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Chronic Kidney Disease: Vitamin D Treatment Regimens and Novel Assay Development for Kidney and Cardiovascular Function Biomarkers

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CHRONIC KIDNEY DISEASE: VITAMIN D TREATMENT REGIMENS AND NOVEL ASSAY DEVELOPMENT FOR KIDNEY AND CARDIOVASCULAR FUNCTION BIOMARKERS

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DEDICATION

I dedicate this thesis to my loving family, Hayat, Maroun, Serge and Ralph. Your love and continuous support gave me the strength and courage to pursue my dream and to make it come true. You are simply the best family a person can have. I love you all very much.

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CHRONIC KIDNEY DISEASE: VITAMIN D TREATMENT REGIMENS AND NOVEL ASSAY DEVELOPMENT FOR KIDNEY AND CARDIOVASCULAR FUNCTION BIOMARKERS

JOE M. EL-KHOURY

ABSTRACT

Chronic kidney disease (CKD) is highly prevalent in the US population and has high incidence of cardiovascular and all-cause mortality. A known complication of CKD is secondary hyperparathyroidism that is caused by bone and mineral imbalances, including vitamin D deficiency. Supplementation of CKD patients with vitamin D is based on guidelines issued by the Kidney Disease Quality Outcomes Initiative (K/DOQI), which recommend administration of vitamin D_2 in variable doses depending on the severity of vitamin D deficiency. Retrospective and pilot studies have shown that vitamin D_2 was not as effective as vitamin D_3 in treating vitamin D deficiency. In Chapter I, we investigated the effectiveness of vitamin D_2 versus vitamin D_3 treatment in resolving vitamin D deficiency in the pre-dialysis CKD population. This study was a double blinded, randomized, single center study that involved 22 CKD subjects. Data showed that vitamin D_3 elicited a more rapid increase in 25-hydroxyvitamin D (25OHD) levels than vitamin D_2, but both forms became equivalent in terms of the number of people who reached target 25OHD levels by the end of study.

Glomerular filtration rate (GFR) is the best overall index of kidney function. GFR is determined by measuring the urinary clearance of a radioactive exogenous biomarker, such as iothalamate, or estimated (eGFR) by measuring creatinine and adjusting for race, gender and age using equations. There are several known limitations to using creatinine-based equations and radioactive substances exposure for eGFR and GFR determinations. In the remaining Chapters, solutions are proposed for measurement of GFR and eGFR, which involve liquid chromatography-tandem mass spectrometry (LC-MS/MS). Chapter II discusses this technique and the process of development and validation of bioanlaytical methods by LC-MS/MS. Chapter III introduces a LC-MS/MS method for the measurement of L-arginine, symmetric dimethylarginine (SDMA), and asymmetric dimethylarginine (ADMA). SDMA was correlated with biomarkers of kidney function, while ADMA was correlated with biomarkers of cardiovascular disease. Chapter IV introduces a new LC-MS/MS method for the measurement of non-radioactive iothalamate to replace existing radioactive measurements for GFR determination. This method is very simple, fast, sensitive and selective and has shown good correlation with radioactive measurement of GFR.

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UPGRADE: A RANDOMIZED, DOUBLE BLIND STUDY TO EVALUATE THE EFFECTIVENESS OF CHOLECALCIFEROL VERSUS ERGOCALCIFEROL FOLLOWING KIDNEY DISEASE OUTCOMES QUALITY INITIATIVE (K/DOQI) GUIDELINES FOR VITAMIN D THERAPY IN STAGES 3 AND 4 CHRONIC KIDNEY DISEASE (CKD) PATIENTS

I.1. Background and Rational

According to the National Health and Nutrition Examination Surveys (NHANES) data set 1999-2004, 13.1% of the US population has chronic kidney disease (CKD), with 80,000 newly diagnosed cases each year [1,2]. Hypovitaminosis D is very common (> 86%) in pre-dialysis CKD patients [3-5], constituting 16.2 million of the US population [1,2]. This vitamin D deficiency/insufficiency is treated according to the 2003 Kidney Disease Outcomes Quality Initiative (K/DOQI) Clinical Practice Guidelines for Bone Metabolism and Disease in Chronic Kidney Disease published by the National Kidney Foundation (NKF) [6]. These guidelines recommend administration of ergocalciferol

50,000 IU at either monthly or weekly intervals for 24 months depending on severity of vitamin D deficiency (mild/severe) or insufficiency. After cessation of therapy, it is recommended that patients are supplemented using vitamin D-containing multivitamins with annual reassessment of their vitamin D status. However, this regimen is believed to be inadequate in treating almost half of vitamin D deficient/insufficient CKD patients [7,3]. As a result, many physicians no longer adhere to K/DOQI guidelines, with one study identifying over 36 discrete vitamin D prescribing regimens in a single medical center [8]. Hence, there is a clear need to evaluate current K/DOQI guidelines, and establish effective treatment strategies.

