IMPROVEMENT AND VALIDATION OF THE HEAVY METAL BLOOD 4 PANEL ASSAY USING ISOCRATIC PUMP DIRECT INJECTION ON LIQUID CHROMATOGRAPHY ICP-MS

by

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STATEMENT OF THESIS APPROVAL

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ABSTRACT

This thesis presents the validation results on a modified heavy metal panel assay known as HYMET4 Blood Panel. It lays out the importance of metal testing in the clinical setting, and presents the underlying motivations that compelled the improvement of the current assay.

We validated the measurement of arsenic, cadmium, lead, and mercury in whole blood using an Agilent Isocratic HPLC system and autosampler as the sample introduction system, herein referred to as Isocratic Pump Direct Injection (IPDI). The autosampler accommodates two 45 vial holders that increases sample throughputs. Sample preparation and introduction have been modified as well as data analysis parameters. Validation studies conducted were imprecision, sensitivity, accuracy, Analytical Measurement Range (AMR) or linearity, recovery, and carryover. EDTA, gold, DMSA, and DMPS have been used to study mercury stability in solution.

The data from the validation studies of all four metals in the panel are analyzed. The results of the imprecision, sensitivity, accuracy, AMR, recovery, and carryover studies are as follows and are well promising. Of the four chelators used for the mercury stability study, DMPS gave the best results.

The validation results suggest that the modifications made to the HYMET4 Blood Panel assay have substantially improved the assay. The successful validation of the modified assay also suggests that the improved assay will increase the laboratory

throughput with the use of the two 45 vial holders. It will increase sensitivity of the results using the newer analytical system. It will also reduce current sample volume by one fifth, which leads to laboratory cost saving and patient specimen volume reduction. It has been proposed based on comparison data that the use of Cetac autosampler will give a more robust touch to the improvement process of the assay. Cetac autosampler is engineered uniquely for use in trace element testing as opposed to the IPDI system engineered for biochemical analysis.

Dedicated to:

Bright

Majoie

Olivia

Wisdom

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1. INTRODUCTION

"From a public health point of view, the term heavy metal usually refers to a metal or semi-metal that has the potential to cause human or environmental toxicity" (World Health Organization, 2014). The primary location of heavy metals is the crust of the earth, but metals are released on the earth surface through volcanic and mining activities. In today's world, heavy metals have become ubiquitous due largely to human action. Metals that are rarely found in the air or on the surface of the earth are now deposited in many places other than their natural location. The advent of industrialization has increased the preponderance of heavy metals on the earth surface and in the environment. This increase in environmental pollution is the cause of many growing health issues around the world, especially in the industrialized countries where many industries manufacture products that contain these toxic metals. The implications to the workers in such industries are acute or chronic exposure to heavy metal vapors. Heavy metals like lead, cadmium, mercury, and arsenic are common in our environment. They are found in volcanic areas, mining sites, and soil, in water and even in household items such as cosmetics products.

The Occupational Safety and Health Administration (OSHA) requires periodic medical examinations to monitor industrial workers for potential exposure. The World Health Organization (WHO) has set guidelines on heavy metals exposure to protect anyone who may have had exposure (World Health Organization [WHO], 2001). These

regulations and recommendations are put in place because some heavy metals are toxic to humans. In addition to WHO and OSHA requirements, physicians can request heavy metal testing as part of a diagnostic evaluation for an individual who presents with clinical signs and symptoms of heavy metal toxicity.

We incorporate metals into our body mostly through inhalation, ingestion, or physical contact. Since the body is unable to metabolize them, they accumulate in our tissues and become toxic upon chronic exposure.

Only a few of the many metals that exist in nature are metabolically essential for our body. Metals such us copper, manganese, chromium, molybdenum, selenium, iron, zinc, and sulfur play a catalytic role in biochemical processes and are only needed in trace amounts.

By mimicking the structure of beneficial metals, biologically toxic metals such as mercury (Hg), lead (Pb), arsenic (As), and cadmium (Cd) can interfere with the enzymatic activities of cells. They do this by binding to proteins or lipids that traffic through the body. This physiological interference of heavy metals causes neurotoxicity and carcinogenicity (WHO, 2014).

Due to the seriousness of metal toxicity, it is clear that a sensitive heavy metal panel assay is needed for accurate metal detection in samples. Some toxic metals present similar signs and symptoms. For example, mercury, lead, and arsenic toxicity affects the central nervous system. Mercury, lead, and cadmium have the ability to affect the kidney and the liver. Cadmium can affect the skin, the teeth, and the bones (WHO, 2011b). This makes the accurate identification of the specific metal a challenge to the physician unless a good patient history is available. For this reason, upon suspecting a metal-induced

ailment with a patient history, it is advisable to test a panel of metals for accurate and timely diagnosis.

The Trace and Toxic Element Department at the ARUP Laboratories is dedicated to accurate and timely testing of heavy metals. One of their test menus is called HYMET4 Blood. The HYMET4 Blood assay is a Heavy Metal Blood Panel Assay that is currently in production in the Trace and Toxic Elements (TTE) laboratory for the testing of lead, cadmium, arsenic, and mercury.

The aim of this thesis research is to modify the HYMET4 Blood Panel by focusing on the pre-analytical and analytical processes of the test procedure. After exploring the reasons behind the modification of this panel, and detailing the means of achieving the areas of improvement, the results from the validation studies are explained. Following the required validation of the improved assay, we hope to have a more robust testing method for this panel in the clinical setting.

2. REASONS FOR THE HYMET4 BLOOD IMPROVEMENT

Similarities of signs and symptoms of toxic metals exposure make specific diagnosis a challenge for the clinician. For this reason, it is ideal for the healthcare provider to request a panel of metal tests if multiple metals are suspected and in the absence of a clear patient history. Since metals are present in trace amounts, the need for sensitive testing methods is critical. Current analytical systems can accurately quantify the amount of trace elements in a sample even at parts-per-trillion. Even though sample types can range from environmental samples such as water, sediment, or air to biological samples like whole blood, plasma, serum urine, nail, and hair, the focus of this project is on whole blood.

To improve the accuracy of heavy metals testing is to increase the sensitivity of the analytical unit and to improve sample preparation and introduction. From sample preparation to sample introduction through the analytical unit to the generation of the results, trace element detection can be improved by modifying some of the analytical steps. Our analytical system for the test was upgraded from Perkin Elmer to Agilent.

Areas of modification included the following.

This project proposal was presented and focused on the following with their respective benefits:

- Sample volume reduction by one fifth.
- Mercury chelation to stabilize Hg in solution.

- Use of the 96-well plate to increase the laboratory sample throughput.
- Use of the Agilent LC autosampler with the Isocratic Pump Direct Injection (IPDI) system for discrete volume pipetting.
- Use of the air gap injection program on the IPDI system to avoid sample loss to the mobile phase.
- Laboratory resources management for cost saving.
- Use of Agilent ICP-MS as an analytical system for a more advanced testing.

A number of instruments can measure the concentration of trace metals in a variety of samples. Acceptable sample types in a clinical setting for trace element testing are whole blood, serum, plasma, hair, urine, and tissues. The available analytical instruments vary in size from handheld to bench top. Some are automated point-of-care testing devices and others are sophisticated analytical instruments. All these instruments have varying degrees of sensitivity and accuracy based on their specific principles of detection (WHO, 2011a). Some point-of care-testing devices have high imprecision but with a fast turnaround time and low sample volume compared to inductively couple plasma mass spectrometry (ICP-MS). According to WHO, ICP-MS technology is the most sensitive method for trace element detection with detection down to parts-per-trillion (ng/L) for the majority of elements in a sample (WHO, 2011a).

After the necessary improvements are made, the validation study is conducted to confirm the feasibility and practicality of the modified assay. The complete research phase validation ensures that primary production validation can be carried out with success and that the test can be put into production after all the necessary CAP and CLIA requirements are met by the TTE laboratory.

Before explaining the methodology used for the validation study, we will first explore the background of each of the elements in the HYMET4 Blood Panel. The following sections will give an overview of each element on the HYMET4 Blood Panel with their possible sources of exposure and the signs and symptoms associated with their toxicity.

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3. HEAVY METAL BLOOD 4 PANEL OVERVIEW

3.1 Lead

3.1.1 Background and Sources of Exposure

Lead is a toxic metal primarily found in the earth. Due to human activities, lead is now a major pollutant of the environment as it is found in many of the products that we use. Some industrial workers are permanently exposed to lead at their working place (Occupational Safety and Health [OSHA], 2015). Battery manufacturers, demolition workers, pipe filters, welders, construction workers, printing servicers, and jewelers are all chronically exposed to lead.

Lead is a major concern for health care organizations mainly due to the danger it poses to children. Homes built before 1978 were painted with lead-based paint and children living in those homes become exposed through inhalation or ingestion of lead chips (Center for Disease Control [CDC], 2015). According to the Center for Disease Control (CDC), "At least 4 million households have children living in them that are being exposed to lead" (CDC, 2015). Exposure to lead paint is the most common way for children to be exposed to the toxic metal.

