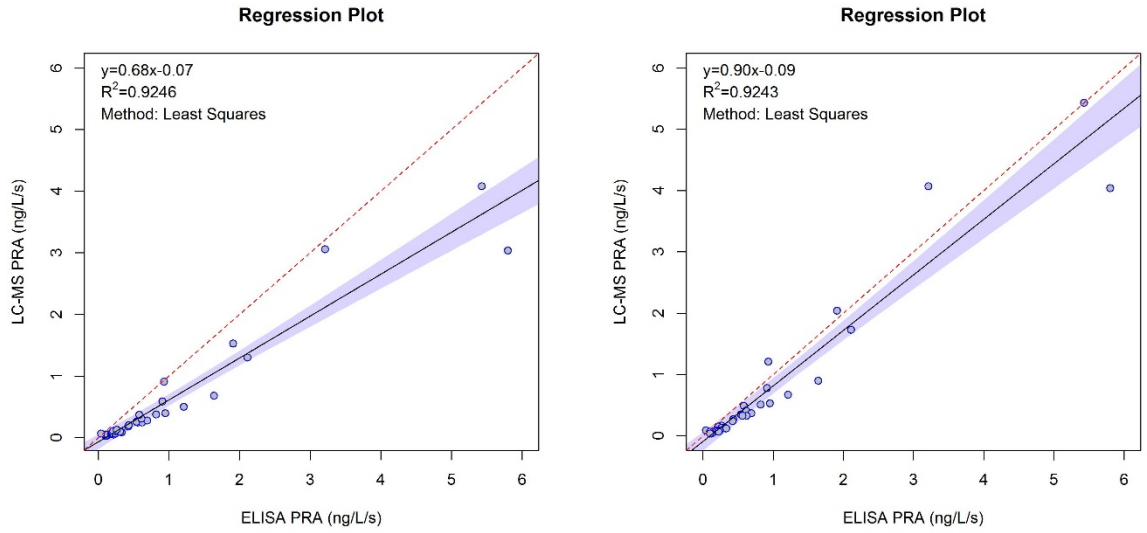


Figure 9. Regression analyses before (left) and after (right) instrument calibration using ELISA calibrators

After re-calibration of both iMALDI and LC-MS/MS methods with DBC ELISA PRA calibrators, slopes of 0.57 and 0.90, respectively.

The correlation of Bio-Rad EQAS monthly external quality control with iMALDI, and with LC-MS/MS method revealed a slope of 0.74 and 0.9, respectively.

7. Results summary and discussion

The automated, high-throughput iMALDI PRA assay showed strong correlation with two currently employed clinical methods for PRA determination. The results of method comparison studies are summarized in Table 7 and Table 8. This method has shown its application on two MALDI-TOF instruments from different manufactures with excellent correlation.

Table 7. Summary of method comparisons with ELISA PRA

	iMALDI	**4800**	**LC-MS/MS**
ELISA (before re-cal)	slope = 0.41 R^2 = 0.92	slope = 0.40 R^2 = 0.89	slope = 0.68 R^2 =0.92
ELISA (after re-cal)	**slope = 0.57** R^2 =0.92	N/A	**slope=0.90** R^2 =0.92

Table 8. Summary of method comparisons with iMALDI PRA

	iMALDI
4800	slope = 1.01 R^2 = 0.99
LC-MS/MS	slope = 0.6 R^2 =0.97

The correlation study also revealed a consistent proportional error in the iMALDI method when comparing to both ELISA and LC-MS/MS methods. The expected slopes of regression analyses were 1, but the iMALDI results were approximately 60% lower than the ELISA results, and 40% than that of the LC-MS/MS method.

This error was initially thought to be due to a calibration issue, as each method was calibrated independently with different sets of calibrators. Both MS methods were then re-calibrated using the DBC ELISA calibrators to correct for this proportional error. After re-calibration, the LC-MS/MS method showed a slope of 0.9 suggesting that most of the error was due to instrument calibration. The other 10% error was thought to be due to methodological differences at various concentrations. Two methods had different reagents and reaction conditions.

DBC ELISA PRA assay is a competitive immunoassay, whereas LC-MS/MS assay uses an off-line solid phase extraction followed by liquid chromatography. Since there currently is no reference method for PRA assay, DBC ELISA assay could have had a 10% overestimation of all results obtained.