This study aimed to evaluate the adequacy of the current K/DOQI guidelines in leading to replacement of 25-hydroxyvitamin D (25OHD) levels. In addition, this study aimed to compare ergocalciferol in a head-to-head fashion with cholecalciferol treatment to better understand which vitamin D analogue is more effective in treating hypovitaminosis D in CKD patients. Because the K/DOQI guidelines are being reviewed and adopted by the international scene through Kidney Disease: Improving Global Outcomes (KDIGO), we expect that our findings will be relevant to the mission of NKF and will guide nephrologists worldwide in designing treatments for CKD patients, stages three and four, with vitamin D deficiency/insufficiency. In this study we have explored how 25OHD levels vary with ergocalciferol or cholecalciferol treatment per K/DOQI dosage guidelines and that what we have learned about these treatments will contribute to the formation of new K/DOQI and KDIGO clinical practice guidelines, impacting millions of CKD patients worldwide. Our focus was on the ability of each analogue to normalize

25OHD levels (>31 ng/mL) for treated CKD patients. We also present literature data suggesting that ergocalciferol is less effective when compared to equimolar cholecalciferol in raising vitamin D to sufficiency levels or suppressing PTH levels in CKD patients, stages three and four. Therefore, our theory was that this proposal will likely lead to acknowledging that treatment following the K/DOQI Clinical Practice Guidelines for Bone Metabolism and Disease in Chronic Kidney Disease is not highly effective, and new guidelines should be formulated that incorporate cholecalciferol instead of ergocalciferol for treatment of 25OHD deficiency/insufficiency. These outcomes have already been demonstrated for the vitamin D deficient Cystic Fibrosis population in separate studies, while here we attempted to resolve them in the same fashion but in a single study for the pre-dialysis CKD population.

I.1.1. K/DOQI definition and classification of CKD

Current definition and classification of CKD patients are based on NKF KDOQI guidelines published in 2002 [9]. The K/DOQI working group defined chronic kidney disease as either a glomerular filtration rate (GFR) less than 60 mL/min per 1.73 m^2 or the presence of kidney damage for a period of three months or more [9,10]. In addition, CKD patients are classified according to their GFR into five different stages: Stage 1- GFR greater than 90 mL/min/1.73 m^2 with other evidence of kidney damage, Stage 2- GFR 60-89 mL/min/1.72m^2 with other evidence of kidney damage, Stage 3 – GFR 30-59 mL/min/1.73m^2, Stage 4 – 15-29 mL/min/1.73m^2 and Stage 5 - GFR of less than 15 mL/min per 1.73 m^2 or renal replacement therapy. GFR can be accurately measured or

estimated (eGFR) using creatinine-based equations. In this study, eGFR is calculated using the Modification of Diet in Renal Disease (MDRD) equation [11].

I.1.2. Vitamin D physiology, deficiency, and complications in general and CKD populations

Unhydroxylated (inactive) vitamin D exists in two forms that simply differ by their side chain: ergocalciferol, also known as vitamin D_2, or cholecalciferol, also known as vitamin D_3 (Figure I.1). Cholecalciferol is synthesized from 7-dehydrocholesterol present in human skin upon exposure to UVB radiation [12], while ergocalciferol is synthesized from ergosterol in yeast and plants and is obtained by humans through their diet [13]. Upon entering the circulation, vitamin D (D_2 or D_3) binds to the vitamin D binding protein (DBP), where it is transported to the liver and hydroxylated by the enzyme Vitamin D 25-hydroxylase to 25OHD (calcidiol). This is the most abundant form of vitamin D in serum, with a half life of 2-3 weeks; it is used as a measure of the adequacy of a patient's vitamin D stores [14]. However, vitamin D does not exert its pleiotropic effects until 25OHD is hydroxylated once more by 25-hydroxyvitamin D-1α-hydroxylase, mainly in the kidneys, to 1,25-dihydroxyvitamin D ($1,25[OH]_2D$, calcitriol). This active metabolite has a very short half life of 4-6 hours. It can enter the cell, bind to the vitamin D receptor (VDR) and subsequently lead to gene expression [15].