Another means of lead exposure is through household drinking water, especially that found in old houses with pipes made from materials containing lead. As water runs through those pipes, the lead in the material used to make the pipes is released into the water as it comes out through the faucet. Contaminated drinking water is the most

common way for adults to get lead into their bodies. With their fast metabolism, children tend to absorb lead faster than adults, about 50% compared to 10% for adults (Mott, Fore, Curtis, & Solomon, 1997). Even though there is no safe level of lead in the blood, the CDC cut-off for lead concentration in the blood is 5ug/dL.

3.1.2. Signs and Symptoms

Lead is toxic to many parts of the body, including the central nervous system (CNS), liver, and kidney (WHO, 2011a). The cardiovascular system can also be affected. Lead has the ability to cross the blood-brain barrier because it can mimic a calcium ion. Calcium mimicry allows lead to interfere with the biological activities of the body like red blood cell formation and bone growth (Sanders, Liu, & Buchner, 2009). Lead can also mimic other ions needed for biochemical metabolism such as zinc and iron (Lead poisoning, 2015). With its ability to cross the blood-brain barrier and to interfere with cellular functions, lead can damage the brain, especially the developing brain, causing neurological disorders. Lead toxicity affects the development of growing children, and even unborn children whose parent has been exposed. Once into the body, lead will deposit on various parts of the body, interfering with the normal developmental processes of those organs. Lead's effects on children can be so severe as to impair their cognitive development, slow down their physical growth, and stunt the growth of their nervous systems.

Lead testing is necessary for diagnosis and for proper treatment of those who are exposed. Prompt and accurate testing in children will prevent developmental retardation and other growth delays.

3.2 Cadmium

3.2.1 Background and Sources of Exposure

Like other metals, cadmium was originally found in the crust of the earth. Cadmium pollution of the environment came through volcanic activities, mining, and other human activities. These natural and human activities have increased the cadmium content of the soil, and its uptake by green leaves is very efficient. Thus, plants that grow on cadmium-polluted soils have a very high level of cadmium content as compared to plants that grow on unpolluted soil (Keil, Berger-Ritchie, & McMillin, 2011; Satarug et al., 2003). This implies that even though cadmium is a nonessential element in plant metabolism, plants can extract cadmium from the soil through a biochemical process. The biggest concern of public health authorities in almost every country in the world is the increasing prevalence of cadmium in agricultural foods (Robson, Braungardt, Rieuwerts, & Worsfold, 2014). Food grown on cadmium-polluted soil is the biggest source of exposure for the general public. As we depend on rice and potatoes for our carbohydrate intake and on vegetables grown on polluted soil, the amount of cadmium in our bodies has increased to a toxic level. Tobacco leaves produced on cadmium-polluted areas show significant increases in the concentration of cadmium (Vögeli-Lange & Wagner, 1990). It is not surprising that cigarette smokers have increased cadmium toxicity. Unlike lead, everyone is exposed to cadmium toxicity by the ingestion of leafy plants or vegetables that have been grown on soils that have a high level of cadmium content. Tobacco smokers are at high risk as they can incorporate more than the daily recommended amount of 30 ug (Lewis, Coughlin, Jusko, & Hartz, 1972; Satarug, Haswell-Elkins, & Moore, 2000). Drinking water can contain an elevated level of cadmium from the ground.

As per the U.S. Environmental Protection Agency (EPA), 5 parts per billion (ppb) is the safe level of cadmium content in our drinking water (U.S. Environmental Agency [EPA], 2013). Cadmium can also be found in batteries, paint, coatings, pigments, alloys, and even toys.

3.2.2 Signs and Symptoms

Cadmium poisoning occurs when someone inhales cadmium-polluted air or has had an acute or chronic exposure to cadmium through smoking, eating contaminated crops, or drinking contaminated water (Bernhoft, 2013). Cadmium has no known physiological benefit in our bodies. It is a carcinogenic element because it can initiate oncogenesis by rendering inefficient the tumor necrosis factor cytokines, thereby increasing the rate of tumor formation in the body (Waalkes, 2000). Cadmium inhalation causes bronchitis. Chronic exposure to cadmium will cause kidney dysfunction and lung cancer (OSHA, 2003). As cadmium enters the body, it complexes with albumin in the liver (Keil et al., 2011) where it can redistribute and accumulate in different areas of the body, especially in the bones, causing low bone density. With its structural similarities to zinc, cadmium will mimic zinc in the body by binding to the zinc binding site on proteins, thus altering cellular functions. Cadmium induces the production of reactive oxygen species and initiates a cascade of molecular disturbances in the body (Chmielowska-Bąk, Izbianska, & Deckert, 2013). Cadmium bio-toxicity has been linked to organ damage of the reproductive, respiratory, and renal systems (Godt et al., 2006).

Due to the severity of the toxicity, the need for accurate and sensitive testing for cadmium must be advocated.

3.3 Arsenic

3.3.1 Background and Sources of Exposure

Naturally found in the earth, arsenic is a toxic metal of growing concern for pollution due to mining and its widespread use as a wood preservative and pesticide (National Institute of Health [NIH], 2014). Most of the arsenic found in the United States is used in the production of wood preservatives. Some can also be found in the fabrication of paint, dyes, metals, drugs, soaps, and semiconductors (EPA, 2012). Arsenic can be found in food grown on polluted soil, and in ground water due to water runoff that runs down from mining sites. Most arsenic is released into the ground water from rocks, but rain water also transports arsenic from the air into ground water, therefore increasing the pollution level of standing water. Arsenic can also be found in our drinking water. Drinking water concentrations of arsenic in the western regions of the United States are above the recommended level set by the EPA (Lewis, Southwick, Ouellet-Hellstrom, Rench, & Calderon, 1999). This is due to the release of arsenic by the Rocky Mountains in the region because mountain soil contains higher than normal level of arsenic. Arsenic toxicity can be caused by chronic inhalation of arsenic in the air, through the use of pesticides, or wood preservatives. Chronic exposure to arsenic through drinking water is the most common source of arsenic intoxication, as was the case in Bangladesh (Uddin & Huda, 2011).

3.3.2 Signs and Symptoms

Apart from its gas form, arsenic presents in several states: As (-3), As (0), the "pentavalent (As^{5+}, arsenate), and trivalent (As^{3+}, arsenite)" (Kaur, Singh, & Goel, 2011).

The most toxic species is the inorganic form arsenite (As^{3+}), which is highly soluble in water (Agency for Toxic Substances and Disease Registry, 2010). Inorganic arsenic is a known carcinogen. It can disrupt molecular processes by binding strongly to molecules and causing metallic carcinogenesis. Inorganic arsenic will cause aberrant DNA formation and production of reactive oxygen species (Sigel, Sigel, & Sigel, 2011). Arsenic has the ability to mimic phosphate, thereby inserting into DNA during DNA formation (Tawfik & Viola, 2011).

Consistent drinking of water that contains higher than recommended levels of arsenic can induce systemic health issues (Utah Department of Health, 2015).

A sensitive detection method is therefore needed for arsenic testing in order to reduce exposure before symptoms appear.

3.4 Mercury

3.4.1 Background and Sources of Exposure

Along with arsenic and lead, mercury is the third most dangerous toxic metal listed by the US Government Agency for Toxic Substances and Disease Registry (Bernhoft, 2012). Like the other toxic metals, mercury is also primarily found in the crust of the earth and it is through atmospheric discharge, erosion, mining, combustion, industrial discharge, and urban discharge that the environment is polluted with mercury. Mercury is a very toxic element that is of high concern for public health authorities in industrialized and nonindustrialized countries alike (Alo & Olanipekun, n.d.). Nevertheless, mercury levels in the air are higher in industrial countries than in other parts of the world. This is due to mining and to the production of mercury compounds

like the light bulb, thermostat, paint, batteries, thermometer, pesticides, toys, and medical products (Bernhoft, 2012; Rice, Walker, Wu, Gillette, & Blough, 2014). According to the Food and Drug Administration's (FDA) regulatory information, some consumer-used drugs contain mercury (North Dakota Department of Health, 2010). Vaccines as well as plasma-derived product like Immune Globulin contain mercury as preservatives (U.S. Food and Drug Administration [FDA], 2015). This shows how useful mercury can be and also how we are exposed to it in multiple ways. However, despite the presence of mercury in items used every day, the biggest route of exposure to mercury is inhalation of mercury-polluted air, dental amalgam, and ingestion of contaminated seafood. Even though there is no safe level of mercury exposure, the level of toxicity depends on the form of exposure, the dose, and the length of time of exposure.

Mercury can be presented in three different forms: elemental mercury that is pure mercury without any other element bound to it, organic mercury which is mercury bound to another non-carbon-containing element, and organic mercury which is mercury bound to a carbon-containing element. Elemental mercury is volatile at room temperature and the route of exposure is through inhalation with the brain being the target organ (New York State Department of Health, 2013). Exposure to elemental mercury is likely to take place at the site of mining or industrial sites where the vapor is inhaled. The exposure to organic mercury usually occurs through inhalation at a polluted site or usage of products containing mercury like medication, vaccines, and body lotions. Eating contaminated fish is another way that people are exposed to organic mercury like methyl mercury. Fish can contain a higher level of mercury than their environment depending on where they are in the food chain. The bigger fish will contain more mercury than the smaller fish in the

same ecosystem and this is because the bigger fish eat the smaller fish and retain the mercury of the smaller fish. Exposure to inorganic mercury is mainly though the ingestion of contaminated seafood.