On the other hand, re-calibration of iMALDI method increased the slope from 0.41 to 0.57 suggesting that most of the difference between these two methods are not due to calibration. The workflow of iMALDI does not require elution of analyte from the antibody-bead conjugate. Calibration material, along with internal standards, are prepared in PBS (with 0.015% CHAPS) solution followed by antibody capture. Patient's samples were incubated with generation buffer for 3 hours followed by antibody capture. Different workflow and different incubation conditions might have affected calibrator recovery in the iMALDI method that led to differential ionization or detector saturation.

CAP performance specifications for analytical validation requires accuracy, precision, analytical sensitivity, analytical specificity, reportable range and reference range. Previous experiments also have demonstrated clinically suitable analytical sensitivity, specificity, reportable range, precision and a high-throughput of up to 360 samples per run.

Table 9. Other performance characteristics of iMALDI PRA assay

	iMALDI PRA assay	**Guideline**
Precision	(1.5 ~to 14.3% CV)	<15% (FDA guidelines for bioanalytical method validation)
Reportable range	(0.04 ~ 5.3 ng/L/s)	0.06~0.08 ng/L/s (Endocrine Society Clinical Practice Guidelines)

Analytical accuracy of the high-throughput iMALDI PRA assay needs to be further evaluated. The current PRA assay lacks reference method, and internationally recognized standardized reference material. However, there is a non-WHO reference material from the National Institute for Biological Standards and Control (NIBSC) which allows for common high-quality

celebrants. Since MS-based PRA assay lacks standardization, and the iMALDI assay is a novel method, another possibility is to develop analytical accuracy is to validate its own reference ranges. Although this would require at least 120 specimen from healthy population, which can be challenging to define. Reference ranges for clinical PRA assays are summarized in Table 10. Finally, a simpler approach would be to apply a mathematical correction factor to the iMALDI PRA assay to adjust the calibration slope to match with that of the DBC ELISA method.

Table 10. Current reference ranges for PRA

	ELISA (Jewish General Hospital)	**LC-MS/MS (St. Paul's Hospital)**
supine:	0.03-0.86 ng/L/s	<0.45 ng/L/s
standing:	0.45-2.06 ng/L/s	0.10-1.10 ng/L/s

Clinical implementation of the high-throughput iMALDI PRA assay will still require a proper cost-effective study, but through optimization of reagents, protocols, robotic systems, and software, the entire iMALDI platform for PRA determination has been automated into a robust, and user-friendly diagnostic platform applicable for use in clinical laboratories.

8. Conclusion

The diagnosis of PA relies on biochemical evidence of relative aldosterone excess and confirmation of abnormal aldosterone production using suppression testing. Excessive and autonomous aldosterone secretion is characteristically accompanied by low or undetectable renin due to feedback inhibition from sodium excess and increased blood pressure. Many patients with PA do not have increases of aldosterone above the reference interval. Rather, they have increases relative to AngII stimulation, the surrogate measure of which is plasma renin activity (PRA). The aldosterone-to-renin ratio (ARR) is generally considered the best first-line screening test for hypertensive patients in whom there is clinical suspicion of PA.

An immuno-Matrix Assisted Laser Desorption/Ionization (iMALDI) method has been developed using anti-AngI antibodies, which are incubated with plasma sample, washed, placed directly on a MALDI target, and analyzed by mass spectrometry (MS).

This project describes a 3-way comparison of methods for determining PRA: a prevalent clinical method, using automated immunoassay (ELISA); an LC-MS/MS method involving on-line HPLC separation and electrospray ionization for quantitation of angiotensin, and an iMALDI method which does not involve either HPLC separation, but instead utilizes antibody capture of angiotensin.

Current immunoassays rely solely on a single principle of antibody binding, and therefore, lack the much needed specificity for clinical diagnostics. The combination of immunoaffinity and MALDI-TOF MS offers excellent specificity by inheriting the principle of MS analysis to accurate quantification of the target of interest. With automated liquid handling system, the immuno-MALDI MS operates as a high-throughput and clinical diagnostics tool applicable for early diagnosis, discrimination of differential diagnosis and monitoring of therapeutic efficacy.

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