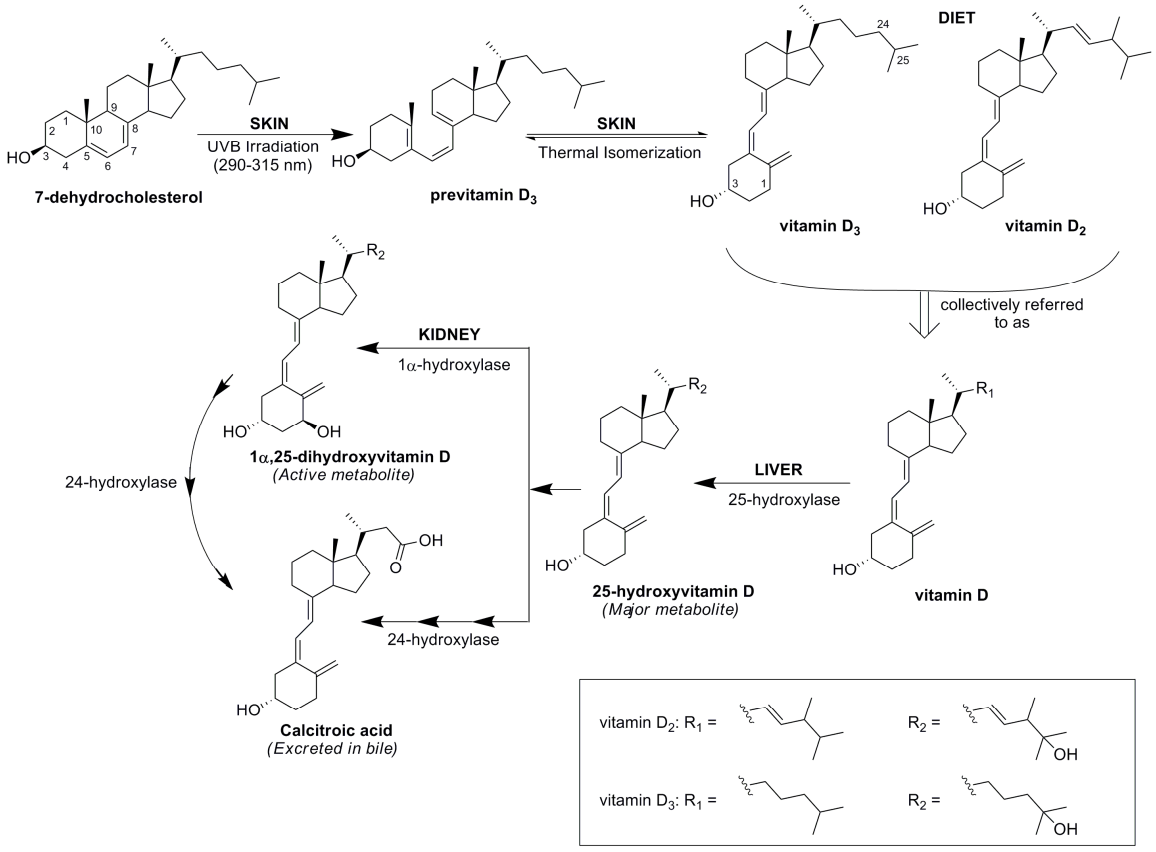

Figure I.1: Synthesis, activation and excretion of vitamin D. Reproduced from El-Khoury et al. [16].

Unlike CKD, the exact definition and classification of vitamin D deficiency remains controversial. The 2011 public health report on dietary intake requirements for calcium and vitamin D from the Institute of Medicine [17] (IOM) recommend 20 ng/mL as the target 25OHD concentration for the general population, while the Clinical Practice Guidelines for the Evaluation, Treatment, and Prevention of Vitamin D Deficiency published by the Endocrine Society recommend 30 ng/mL [18]. As summarized by Rosen et al. [19], there are several reasons for this discrepancy, mainly that 25OHD > 30 ng/mL does not confer any additional health benefits over 20 ng/mL.. Therefore, for the purpose of this study the targets we use are outlined in Table I.1 and defined for CKD patients by K/DOQI in their 2003 clinical practice guidelines for bone metabolism and disease in CKD [6]. Based on these recommendations, CKD patients presenting with a 25OHD value of 15 ng/mL or below are considered vitamin D deficient, while patients with values ranging between 16 ng/mL and 30 ng/mL are considered vitamin D insufficient.

Levels of 25OHD below 30 ng/mL have been associated with increased risk of falls [20], osteoporotic fractures [21-23], cancer [24-27], diabetes [28], hypertension [29], auto-immune diseases [30-32], congestive heart failure [33], and all-cause and cardiovascular mortality [34].

Table I.1: K/DOQI Recommended Supplementation for Vitamin D Deficiency/Insufficiency in Patients with CKD Stages 3 and 4.

Serum 25OHD, ng/mL (nmol/L)	**Ergocalciferol Dose (Vitamin D_2)**	**Duration (months)**
<5 (13)	50,000 IU/week orally ×12 weeks then monthly **OR** 500,000 IU as single intramuscular dose	6 months
5-15 (13-38)	50,000 IU/week orally × 4 weeks then monthly	6 months
16-30 (40-75)	50,000 IU/month orally	6 months

It was widely believed that the kidneys were the only sites of 1α-hydroxylation of calcidiol, which is why in the presence of CKD cholecalciferol and ergocalciferol supplementation received little attention [35]. Recent clinical studies have shown that 86-89% of CKD patients are vitamin D deficient [3,4], and that low levels of 25OHD were associated with low levels of $1,25(OH)_2D$ in CKD patients, independent of CKD progression [4,36]. These findings are explained by the fact that a wide variety of tissues in the human body, including immune cells, express VDR and/or 1α-hydroxylase and therefore respond to $1,25(OH)_2D$ and/or can synthesize it locally from 25OHD, independent of renal conversion [37]. The high prevalence of hypovitaminosis D within the CKD population was explained by lack of sun exposure, malabsorption, inadequate intake, proteinuria [38], decreased synthesis of vitamin D_3 in the skin due to impaired response to sunlight [39], advanced age [40], increased skin melanin levels [41], and progressive loss of 1α-hydroxylase enzyme effectiveness in converting 25OHD to $1,25(OH)_2D$ with progression of CKD [39]. The resulting deficiency in vitamin D, along with progressive loss of GFR in CKD patients, leads to stimulation of parathyroid hormone, a disorder known as secondary hyperparathyroidism (SHPT) [42]. SHPT is a common complication in CKD defined by elevated parathyroid hormone (PTH) levels, and is associated with mineral and bone disorders (MBD) such as renal osteodystrophy, which can lead directly to increased risk of bone fractures as well as an association with increased mortality [43,42]. Hence management of vitamin D deficiency, SHPT, and mineral homeostasis (calcium, phosphorus, and calcium-phosphorus product) is critical for CKD patients to reduce all-cause and cardiovascular mortality.