3.4.2 Signs and Symptoms

One of the mimics of mercury is when methyl mercury binds to cysteine compound to mimic methionine. The mimicry is due to the structural similarities between the methyl-mercury-cysteine species and the amino acid methionine (Hoffmeyer et al., 2006). The bioavailability of mercury in the human body depends on the specie and the form of toxicity. Depending on its source and nature, mercury can accumulate in different areas of the body. Inhaled mercury vapor can readily enter the blood stream and accumulate in the brain, where the half-life can be up to 20 years due to its capacity to bind to lipid (Friberg & Mottet, 1989). Different species of mercury are handled differently by the body and the areas of accumulation differ in the body. This leads to differences in toxicity and symptoms presented. Dimethyl mercury is one of the most dangerous of all the mercury compounds as it can easily cause death upon exposure of a very minimal amount (Joshi, Mittal, Shukla, & Srivastav, 2012). Inhaled elemental mercury, Hg(0), is mostly stored in the brain while the inorganic mercury $HgCl_2$ is stored mainly in the kidneys (EPA, 2016; Heller, Zieve, & Black, 2014). Organic mercury is mostly contained in the red blood cells and it can cross the blood brain barrier by bonding to compounds like L-cystein and mimicking their activity, therefore fooling the body and getting into the brain. Mercury poisoning can result in a wide range of symptoms, from mental retardation, neurological deficit, vision loss, abnormal muscle tone, and

developmental delay, to even death. It is noteworthy to state that most asymptomatic people have a high level of mercury in their blood (Hightower & Moore, 2003). This is of concern regarding childbearing women in that group as their unborn children can be seriously affected. Methyl mercury ingestion through fish consumption has been strongly associated to heart muscle defect (Guallar et al., 2002; Rice et al., 2014).

Viewing the burden that the four elements in the HYMET4 Blood Panel place on the body, and knowing that they are only present at trace levels in the body, the need for a sensitive and accurate testing system for the panel is clear. The confirmation of the accuracy of the testing method is regulated by CLIA88 (Clinical Laboratory Improvement Act of 1988) a laboratory regulatory body overseen by CAP (College of American Pathologists) and CMS (Center for Medicaid and Medicare Services).

4. LABORATORY TESTS REGULATIONS

As we aim to improve the current HYMET4 blood testing methodology, we must validate the modified assay for compliance. Laboratory assay validation is a requirement of CLIA, the Clinical Laboratory Improvement Act, for new and modified clinical laboratory tests. Enacted by the US senate in 1988, CLIA is a set of regulations concerning clinical laboratory practices (Burd, 2010). "The Centers for Medicare & Medicaid Services (CMS) regulates all laboratory testing (except research) performed on humans in the U.S. through the Clinical Laboratory Improvement Amendments (CLIA)" (U.S. Centers for Medicare and Medicaid Services, 2015). Ensuring quality of laboratory tests in all CLIA-certified laboratories is the objective of the CMS. The College of American Pathologists (CAP) is the accrediting agency of medical laboratories in the US and they require compliance with all CLIA regulations, including laboratory tests validation. Laboratories must comply with the CLIA law to stay in practice and to receive reimbursement from CMS.

Validation is defined as a way for a laboratory to prove that a test procedure is accurate and trustworthy and that the inherent results are accurate, and therefore can be used for patient treatment. To prove the validity of a clinical test, the laboratory must conduct a set of studies that will verify the reproducibility and the repeatability of the test system despite the analyst and the location of testing (Burd, 2010). Reproducibility studies refer to interlaboratory precision of the test and repeatability studies ensure

within-laboratory precision (González, Herrador, & Asuero, 2010). The analytical system must be tested regarding its sensitivity toward the analyte in the sample and also its specificity in the detection of the analyte. In addition, reportable range and reference intervals must be studied as well as other performance characteristics such as recovery and carryover, which may be of clinical significance to the test results (Oliver, 2010).

To be in compliance with CLIA, the improved HYMET4 Blood assay was validated through the following set of studies: imprecision, accuracy, sensitivity, recovery, carryover, and AMR. Studies of the reportable range and reference intervals were not carried out as these were done at the initial introduction of the assay into production. The objective of each one of these studies is explained and the protocol of carrying them out is laid out on the next pages.

5. MATERIALS AND METHODS

5.1 Materials

5.1.1 Instrument Configuration

The ICP-MS instrument used for the determination of the trace elements in this validation was an Agilent ICP-MS 7700X calibrated and tuned for the detection of a wide range of elements, including lead, cadmium, arsenic, and mercury. The carrier gas was argon. Helium gas was used in collision mode to reduce polyatomic interferences when needed as in the analysis of arsenic.

The sample introduction system was the Agilent 1260 Infinity Binary LC, used for direct sample introduction and referred to as IPDI. The configuration of the LC system in this application consisted of an isocratic pump stacked to the autosampler with the solvent rack on top. The autosampler was connected to the ICP-MS via the nebulizer by a capillary. The autosampler chamber can either hold 2 96-well plates or 2 sets of 45 1-ml vials.

5.1.2 The Isocratic Pump Direct Injection

The pump consisted of 2 pistons that move in and out, allowing a precise amount of liquid to flow through. The solvent flows in the inlet valve from the bottle through the first piston into the damper where it is drawn by the second piston into the metering device. This ensures a discrete volume (based on the flow rate) flows through the sample

loop and the needle into the capillary which is connected to the nebulizer during the mainpass position of the injection valve (Agilent Technologies, 2015). When the injection valve switches to bypass position during sampling, the pump directs the flow of solvent into the capillary.

5.1.3 The Agilent LC Autosampler

As mentioned above, during the sampling sequence, the injection valve of the autosampler switches to the bypass position. The needle is lowered into the sample vial and aspirates the required volume of sample. The needle then moves to the wash station to clean its outside in order to minimize carryover. The sample loop then closes and the injection valve switches to mainpass in order to inject the sample into the capillary and also to redirect the flow of solvent back into the mainpass (Agilent Technologies, 2015) which leads into the nebulizer. The flow rate of the solvent pushes the sample into the analytical system. With the air gap injection program, a certain amount of air is injected between the sample and the solvent to prevent mixing. The sample flow rate was set at 0.100 ml/min and the sample volume was 90µL.

5.1.4 The ICP Torch

The sample is introduced into the nebulizer by the autosampler where it is mixed with argon gas as the carrier. The nebulizer aerosolizes the sample into fine droplets into the spray chamber where large droplets fall to the drain. The small aerosols leave the spray chamber and enter the ICP torch via the transfer tube. In the torch, the sample is dried and atomized in the plasma. This plasma is generated by a radio frequency (RF)

power that is applied to the metal coil that surrounds the torch, creating a magnetic and electrical field in the torch through which flows the argon gas containing the sample. A discharge in the argon contained in the torch causes the argon atoms to become positively charged through the loss of an electron. The released electrons accelerate in the magnetic field and collide with other atoms, causing them to lose electrons. These collisions of accelerated electrons with argon atoms generate a plasma temperature of about 6000 K which provides the necessary energy for complete ionization of atoms in the sample (Wolf, 2005). The ionized sample enters the mass spectrometer through the interface cones.

5.1.5 The Mass Spectrometer

In the mass spectrometer, the specific element of interest is selected by the quadruple mass filter based on the mass-to-charge ratio and sent to the detector where the intensity is multiplied, quantified, and displayed on the monitor. In collision mode, helium gas collides with the polyatomic molecules of the sample in the collision cell causing them to lose energy, thereby reducing polyatomic molecule interference and improving speciation, which is required for arsenic.

5.1.6 Data Analysis

The Mass Hunter software was used for data acquisition and analysis of the elements of interest. Data were collected during spectrum mode. The analysis of the lead element was done by summation of the most abundant isotopes. The following were the targeted masses of each element on the panel.

- Lead 204, 206, 207, and 208
- Arsenic 75
- Mercury 201
- Cadmium 111

5.1.7 Reagents

Matrix Matched Diluent (MMD) was used for all dilutions. MMD contains 0.5% nitric acid, 0.05% Triton X-100, 1000 µg/L each of beryllium and gold, 50 µg/L gallium, 50 µg/L iridium, and 25 yttrium µg/L. All were mixed in clinical laboratory reagent water. The matrix used was goat whole blood. Four commercially available multianalyte standards materials containing all four elements were used to quantify the isotopes. Also, four multianalyte control materials were used to assess the acceptability of the calibration curve. The internal standard, Indium—used to compensate for ion suppression, was added to the diluent. The mobile phase that carries the sample was composed of 1% ethyl alcohol, 3 mM $NaNO_3$, 0.2 mM EDTA disodium salt, 2 mM NaH_2PO_4 , and 10Mm $NaCH_3COO3H_2O$. Clinical Laboratory Reagent Water (CLRW) was used for the preparation of the blank.

5.2 Mercury Chelation

Among the four elements on the panel, mercury is the only one that tends to quickly diminish in concentration over time while in solution. The loss of mercury in solution mostly affects the accuracy of its measurement. To reduce this loss of mercury, gold is being currently used as a chelator. Mercury can be lost through various processes

such as adhesion on the wall of the container and volatilization. In low concentration solutions and in the presence of mild reducing agents, Hg^{2+} will gain an electron and become Hg^{+}, which will transform into volatile metallic mercury and escape from the solution (Yu & Yan, 2003). A detailed explanation of this phenomenon is beyond the scope of this project. The loss of mercury in a laboratory setting is mostly due to its adsorption ability on the wall of the containers and turbines of the analytical instrument. This is more pronounced on polyethylene surfaces than glass surfaces (Gaines, 2016). Mercury loss can also partially be caused by the reaction of mercury with some elements that are present in the analytical environment (Batema, 2015). To mitigate mercury loss, a chelation experiment was conducted to find the best chelate for mercury because the current chelate, gold, used in the laboratory was not proving effective at preventing mercury loss during analysis. The mercury standards were prepared from a secondary standard of HgN (100µg/ mL of Hg in 10% HNO_3) purchased from Organic Ventures in Lakewood, New Jersey. To prevent quality controls failure, the laboratory currently prepares the working calibrators and controls weekly as a way of preventing run failure because of the limited ability of gold to reduce loss. In addition to its ability to adsorb on the wall of plastic containers, this loss of mercury in solution is due to its capability to selectively react with other elements in the testing environment. The rate of mercury adsorption is based on its concentration in the solution, the elemental constituents of the container, and the temperature of the environment (Luo et al., 2004). As part of the routine laboratory operation, glass containers are currently used to store the prepared working standards and controls, but testing specimens are prepared in plastic vials. To lessen the burden of constant preparation of working solution, a chelation study was

found necessary. During the chelation study, we experimented with Ethylenediaminetetraacetic Acid (EDTA), Dimercapto Succinic Acid (DMSA), and dimercaptopropane sulfona (DMPS) in addition to gold to find the strongest chelator. A stronger chelator will help reduce the frequency of preparing mercury working calibrators and controls materials by the laboratory staff. The abilities of these compounds, with the exception of gold, to stabilize mercury are due to the presence of sulfur groups. Mercury has a high affinity for the sulfur groups (Sugiura, Tamai, & Tanaka, 1978). These thiol groups form strong bonds with mercury therefore preventing the mercury molecules from reacting easily with other elements in the analytical environment (Sears, 2013).

Two concentrations of mercury, 10µg/L and 20µg/L, were prepared for the experiment. Each of the above two concentrations of mercury contained 1mg/ml and 2 mg/ml of each chelate. This means each chelate had a total of four samples that were prepared and tested. All samples were analyzed in the production lab for a period of 20 days and the data collected are shown in Figure 1.

From the data, DMPS and DMSA showed less mercury loss compared to the others. Throughout the project, DMPS has been used to chelate mercury because it showed higher solubility in solution than DMSA.

5.3 Working Calibrator Preparation

The working calibrators were made from a combination of four individual stock solutions. Four concentrations of calibrators were prepared containing cadmium, mercury, lead, and arsenic. Goat whole blood was spiked with a calculated amount of each analyte from the stock solution, purchased from Organic Venture, to make the

desired concentrations. The same batch of working materials was used during the project. To keep mercury in solution, 0.1% of DMPS was added to each prepared solution. Table 1 lists the concentrations of each analyte.

5.4 Preparation of the Control Materials

The same procedure used to prepare the calibrators was used to prepare the controls. The matrix used was goat blood. Calculated volumes of the stock solutions, from Organic Venture, were directly added to the matrix diluent to prepare the desired concentrations, and 0.01% of DMPS was also added. Table 2 shows the values of the working controls.

5.5 Methods

5.3.1 Current Assay Preparation

The current Standard Operating Procedure (SOP) requires a total prepared sample volume of 5mL. An aliquot of 1mL of that volume was pipetted by the auto sampler for analysis. The eluate was prepared by a 50-fold dilution of the patient sample. A volume of 100 µL of patient whole blood was added to 4800 µL of MMD containing the internal standard. Nitric acid (1% in solvent, 100 µL) was added to the diluted sample to increase the solubility of the elements, hence avoiding their adhesion to the wall of the capillary, nebulizer, and spray chamber. Current sample introduction is by flow injection by a peristaltic pump. Table 3 shows a summary of the current sample preparation.

5.3.2 Modified Sample Preparation

The total volume of sample prepped for the modified assay was 1 ml. The sample was prepped in a 1 ml polypropelene vial and capped with a snap cap preventing evaporation and allowing easy needle puncture. All working calibrators, controls, and spiked samples were in the same matrix of goat blood. Patients' samples were in whole blood. A 1/50 dilution was made for all samples to be assayed on the instrument. The diluent was an acid-based matrix match diluent made in-house. The diluent was made of Nitric Acid, Yttrium, Gallium, Beryllium, Iridium, and Trition-X dissolved in CLRW.

Sample preparation was the same for all calibrators, controls, spike samples, and patient specimens as they were all in the same blood matrix.

Each sample was prepared in the following steps:

- Pipette 960ul of Matrix Matched Diluent into the 1 ml snap cap vial.
- Add 20ul of 1% nitric acid.
- Aliquot 20ul of specific calibrator, control, and samples as needed into the vial. Add 20ul of goat blood for the pool or 20ul of water for the blank as needed. Table 4 presents a summary of the modified sample preparation.
- Cap the vial and vortex well.
- Load the prepped samples on the LC in the following order: blank, pool, Calibrators 1-4, Control 1, Control 2, patients.

Spike samples and PT samples were treated as patients. Table 4 shows how each sample was prepared.

Figure 2 shows the HPLC sample introduction system connected to the Agilent

ICP-MS instrument used for the study. The air gap program on the Agilent HPLC is a highly useful technique used to increase sensitivity and accuracy of the results. The air injection between the sample and the mobile phase prevents the mixing of the sample with the mobile phase.

This technique helps to prevent sample lost due to the mixing of the sample with the mobile phase. It helps to avoid a trailing peak of the spectrum and thus gives more accurate ions counts. A needle wash step also allows preventing carryover. The acquisition method for the ICP-MS instrument was set as displayed in Figure 3.

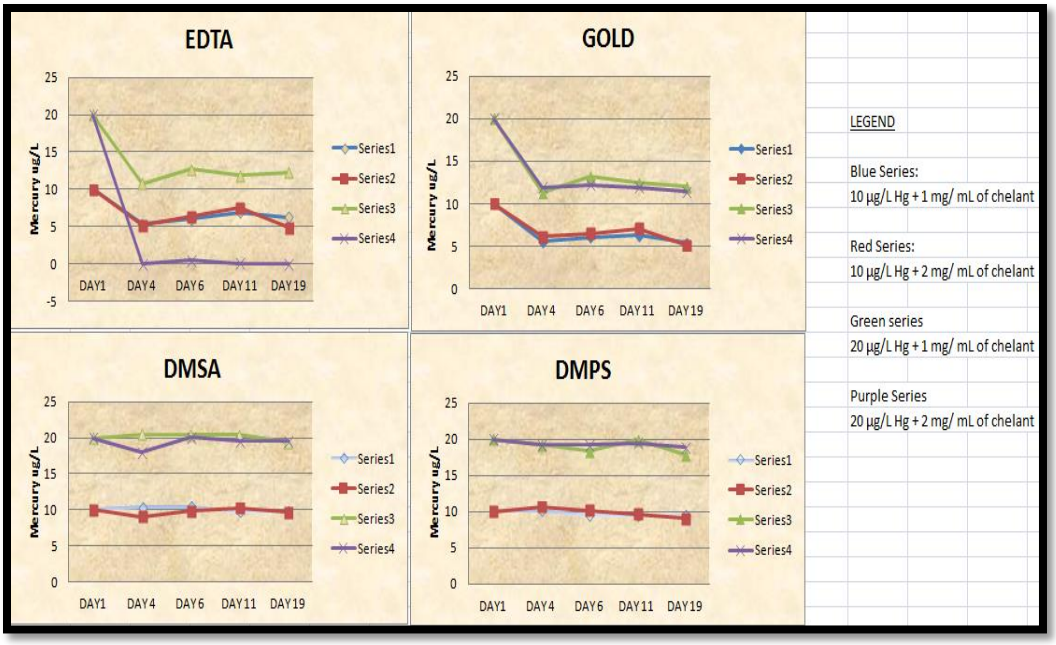

Figure 1: Mercury chelation results data plot. Data showing EDTA, GOLD, DMSA, and DMPS. Mercury shows more stability with DMSA and DMPS at all concentrations.

Table 1: Working calibrators' concentrations.

	As (µg/L)	Cd (µg/L)	Hg (µg/L)	Pb (µg/dL)
STD1	10	1	2.5	2
STD2	50	5	5	10
STD3	100	10	15	20
STD4	250	25	80	50

Table 2: Concentration of controls.

	As (ug/L)	Cd (ug/L)	Hg (ug/L)	Pb (ug/dL)
	Concentration	Concentration	Concentration	Concentration
QC1	10	2	5	2
QC2	16	5	15	6
QC3	55	20	57	20
QC4	100	37	99	76

Table 3: Current sample preparation with required volumes.

Four of each calibrator, controls and patient specimen. (Arsenic in blood and urine by Inductively Coupled-Mass Spectrometry)

	Nanopure water	1% Nitric acid	Goat whole blood (matrix)	Matrix matched diluent (MMD)	Total volume
Blank	100ul	100ul		4800ul	5 ml
Calibrators (100ul)			100ul	4800ul	5 ml
Controls (100ul)		100ul		4800ul	5 ml
Spikes and Patients (100ul)		100ul		4800ul	5 ml

Table 4: Modified sample preparation and volume required for each sample type.