I.1.3. K/DOQI guidelines for vitamin D deficiency and SHPT

As shown in Table I.1, guidelines from K/DOQI for treatment of vitamin D deficiency and SHPT in patients with stages 3 and 4 CKD recommend administration of 50,000 IU of vitamin D as ergocalciferol weekly for 4 or 12 weeks depending on vitamin D deficiency, and 50,000 IU monthly thereafter for a total duration of 6 months **[Guideline 8.2]**[6]. However, at the time of inception of these guidelines cholecalciferol was not available in such large doses, and ergocalciferol was thought to be the safer vitamin D sterol [44]. As a result, the released K/DOQI guidelines advocated the use of ergocalciferol over cholecalciferol, yet without controlled comparisons in humans to support that decision. Today, cholecalciferol supplements are available in doses as high as 100,000 IU, and such megadoses have been safely and efficaciously administered orally to CKD patients [45-47]. In one study up to 10,000 IU/day for 5 months of cholecalciferol did not cause toxicity and has been recommended as the safe upper limit in healthy adults [48]. Moreover, recent clinical studies evaluating modified versions of K/DOQI, have shown that ergocalciferol is only modestly effective in treating vitamin D deficiency and SHPT [7,3]. However, to date, no study has compared the efficacy of ergocalciferol to cholecalciferol in a head-to-head manner when following the K/DOQI guidelines. Nevertheless, limited data exist suggesting cholecalciferol is more efficacious at replacing vitamin D levels and there is no evidence to preferentially support ergocalciferol. It is very possible that the recommendation to use ergocalciferol is resulting in under replacement of vitamin D stores and contributing to the inadequacy of the K/DOQI dosing guidelines in replacing vitamin D. In addition, K/DOQI guidelines recommend continued supplementation with a vitamin-D-containing multi-vitamin

preparation for patients replete with vitamin D by the end of the treatment **[Guideline 8.3e]**[6]. Today, multivitamins contain only 400 IU of vitamin D (D_2 or D_3) [13], while studies have shown that more than 1000 IU/day of vitamin D is needed to maintain serum levels of 25OHD greater than 30 ng/mL [49,50]. Hence, patients currently taking multivitamin preparations as K/DOQI requires might not maintain their 25OHD levels in the required range. This is often seen clinically as vitamin D levels are frequently observed to drop after switching from high-dose to the low-dose vitamin D supplementation. As a result, there is a need to evaluate the efficacy of this dose at the end of the treatment to adequately maintain 25OHD within sufficiency levels.

I.1.4. Ergocalciferol in pre-dialysis CKD patients: clinical trials

Clinical studies on the efficiency of ergocalciferol in raising 25OHD and suppressing PTH in the CKD population have shown consistent results. In a prospective, nonrandomized, observational analysis using 52 stage 3 and 4 CKD patients with vitamin D deficiency/insufficiency and SHPT treated per a *modified* version of the K/DOQI guidelines using ergocalciferol, Zisman *et al.* [7] has shown that 60% reached recommended 25OHD levels by the end of treatment. In addition, 54% (stage 3) and 20% (stage 4) reached target $1,25(OH)_2D$ levels, with 13.1% and 2.0% decreases in PTH, respectively. The authors concluded that with progression of CKD, there is decreased potential of improving PTH levels in spite of correcting calcidiol levels, probably due to decreased renal mass and ultimately decreased calcitriol production. It should be noted that ergocalciferol dosing was not as intense as recommended by K/DOQI, with the maximum duration of weekly interval being 4 weeks, regardless of 25OHD level. Other

studies yielded similar findings, for instance Deville *et al.* [51] using 85 stage 3-5 (excluding those on dialysis) CKD patients has shown that despite an overall decrease in iPTH using ergocalciferol for 90 days in doses ranging from 800 IU/day to 100,000 IU/week, only small numbers achieved K/DOQI target PTH levels. Also, Al-Aly *et al.* [3] retrospectively studied 66 stage three and four CKD male patients who were administered 50,000 IU/week ergocalciferol for 12 weeks and then 50,000 IU/month for a total of 6 months. The authors observed that almost half of patients (~45%) had a trivial or no increment in 25OHD at 6 months, and PTH levels decreased by 16.8% post-treatment. It remains unclear how effectively 25OHD and PTH levels respond at the different dosing intervals and whether control was achieved at the more frequent interval then lost again by the end of the study after the monthly dosing interval period. This is suspected clinically by the authors as it often takes more than 12 weeks of weekly, high-dose ergocalciferol therapy to fully replace 25OHD levels and the levels will frequently fall again within 4-8 weeks after once-monthly dosing is instituted. In conclusion, intermittent or high doses of ergocalciferol as recommended by K/DOQI may not be effective in raising 25OHD levels or suppressing PTH, and alternative options should be investigated. It is unclear whether this is due to inadequacy of the ergocalciferol formulation, the dosing pattern as recommended by K/DOQI or both.