	Matrix Matched Diluent (µL)	1% Nitric Acid (µL)	Sample volume aliquot (µL)	Goat Blood (µL)	CLRW (µL)	Total sample prepped volume (mL)
Blank	960	20	-	-	20	1
Pool	960	20	-	20	-	1
Calibrators	960	20	20	-	-	1
Controls	960	20	20	-	-	1
Spikes samples	960	20	20	-	-	1
Patients samples	960	20	20	-	-	1

Figure 2: Agilent ICP-MS and HPLC complex.

LC autosampler:	
Flow rate: 0.100ml/min	
Injection Volume: 90ul	
Air gap injection with needle wash	
Acquisition parameters:	
Spectrum mode	
Peak pattern: 1 point	
Replicate: 3	
Sweeps: 100	
Mass monitored:	
As 75 in Helium mode	
Cd 111	
Hg 201	
Pb 206,207,208	
ISTDS: 89 (Y) and 193 (Ir) in no gas ;	
193 (Ir) Helium mode	

Figure 3: Instrument setting for data collection. (These settings are experimental settings that gave the best results during the project)

6. MODIFIED ASSAY VALIDATION

The major experiments for the clinical laboratory test validation protocol consisted of the following studies: imprecision, accuracy, sensitivity, recovery, carryover, and AMR. This group of studies was selected from a panel of recommended studies for laboratory assay validation (Dufour, 2008; Taverniers, De Loose, & Bockstaele, 2004). Since the goal of this project was to evaluate the feasibility and the success of the assay modifications, only the most critical studies have been carried out. We must note that all the above studies have no criteria for acceptability since they are used as proof of principle for the production validation of the modified assay. Each of the listed studies is explained in detail below. The analysis of the data was done using an Excel program designed for research statistics.

6.1 Imprecision

Imprecision is the degree of variability among the replicated results. In order to accurately compare the study results, an imprecision study is first carried out to determine the variation among the replicates (Sarewitz, 2013). Inherent variations due to the physical analytical instrument as well as variations due to differences in performing analysts are therefore determined. The rationale in performing the imprecision studies is to determine the repeatability (intra-assay) and the reproducibility (interassay) of the measurements.

6.1.1 Intra-assay

During sample analysis, errors can occur from many sources, including inherent errors from the analytical procedure. From sample preparation to the instrument performance, the laboratory needs to reduce as much as possible the sources of error that can affect the test results. And intra-assay experiment studies variances between the replicates of an analyte (Reed, Lynn, & Meade, 2002). It is an important step in a validation study. It verifies the repeatability of the replicates from the instrument. A minimum of 20 replicates are required to verify the intra-assay result during a validation. The CV (coefficient of variation) calculated from the resulting values of all replicates is used as a comparative figure for acceptability or rejection of the instrument's performance. In this validation experiment, we used two levels of standards materials. Each material was prepared and aliquoted 20 times to determine the CV. The CV = $\frac{SD}{Mean}$ with SD being the standard deviation of the data set.

6.1.2 Interassay

Unlike the intra-assay study that uncovers possible errors related to the analytical procedure, the interassay experiment is conducted during assay validation to discover any error that can originate from the change in analyst, location, reagent stability, or temperature. It is a reproducibility check experiment to validate comparison of results between runs or between laboratories. The goal here is to confirm that the results of the same samples assayed on different days, possibly at different locations by different analysts, will agree within the specifications set during the development of the test (Mandle, 2015). This study is to be conducted interlaboratory and/or interday. Like the

intra-assay, a minimum of 20 data points is required to calculate the variance between the replicate and the various runs. The main difference from the intra-assay is that the 20 replicates are to be assayed on several different days in order to include as much variability as possible. For this validation, we tested four replicates per run for 5 days, spread out over 19 days. The intra-assay and interassay imprecision studies are done using the multi-analytes controls QC2 and QC3 as low and high concentrations. The same calibration materials used for the intra-assay study were used in this experiment. The CV was calculated from the 20 collected data points using the same formula as above.

6.2 Accuracy

Accuracy is the agreement of a result to its true value or its expected value. It is sometimes called patient comparison, if only patients' samples are used in the study (Mandle, 2015). The accuracy study is more informative if, in addition to patients' results, samples from previous proficiency testing (PT) and certified standards are used, because they increase the acceptance and confidence level of the study. They also allow a true comparison of the performance of the two analytical systems. An accuracy study is conducted to detect system errors related to the analytical unit (Sarewitz, 2013). The accuracy study, along with the imprecision study, is used to establish the total allowable errors of the test on the specific instrument, thereby establishing the performance criteria of the assay. System error is comprised of two sets of errors: proportional errors and constant errors. The magnitude of the proportional error on any given analyte depends on the concentration of the analyte in the sample. Therefore, proportional errors tend to be

insignificant at lower concentration but very remarkable at higher concentration. This type of error is represented by the slope of the curve derived from the study data. The ideal slope is 1 and signifies perfect agreement between the new results and the existing results. It showed the difference in performance between the two analytical systems. Any increase or decrease from the original result is translated by a higher or lower result than the slope of one.

Constant errors are also errors that originate from the analytical unit, but do not depend on the size of the analyte. They are represented by the value of "b" in the equation of the curve. The R^2 derived from the accuracy study shows the degree of confidence in predicting the new result from the equation of the curve giving the existing value. A minimum of 40 samples spanning the AMR are needed to perform the accuracy study. A combination of patient samples, spikes, and PT samples has been used in the current project. These 40 samples are run in batches of 8 over 5 different days. The coefficient of determination and the equation of the curve are determined by plotting the expected results, which are the research laboratory results, and the reference laboratory results into the Excel program. The accuracy graphs show the results of the reference laboratory versus ARUP. The reference laboratory results are from the Trace and Toxic Elements laboratory at ARUP using the current method. The R & D results are referred to as ARUP results displaying the results from the research project with the new method.

6.3 Recovery

During analysis, some analytes can react in the testing system to form other complexes that are not counted toward the output of the analytical result. These types of

reactions or changes in the nature of the analyte cause the output result of the instrument to be inaccurate as all the analyte was not counted for toward the total ion counts. This usually causes false low results (Booth & Kadavil, 2013). In other cases, other components of the testing system will either structurally or functionally resemble the analyte of interest and therefore will be counted toward the total analyte present in the specimen. This will cause a false increase in the output result of the instrument.

In order to ensure neither one of the above instances is occurring in the testing system, a recovery study must be carried out in order to check the efficiency of the analytical process. A recovery study allows us to compare the output amount of our analyte to the input amount, and to rule out any suppression or enhancement in the system. As in other analytical chemistry testing, mass spectrometry detection uses Internal Standard (ISTD) to normalize ion counts in order to prevent falsely low or high results. There are two types of recovery experiment: the analytical recovery and the clinical recovery.

The analytical recovery experiment compares the output on the instrument to the known input amount in the sample. It is calculated by dividing the output concentration or ion count by the expected concentration or ion count.

For clinical recovery, a normalization ratio referred to as a correction factor is applied to the calculated percent recovery. Its purpose is to correct for any loss or enhancement that may have occurred in the analytical unit during analysis. The calculation of the normalizing ratio is based on the discrepancy between the recovered ions counts and the expected ions counts of the ISTD.

A standard or certified material is an analyte with known ion counts that is usually

called the expected ion counts. In a recovery study, a standard material would be used to validate the recovery of the analytical instrument under study. The ion counts recovered by the instrument are the resulting ion counts. The calculation of the percent recovery is a figure of merit for the performance of the unit. The ISTD is a certified analyte with a known or expected ion count that is added to the testing medium to monitor for loss or enhancement in the system (Dolan, 2012). In clinical chemistry, ISTD are added to samples to validate the accuracy of the results of the analyte of interest. The ISTD is a compound that behaves like the analyte but does not interfere with the analyte detection. In trace element testing, ISTD is added to the matrix match diluent. A known amount of ISTD is then added to every sample before analysis. Since the ISTD behaves like the analyte of interest, anything that affects the ISTD would also affect the analyte of interest. For this reason, the results of the analyte of interest would need to be adjusted to reflect the true results.

The calculation is as follows : $\%\, recovery = \frac{Resulting\ ions\ counts}{Expected\ ions\ counts}$

6.4 Sensitivity

Analytical sensitivity determines the lowest concentration of analyte that an analytical instrument can measure with acceptable accuracy and precision (Armbruster & Pry, 2008). The sensitivity study combines the studies of the limit of the blank (LoB), the limit of detection (LoD), and the limit of quantification (LoQ) (Mandle, 2015). The determination of these parameters depends on the analytical system (Saadati et al., 2013). In this project, only the LoB and the LoQ were studied. The study of the LOQ and LOB was done similar to the inter-assay imprecision study.

The limit of the blank is the response of the analyzer to a blank solution. This level of response is considered as the background noise in the analytical system and will automatically be deducted from all samples. In trace elements testing, the matrix diluent is considered the blank solution and was used for the determination of the LoB value. The study was done just as the interassay study but with the blank solution instead. The mean and the SD of the blank data set were calculated. The value of the LoB was determined as follows: $LoB = Mean + 1.66\,SD$

The LoD is the concentration at which the analyte can be quantified with poor predictable accuracy and precision (Armbruster & Pry, 2008).