I.1.5. Cholecalciferol in pre-dialysis CKD patients: clinical trials

Clinical studies investigating the efficiency of cholecalciferol in raising 25OHD and suppressing PTH in the CKD population suggest it may be more efficacious than ergocalciferol. In a randomized study including 87 stage 2-4 CKD patients with vitamin

D deficiency/insufficiency, Oksa *et al.* [52] has shown that treatment with either 5,000 IU/week or 20,000 IU/week of cholecalciferol for 12 months corrects vitamin D deficiency/insufficiency, with the higher dose being more efficacious (39% versus 75% correction, respectively). In a small pilot study using 20 patients, Chandra *et al.* [53] has shown that treatment with 50,000 IU/week of cholecalciferol for three months results in 90% of stage 3-4 CKD patients becoming vitamin D sufficient at the end of treatment, with 31% decrease in serum PTH levels. In comparison with results from Zisman *et al.* [7], cholecalciferol seems superior in raising 25OHD levels and PTH suppression, though a higher cumulative dose of vitamin D was used in Chandra's study. In addition, in the DECALYOS II study (62), Kooienga studied 610 CKD patients with vitamin D deficiency who were treated with over the counter vitamin D (800 IU cholecalciferol) and calcium supplementation. PTH levels in patients with early stage 3 CKD (eGFR > 45 mL/min/1.73m^2) were controlled to goal in 90% after 6 months of treatment. PTH levels in patients with eGFR < 45 mL/min/1.73m^2 were controlled to goal in 75% of patients. This is in the setting of adequate vitamin D replacement in only 41-49% of patients. Though the data supporting cholecalciferol is impressive, it is not adequate to definitively determine that it is a superior formulation to ergocalciferol for the replacement of 25OHD levels and suppression of hyperparathyroidism in CKD patients.

I.1.6. Treatment of hypovitaminosis D in Cystic Fibrosis (CF) patients: An example to follow

In a manner similar to K/DOQI, the U.S. Cystic Fibrosis Foundation (CFF) gathered a panel of experts to develop treatment guidelines for hypovitaminosis D, which were published in 2005 [54]. Their treatment consisted of two regimen courses: 8 weeks of

treatment with ergocalciferol 50,000 IU/week, and if serum 25OHD levels fail to rise to 30 ng/mL, it is followed by an additional treatment of 100,000 IU/week for 8 weeks. Shortly thereafter, a prospective clinical trial involving 66 adult CF patients evaluating the CFF published guidelines concluded that only 8% of patients had corrected 25OHD levels after treatment [55]. In addition, the same conclusions were reached in a retrospective clinical study involving pediatric CF patients, when only 43% reached serum 25OHD target levels using 150,000 IU/week ergocalciferol [56]. The inadequacy of ergocalciferol in treating vitamin D deficient/insufficient CF patients led to the investigation of cholecalciferol as a possible replacement. In a recent prospective clinical study comparing efficacy of treatment using ergocalciferol versus cholecalciferol in CF patients, 60% versus 100% of patients respectively became vitamin D sufficient after treatment with 50,000 IU/week for 12 weeks [57]. The outcome of the study was that cholecalciferol treatment is the more efficacious regimen for achieving optimal vitamin D status.

I.2. Study Design and Procedures

This study was approved by the Cleveland Clinic Institutional Review Board and was conducted at Cleveland Clinic facilities.

I.2.1. Protocol Synopsis

Title: UPGRADE: A Randomized Study to Evaluate the Effectiveness of Cholecalciferol versus Ergocalciferol following Kidney Disease Outcomes Quality Initiative (K/DOQI) Guidelines for Vitamin D Therapy in Stages 3 and 4 Chronic Kidney Disease (CKD) Patients

Indication: Vitamin D deficiency/insufficiency in subjects with stage 3 and 4 CKD not on dialysis.

Primary Objective:

To evaluate:

Percent of subjects achieving 25-hydroxyvitamin D (25OHD) levels > 31 ng/mL (indicates vitamin D sufficiency) in chronic kidney disease subjects with vitamin D deficiency receiving high dose cholecalciferol versus subjects receiving high dose ergocalciferol per K/DOQI dosage and frequency guidelines.

Secondary Objective(s):

To evaluate:

- Changes in absolute serum 25OHD levels over the course of the treatment
- Absolute and percent changes in intact parathyroid hormone (iPTH), calcium (Ca), and phosphorous (P)
- For subjects with corrected 25OHD, the efficacy of daily vitamin D containing multi-vitamin supplementation in maintaining serum 25OHD levels at goal after cessation of high-dose therapy.
- Safety and tolerability of cholecalciferol and ergocalciferol.
- Incidence of symptomatic adverse effects.
- Incidence of hypercalcemia or hyperphosphatemia.