The imprecision and inaccuracy studies might not be clinically acceptable and the response of the analyzer is well above the noise. LoD was not studied as it was not clinically significant for the assay. If we were to determine the LoD, it would be done by dilution study of the lowest calibration standard and the value would be calculated as follows: $LoD = LoB + 1.645\,sd$, with the *sd* = standard deviation of the lowest concentration (Armbruster & Pry, 2008).

The LoQ is the concentration at which the analytical instrument can quantify the analyte with acceptable accuracy and precision (Armbruster & Pry, 2008). A solution with a concentration at the currently acceptable LoQ of the production laboratory was used to verify the new method. Twenty data points were collected over a minimum of 5 days. The CV and the SD of the data set were calculated. The minimum detectable concentration has been used in this study. Statistically, the LOQ should be at least the LOB+ 3SD of the blank in order to avoid an overlap between the count of the blank and the LOQ.

6.5 Analytical Measurement Range

Analytical Measurement Range (AMR) is the range of analyte concentration that can be measured on the instrument without any dilution but with the acceptable imprecision and accuracy (Killeen, Styer, & Castellani, 2011). This is the range of accuracy and precision that can reliably be released by the laboratory based on the performance of the specific analytical system (Mandle, 2015). In this range, any increase in the amount of analyte is expected to cause increases in the result.

We conducted the AMR study by spiking a pool at different concentrations that spanned across the existing AMR with a known amount of analytes made from a standard. Over the course of 5 days, four replicates of each spike sample were prepared each day and run in a single batch. The spikes were then assayed and the observed results were compared to the expected results. The correlation coefficient was calculated along with a linearity curve. The slope of the curve along with the correlation coefficient is used to establish or confirm the measurement range.

6.6 Carryover

The carryover study assesses the percent carryover of the analyte that can occur due to the previous sample's concentration. Most of the time, carryover can be minimized with a washing step between each patient sample. This is a 1-day experiment. Matrix pools are spiked to make concentrations that are very low and very high but are within the AMR. The samples are set up in the following order: H1, H2, L1, L2, H1, H2, L1, L2, H1, H2, L1, L2. The percent carryover is determined as follows: [(AverageL2 – AverageL1) / AverageH1] x 100 = % carryover (Adzitso, Hackenmueller, Ricks, Law, & Strathmann, 2015).

7. RESULTS

The results were collected from the Mass Hunter software and input into excel software for data analysis. The following figures were taken from the original Excel sheets.

7.1 Lead

The intra-assay and interassay imprecision study results are shown in Table 5. Intra-assay imprecision is 7.78% CV for QC2 and 2.78 for QC3. The interassay CV is 3.16% for the low concentration and 3.14% for the high concentration.

The sensitivity studies are focused on the LoB and LoQ studies. The concentration of 2.0ug/dl (which is the current LoQ in production) was used to access the CV on the modified method. The resulting LoQ is 2.27ug/dl, and the LoB is 0.07ug/dl as reported in Table 5.

The accuracy study was done with 40 previously analyzed patient samples that span the AMR, including spikes that were made for the upper range of the AMR and some PT samples.

The correlation coefficient for the accuracy study is 0.987 and is illustrated in Figure 4. This coefficient of determination shows the level of confidence in predicting the current results based on the expected results using the equation $y = 0.931x - 0.193$.

The equation of the graph shows that new results are lower compared to the

expected results. There is a 0.069 x µg/dl proportional low bias and a -0.193µg/dl constant bias in our analytical system in comparison to the analytical system used in the determination of the reference results.

For the AMR study, also known as linearity study, seven spikes are made with a known standard to study the AMR and the $R^2 = 0.985$ shown in Figure 5. The slope of the graph shows that an increase in the lead analyte indeed reflects an increase in ion counts. The reference laboratory results are from the Trace and Toxic Elements laboratory at ARUP. The R & D results are referred to as ARUP results.

The analytical and clinical recovery studies are conducted by spiking matrix solutions to concentrations of 5ug/dl and 80ug/dl. Analytical recovery at the low end is 87% and the clinical recovery at that end is 97%. However, at the higher end, the analytical recovery is 89% returning a clinical recovery of 104% as in Table 6.

Two different concentrations of 1.8ug/dl and 189ug/dl are made from standard materials to assess the carryover in the system. The resulting percent carryover is 1.5 as shown in Table 7.

7.2 Cadmium

The intra- and interassay imprecision studies were done using QC2 and QC3. The intra-assay imprecision was 15.54% CV at QC2 range and 8.66% in the range of QC3 as shown in Table 5. The interassay CV was 6.86% for the low concentration and 6.94% for the high concentration as shown in Table 5. The cadmium sensitivity experiment focused on the LoB and LoQ studies. The concentration of 2.0ug/dL (which is the current LoQ in production) was used to access the CV on the modified method. The resulting LoQ was 3.63ug/L, and LoB was 0.13ug/L and is illustrated in Table 5.

The accuracy study was done with 40 previously analyzed patient samples that span the AMR, including some spikes and PT samples. The correlation coefficient was 0.994. The equation of the line was y = 0.901x + 0.198 showing that our results are lower compared to the expected results from Figure 6. There is a 0.099x proportional low bias and a 0.198 constant bias in our analytical system in comparison to the current instrument. One outlier was removed during data analysis. In order to confirm which analytical unit was accurate, we needed to run a certified standard sample on both instruments and check the results against the expected result of the standard. The degree of confidence (R^2) was 0.994 on the accuracy study.

For the linearity results, $R^2 = 0.947$. The equation of the curve is y = 1.153x +1.183 and was derived from the curve in Figure 7.

The analytical and clinical recovery studies were carried out by spiking matrix solutions to concentrations of 5ug/dl and 36ug/dl. Analytical recovery at the low end was 101% and the clinical recovery at that end was 98%. However, at the higher end, the analytical recovery was 94%, returning a clinical recovery of 100% that is illustrated in Table 8.

Two different concentrations of 2ug/L and 230ug/L were used for the carryover study and the resulting percent carryover was 0.221 as shown in Table 7.

7.3 Arsenic

The intra- and interassay imprecision studies were carried out using the same multianalytes QC2 and QC3. The results for the intra-assay imprecision were 18.50% CV at the low target and 10.54% CV at the high target. The interassay CV was 11.40% for

QC2 and 7.99% for QC3. These results are presented in Table 5. The coefficient of variations for both the intra- and interassay imprecision were within the acceptable limits.

The LOB and LOQ were studied for sensitivity of the analytical system for the arsenic analyte. The concentration of 10.0ug/L (which is the current LOQ in production) was used to access the CV on the modified method. The resulting LOQ was 19.2ug/L and LOB was 1.71ug/L.

A minimum of 40 previously analyzed patient samples, spikes, and PT samples spanning the AMR were used for accuracy study. The correlation coefficient for the accuracy study is 0.980 and is shown in Figure 8.

The equation of the line is $y = 1.190x + 3.385$ showing a 0.190x high proportional bias and a 3.385 constant bias in our analytical system in comparison to the current instrument.

For the linearity analysis, seven spikes were made to study the AMR and the $R^2 = 0.992$. The equation of the curve is $y = 0.979x + 16.721$ seen in Figure 9.

The $R^2 = 0.980$ on the accuracy study is great, but the constant bias of 16.721 in the system signals possible carryover. This may be why the LOQ is higher than the current one.

The analytical and clinical recoveries are studied by spiking matrix solutions to concentrations of 16ug/L and 103ug/L. Analytical recovery at the low end is 83% and the clinical recovery at that end is 110%. However, at the higher end, the analytical recovery is 81% returning a clinical recovery of 100% as presented in Table 9.

Two different concentrations of 10ug/L and 237ug/L were used to study carryover in the system. The rate of carryover in the system was 1.6 and is listed in Table 7. With a

higher bias than currently in production more certified samples need to be evaluated on both instruments to confirm the accuracy of the two systems.

7.4 Mercury

The intra- and interassay imprecision studies were done with QC2 and QC3. From the summary data, the CV for intra-assay imprecision was 9.47% for QC2 and 3.88% CV for QC3. As listed in Table 5, the interassay CV was 6.99% for the low concentration and 5.20% for the high concentration. Both the CVs for the intra- and interassay imprecision meet the specified expectation

For the sensitivity study, the concentration of 3.0ug/L (which is the current LOQ in production) has been used to determine the CV on the modified method. The resulting LOQ is 7.65ug/dl and LOB is 0.44/dl, as presented in Table 5.

About 40 samples from patients, PT, and spike solutions that span the AMR were used in the accuracy experiment. The correlation coefficient was 0.979. The equation of the line is y = 1.020x + 3.181, as shown in Figure 10. There was a 0.020ug/L high proportional bias and a (+) 3.181µg/L constant bias in our analytical system in comparison to the current instrument.

For the linearity analysis, seven spikes have been made to study the AMR. The R^2 = 0.955 based on the first section of the graph in Figure 11. The second graph in the same figure is showing an improved curve due to the removal of an outlier data point.

The slope of the graph shows that an increase in analytes reflects an increase in ion counts. The equation of the curve is y = 0.861x + 0.411.