Hypothesis:

The clinical hypothesis of this study is that a treatment regimen including high dose cholecalciferol will raise 25OHD levels to normal for a greater percentage of treated patients over the six months K/DOQI recommended treatment period compared with a

treatment regimen that includes high dose ergocalciferol, in subjects with stage 3 or 4 CKD with vitamin D deficiency/insufficiency.

Study Design:

This single-center, randomized, double-blind, open-label trial will consist of 3 phases:

1. Patient screening to assess for eligibility for the study. Labs will be considered valid for use to meet inclusion and exclusion criteria if the were completed within 45 days of screening.

   Eligible subjects will be randomized to either:

   - Group A- Vitamin D2 group: high dose of ergocalciferol
   - Group B- Vitamin D3 group: high dose of cholecalciferol

2. A treatment phase lasting 24 weeks
3. A follow-up phase lasting 12 weeks

Primary and Secondary Endpoints:

Primary

Percent of subjects with vitamin D sufficiency (25OHD > 31 ng/mL) at weeks 12 and 24

Secondary

Absolute change of 25OHD levels from baseline to end of study (week 24)

Absolute in iPTH from baseline to end of study (week 24)

Absolute change from baseline to end of study (weeks 6 through 36) in calcium and phosphorus.

The safety of cholecalciferol and ergocalciferol as determined by the nature, frequency, severity, and relationship to treatment of adverse events

Sample Size:

A total of 22 subjects were randomized into this study at a 1:1 ratio:

- Control group (11 subjects)
- Cholecalciferol group (11 subjects)

Randomization was stratified by severity of 25OHD deficiency (insufficient: ≤ 30 to 16 ng/mL, mild deficiency: ≤ 15 to 5 ng/mL, and severe deficiency: < 5 ng/mL)

Summary of Subject Eligibility Criteria

Inclusion criteria:

- Adults ≥ 18 years
- Chronic kidney disease stage 3-4 (eGFR 15-59 mL/min/1.73m^2 body surface area, calculated using the MDRD Study equation GFR calculator)
- Hypovitaminosis D (serum 25OHD < 31 ng/mL)

Exclusion criteria:

- History of liver failure
- History of intestinal malabsorption or chronic diarrhea
- Serum calcium level greater than 10.2 mg/dL
- Treatment with an activated vitamin D formulation (calcitriol, doxercalciferol or paracalcitol) within the past 6 months
- Treatment with phenobarbital, phenytoin, rifampicin, sucralfate, steroids, digoxin, or other medications that could affect vitamin D metabolism
- Primary hyperparathyroidism, active of a prior history of such
- Active malignancy excluding basal cell or localized squamous cell skin cancer
- Subject is pregnant (e.g. positive HCG test) or breast-feeding

- Refusal to use highly effective contraceptive measures (as determined by the investigator) throughout the treatment phase of the study
- Serum phosphorus level greater than 4.5 or treatment with an oral phosphate binder within the past 6 months
- Treatment with cinacalcet or other calcimimetic within the past 6 months
- Anticipated dialysis within 6 months after randomization
- Inability to swallow tablets
- Known sensitivity, intolerance, or other adverse response to the study drugs which would prevent compliance with study medication
- Have an unstable medical condition, defined as having been hospitalized within 30 days before screening, the expectation of recurrent hospital admissions or life expectancy of less than 6 months in the judgment of the investigator
- Subject is currently enrolled in, or fewer than 30 days have passed since subject has completed another investigational device or drug study(s); or subject is receiving another investigational agent(s).

Treatment Group:

The treatment group received cholecalciferol provided in tablets of 1.25 mg, the equivalent of 50,000 IU, and dosing per K/DOQI guidelines.

Control Group:

The control group received ergocalciferol also provided in tablets of 1.25 mg and dosing per K/DOQI guidelines. Pills are green and oval shaped, imprinted with a circled W on one side and “D 92”on the other.

Procedures:

General

Enrolled subjects were assigned to one of two arms of therapy to replace vitamin D. Monitoring was performed via phone interviews only, to include medication tracking, reminders to have labs drawn and to screen for adverse events.

Clinical appointments were made at the discretion of the responsible clinical Nephrologist outside of the study.

All laboratory specimens were drawn either at CCF Main hospital labs or at a CCF family Health center lab to ensure specimens are processed at the CCF Main core laboratory.

Patient Screening

Patients were identified as potential candidates for enrollment by the Nephrology provider caring for them. Once identified, a study investigator made sure the patient meets enrollment criteria of having stage 3 or 4 CKD and low vitamin D levels.

In order to ensure eligibility for the study, laboratory tests were performed within 45 days of enrollment. These tests included results of 25OHD, iPTH and serum creatinine level for GFR assessment. Subjects meeting eligibility criteria were approached for consent and enrollment in the study. Once consented, if not already completed in the past 45 days, $1,25(OH)_2D$, calcium, phosphorus, albumin levels and serum pregnancy test (for all women of child-bearing potential) were ordered. A brief clinical history was collected from the medical chart and from the patient interview. Patients were randomized to a

treatment arm. Women of child-bearing age were instructed not to start the study medication until the pregnancy test is confirmed to be negative.