Spike concentrations of 15ug/dl and 100ug/dl were used in the determination of

mercury recovery. Analytical recovery at lower concentration was 84% and the clinical recovery at that end was 114%. However, at the higher concentration (Table 10), the analytical recovery was 88% and a clinical recovery of 112%.

Concentrations of 5ug/L and 200ug/L were used for the study of mercury carryover in the system. The resulting rate of carryover was 0.08 (Table 7). This low rate of carryover can be credited in part to the success of the chelation of mercury by DMPS as previously seen in Figure 1.

Table 5. Sensitivity and imprecision results for all analytes

ELEMENTS	CONCENTRATIONS	INTRA/INTER IMPRECISION (% CV)	SENSITIVITY LOB/LOQ
LEAD	LOW	2.78/3.16	0.07/2.27(ug/dl)
	HIGH	2.78/3.14	
CADMIUM	LOW	15.54/6.86	0.13/3.63(ug/L)
	HIGH	8.66/6.94	
ARSENIC	LOW	18.50/11.40	1.71/19.21(ug/L)
	HIGH	10.54/7.99	
MERCURY	LOW	9.47/6.99	0.44/7.65(ug/L)
	HIGH	3.88/5.20	

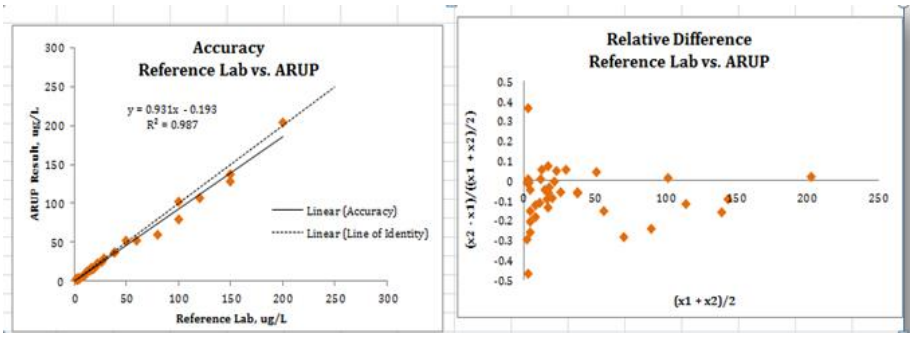

Figure 4. Lead accuracy study results.

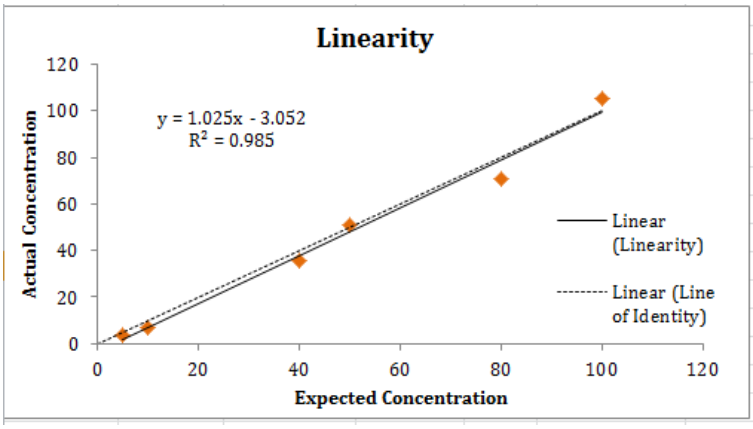

Figure 5: Lead AMR or linearity results.

Table 5: Lead recovery results.

	5ug/L	80ug/L
Analytical recovery (ug/L)	87	89
Clinical recovery (ug/L)	97	104

Table 6: Carryover study results for all four heavy metals

Elements	Lead	Cadmium	Arsenic	Mercury
% Carryover	1.5	.22	1.56	-0.08

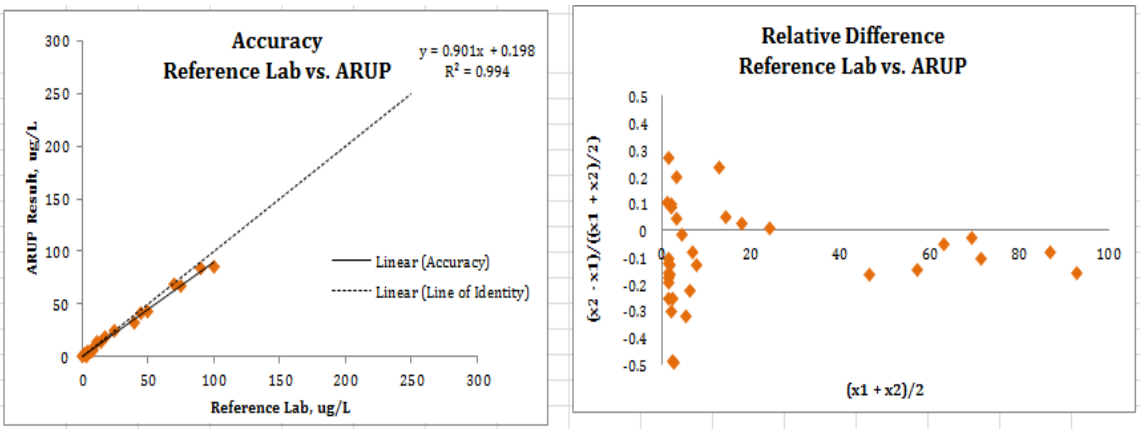

Figure 6: Cadmium sensitivity results. The reference laboratory results are from the Trace and Toxic Elements laboratory at ARUP. The R & D results are referred to as ARUP results.

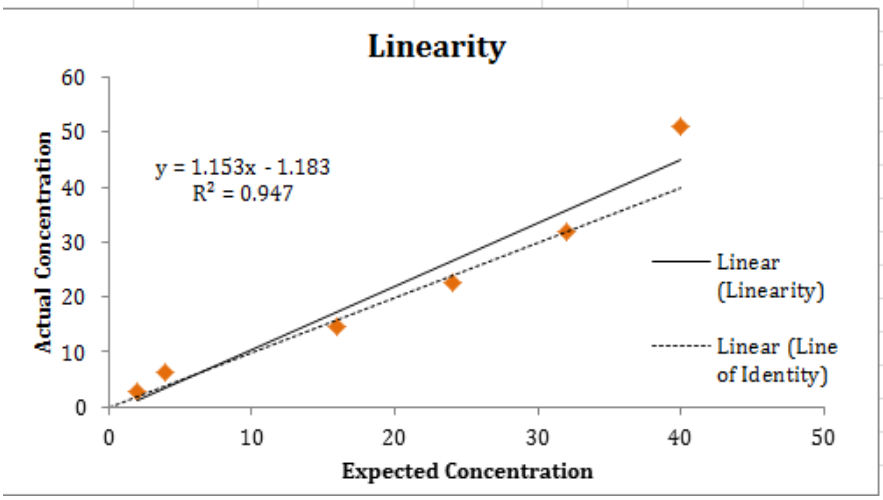

Figure 7: Cadmium AMR data

Table 7: Recovery data for cadmium study

	5ug/dL	36ug/dL
Analytical recovery (ug/dL)	101	94
Clinical recovery (ug/dL)	98	100

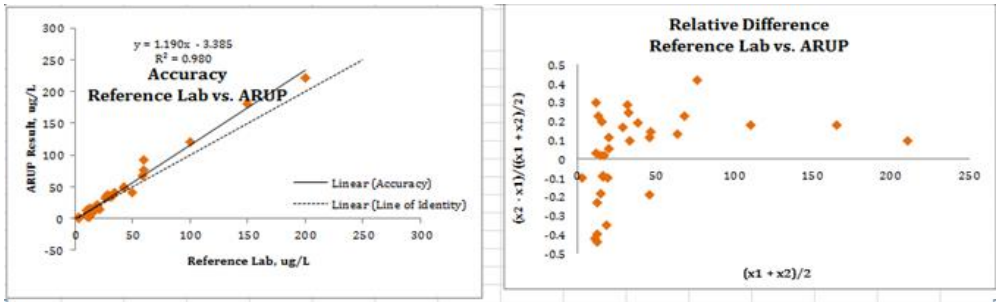

Figure 8: Accuracy results for arsenic. The reference laboratory results are from the Trace and Toxic Elements laboratory at ARUP. The R & D results are referred to as ARUP results.