Randomization was stratified by severity of 25OHD deficiency (insufficient: ≤ 30 to 16 ng/mL, mild deficiency: ≤ 15 to 5 ng/mL, and severe deficiency: < 5 ng/mL).

All other vitamin D supplements were stopped at the time of enrollment.

Study Start Date

The study start date of this study is defined as the day when the study medication is started.

Treatment Phase

The treatment phase was 24 weeks (6 months) in duration. Blood was collected in the laboratory every 6 weeks starting at day 1. $1,25(OH)_2D$, 25OHD, iPTH, and renal function panel were obtained at the week 6, 12, 18, and 24 study visits.

Subjects randomized to the cholecalciferol (D3) treatment group received 1.25mg (50,000 IU) cholecalciferol at an interval dependent on the severity of their vitamin D deficiency/insufficiency similar to K/DOQI guidelines recommendation for treatment of hypovitaminosis D. Subjects with severe vitamin D deficiency (25OHD < 5 ng/mL) received a single dose once weekly for 12 weeks followed by a single dose once monthly for 3 months. Subjects with mild vitamin D deficiency (25OHD: 5 to 15 ng/mL) received a single dose once weekly for 4 weeks followed by a single dose once monthly for 5 months. Subjects with vitamin D insufficiency (25OHD: 16 to 30 ng/mL) received a single dose once monthly for six months. In the event of hypercalcemia or

hyperphosphatemia, changes in vitamin D and/or calcium containing phosphate binder dose were considered in accordance with treatment practice guidelines.

Subjects randomized to the ergocalciferol (D2) group received ergocalciferol following the K/DOQI guidelines recommendation for treatment of hypovitaminosis D, which follows the same dosage pattern as the cholecalciferol treatment group, except ergocalciferol 1.25 mg (50,000IU) is used.

In the event of hypercalcemia or hyperphosphatemia, communication was made with the subject's responsible Nephrologist and any calcium supplements were stopped. If hypercalcemia persists, then the interval of vitamin D administration was decreased in accordance with treatment practice guidelines. Vitamin D was stopped and the patient censored if hypercalcemia persisted despite medication adjustments.

Follow-up Phase

The follow-up phase lasted 12 weeks, with lab visits every 6 weeks to measure $1,25(OH)_2D$, 25OHD, iPTH, and renal function panel. Subjects in both treatment groups with 25OHD serum levels > 31 ng/mL received vitamin D containing multi-vitamin preparations at a vitamin D dose of 10 μg/day (400 IU) for 12 weeks. All other subjects were censored from the study at this stage, and continued to receive recommended treatment for their persistent vitamin D deficiency and/or hyperparathyroidism through their responsible Nephrologist.

Statistical Considerations

The study was originally planned to have 86 subjects randomized to each of the control and treatment groups, which would have given enough power to detect differences in PTH suppression between the two treatments. However, due to the slower than expected enrollment rate, the power calculations were re-examined and the study was adjusted to only detect differences in 25OHD reaching target level (primary outcome). This study was geared to have 80% power to detect differences of 90% in cholecalciferol group versus 60% in control group in percent of subjects vitamin D sufficient (25OHD > 31 ng/mL) after 12 and 24 weeks of treatment, with alpha level of 0.05 (two-sided). The power analysis resulted in a calculated sample size of 11 per treatment group, and 22 total.

Other laboratory variables will be summarized.