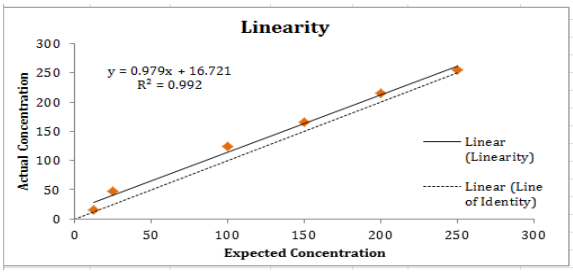

Figure 9: AMR study results for arsenic

Table 8: Arsenic recovery results

	16ug/dL	103ug/dL
Analytical recovery (ug/dL)	83	81
Clinical recovery (ug/dL)	110	100

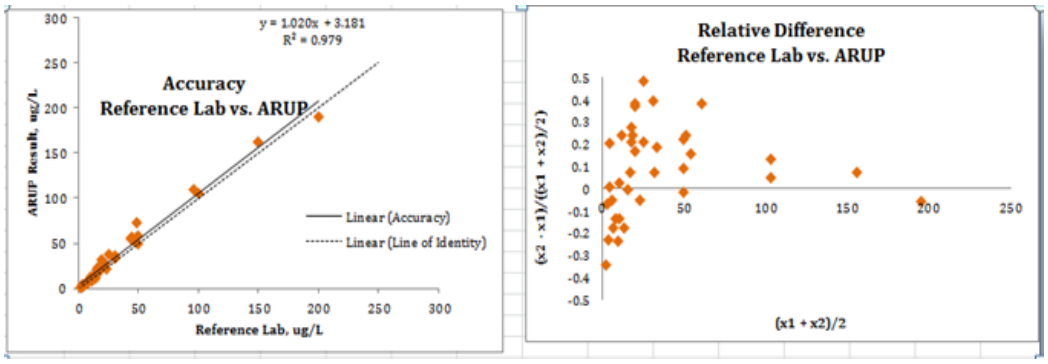

Figure 10: Accuracy study data on mercury. The reference laboratory results are from the Trace and Toxic Elements laboratory at ARUP. The R & D results are referred to as ARUP results

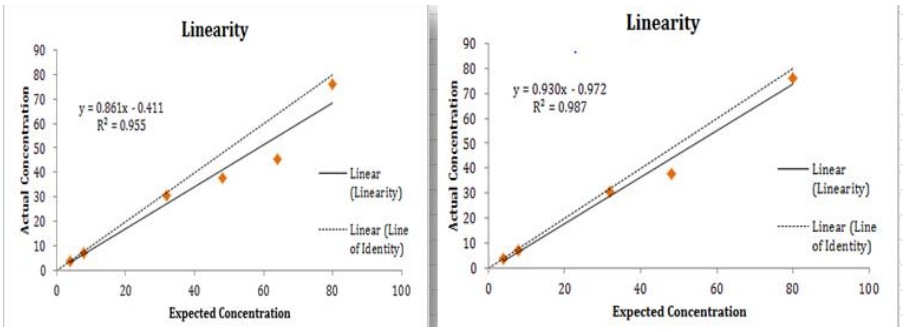

Figure 11: AMR results for mercury. The first graph shows the raw data of the mercury linearity study. The second graph depicts the adjusted curve with the removal of an outlier point.

Table 9: Mercury recovery data

	15ug/dL	99ug/dL
Analytical recovery (ug/dL)	84	88
Clinical recovery (ug/dL)	114	112

8. DISCUSSION

8.1 Lead

The lead percent imprecision studies of the inter- and intraassay are less than 3 which is less than the 10% generally accepted. The limit of the blank is 0.07, which is insignificant compared to the limit of quantification of 2.27ug/L. The current LoQ is 2.0 ug/L. The coefficient of correlation for the accuracy study is 99%. This shows that the reference result could be predicated with 99% accuracy using the equation $y = 0.93x + 0.19$. The measurement range can also be predicted with 99% confidence level using the curve $y = 1.03x + 3.05$. Table 11 shows the summary of the validation results for all four analytes studied.

At the lower concentration, the analytical system recovers 87% of the input lead. The clinical recovery at the low concentration is 97%. At the higher concentration, the percent recoveries are 89 and 104, respectively. The lead percent carryover in our system is 1.5.

8.2 Cadmium

The intra- and interimprecision studies for cadmium as shown in the table are, respectively, 15.54 and 6.86 for the low level of concentration. The CVs at the high level of concentration are 8.66 and 6.94, respectively.

The LoQ of 3.63 is well above the LoB of 0.13 allowing a good distinction between the blank and the lowest concentration measureable.

The accuracy study shows a 99% degree of confidence in predicting the values using the inherent curve with a slope of 0.9 and an intercept of 0.20.

The slope of the curve for the AMR study is 1.15. The intercept for that curve is 1.18 and the correlation coefficient is 95%. Cadmium recovery from the system is 101% for the lower concentration and 98% at the high concentration. The clinical recovery for cadmium which factors the normalization ratio to compensate for analyte enhancement or suppression in the system is 94% and 100% at the low and high concentrations, respectively. A carryover percent of 0.22 was observed with cadmium.

8.3 Arsenic

Arsenic shows a slightly higher imprecision CV. The low concentration is 18.5 for intra-assay and 11.40 for interassay imprecision studies. Likewise, the high concentration CV is 10.54 and 7.99 for intra- and interassay, respectively. The current LoQ is 10 but the LoQ from the study is 19.21. The LoB in the study is 1.71. The degree of confidence in the accuracy study for arsenic is 98% from the curve $y = 1.19x + 3.39$. The equation for the accuracy study shows that the results from our study are higher than the reference results. This may be partially due to the system parameters that were set up during the analysis like the use of the helium gas to reduce interference and increase speciation. Likewise for the AMR, $R^2 = 0.99$ with $y = 0.98x + 16.7$. The analytical recoveries at the low and high concentrations are 83% and 110%, respectively. Their clinical recoveries are 81% and 100%. Percent recovery for arsenic is 1.56.

8.4 Mercury

The mercury imprecision study resulted in a percent CV of 10.54 and 7.99 for the intra-assay and interassay, respectively, at the low concentration. At the high concentration, the CVs are 9.47% and 6.99%. The limit of the blank is 0.44 and the LoQ is 7.65. The current LoQ is 3.0. The degree of confidence for the accuracy study is 98%. The slope of the curve is 1.02 and the intercept is 3.18. The equation of the curve for the AMR study is $y = 0.86x + 0.41$. The analytical and clinical recoveries are 84% and 114% for the low concentration. For the high concentration, the recoveries are 88% and 112%. The mercury carryover is (-o.o8).

The overall validation of the HYMET4 Blood is acceptable as the results are in agreement with the current validation data. The use of IPDI system for discrete sample pipetting is doable in the trace element testing since the results of this validation are comparable to the current validation results on file. The 96-well plates are suitable in the sense that they increased sample throughput and are cost and time efficient.

This is time efficient, because repeat and replicate testing can be done using the same prepared sample. The use of a liquid handler from TECAN will remarkably shorten the technologist's time of pipetting. Also, the cost saving on the supplies due to the use of the 96 well plates instead of the 5 mL vials will considerably benefit the laboratory. We also need to mention the cost saving from the volume cut of the reagents used for testing (Hackenmueller, 2013).

Current concerns are toward the settling of the red blood cells on the TECAN before pipetting. Also, the formulation of the chelator, DMPS, used for mercury chelation is unknown but can possibly interfere with the quantification of the other

elements on the panel. We also need to look into the materials used in the manufacturing of the TECAN probes to ensure they are appropriate for metal testing to avoid possible erosion with long-term usage as has been seen with the Agilent autosampler probe. This erosion scenario has prompted the need for an alternative autosampler that warrants a comparison study with the Cetac brand autosampler which is made specifically for trace element testing. The comparison study of the Cetac autosampler to the Agilent LC system was published by Cetac Technologies and has shown increased efficiency, promising a more robust testing if the Cetac autosampler is used in place of the IPDI (Adzitso et al., 2015)

Table 10: Summary of the validation studies on the four heavy metals

ELEMENTS	CONCENTRATIONS	INTRA/INTER IMPRECISION (%CV)	SENSITIVITY LOB/LOQ	ACCURACY	AMR	ANALYTICAL/CLINICAL RECOVERY	%CARRYOVER
LEAD	LOW	2.78/3.16	0.07/2.27	R^2=0.99 $Y = 0.93x + 0.19$	R^2=0.99 $Y = 1.03x + 3.05$	87/97	1.5
	HIGH	2.78/3.14				89/104	
CADMIUM	LOW	15.54/6.86	0.13/3.63	R^2=0.99 $Y = 0.90x + 0.20$	R^2=0.95 $Y = 1.15x - 1.18$	101/98	0.22
	HIGH	8.66/6.94				94/100	
ARSENIC	LOW	18.50/11.40	1.71/19.21	R^2=0.98 $Y = 1.19x + 3.39$	R^2=0.99 $Y = 0.98x + 16.7$	83/110	1.56
	HIGH	10.54/7.99				81/100	
MERCURY	LOW	9.47/6.99	0.44/7.65	R^2=0.98 $Y = 1.02x + 3.18$	R^2=0.96 $Y = 0.86x + 0.41$	84/114	-0.08
	HIGH	3.88/5.20				88/112	

9. CONCLUSION

Trace element testing is critical for assessing toxic elements. Accurate analysis implies good sample preparation and sensitive analytical processes. The improvement of the assay is tailored to increase that accuracy. The successful validation of the improved HYMET4 Blood assay in the research setting is a proof of principle for the production laboratory, as it will enable validation in the production lab to be undertaken without much concern.

However, the mixing of the sample before pipetting to avoid settling of the red cells is still a challenge. Also, there is the need to investigate the effect of the chelator on the other analytes to assure that none of the elements on the HYMET4 Blood Panel has been used in the formulation of the chelator, DMPS. Once these roadblocks are taken care of and the new process validated, the laboratory will be able to go live with the assay. The success of this improvement will make the trace and toxic laboratory at ARUP one of the only toxic laboratories to use a 96-well plate and a liquid handler in production in a clinical setting.

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