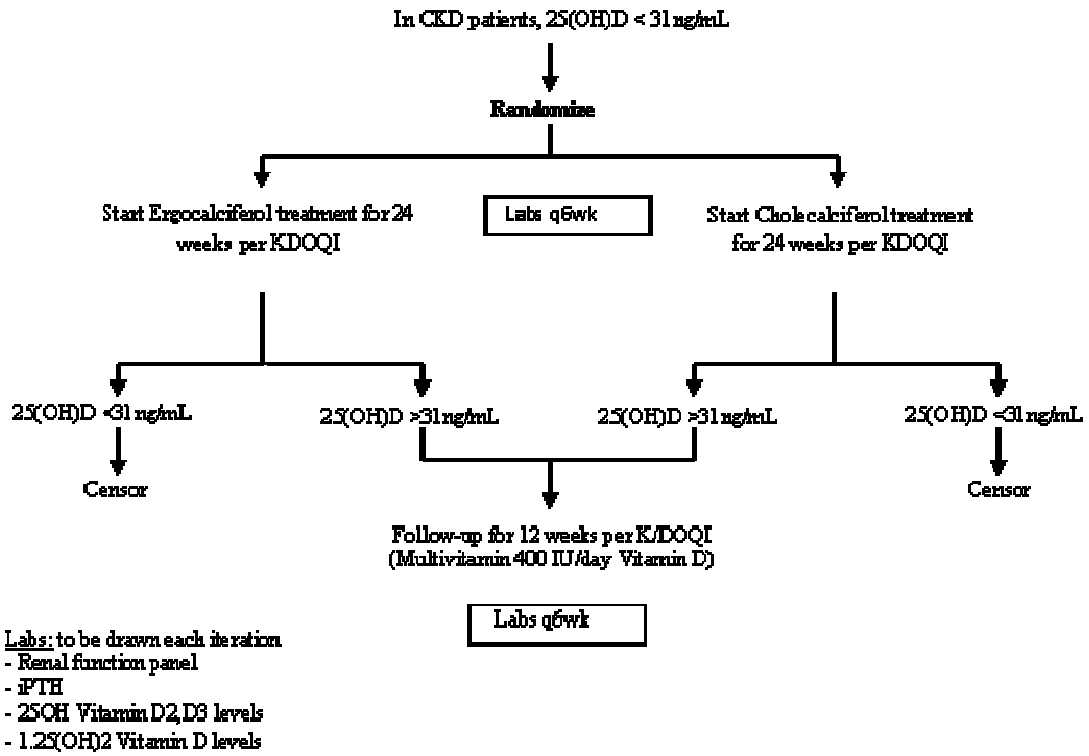

Figure I.2: Schematic illustration of UPGRADE study design.

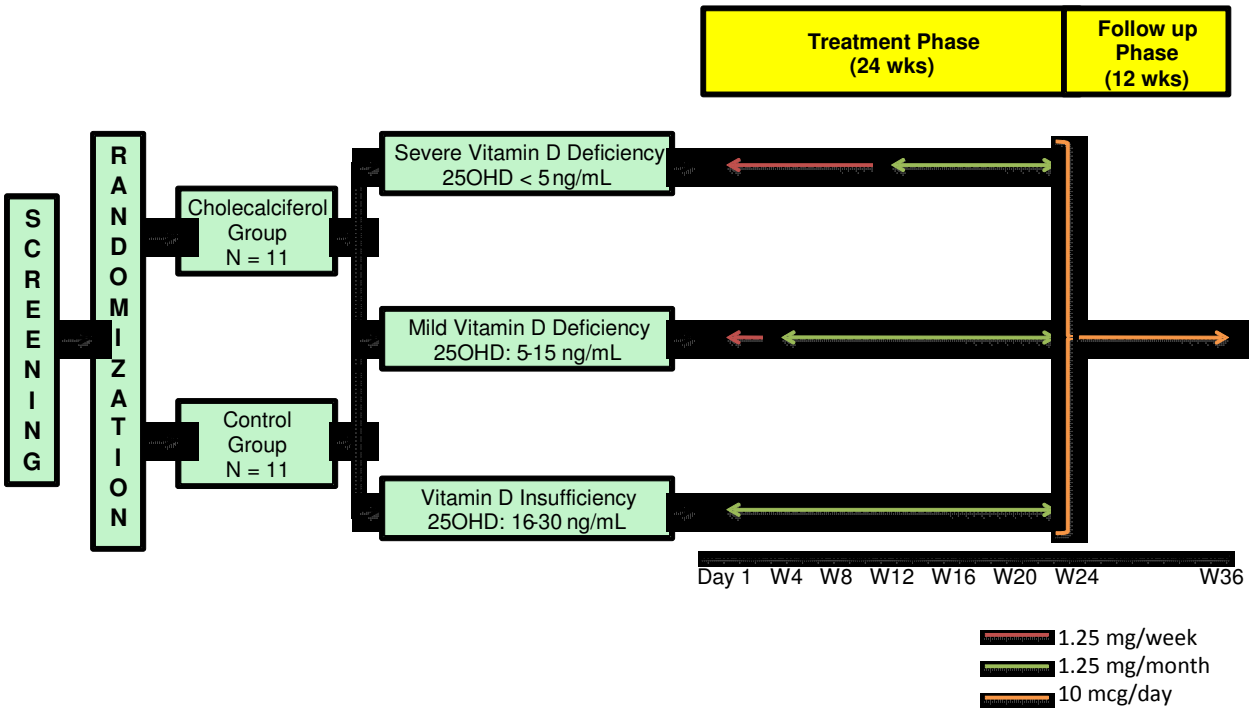

Figure I.3: Schematic illustration showing the vitamin D treatment regimen used in UPGRADE.

Table I.2: Schedule of laboratory blood tests.

Treatments and Procedures		Treatment Phase					Follow-up Phase	
	Screen	Day 1	W6	W12	W18	W24	W30	W36
Informed Consent (a)	X							
Medical History	X							
Serum pregnancy test (all women of child bearing potential)	X							
25OHD level	X	X	X	X	X	X	X (e)	X (e)
iPTH level	X	X	X	X	X	X	X (e)	X (e)
Calcitriol level		X	X	X	X	X	X (e)	X (e)
Renal function panel	X	X	X	X	X	X	X (e)	X (e)
Additional serum samples (b)		X	X	X	X	X	X (e)	X (e)
Drug Dispensing		X (c)				X (d)		

a) To occur before any study procedure
b) Collection of additional serum samples may not be applicable for all subjects
c) Bottles containing 50,000 IU cholecalciferol or ergocalciferol will be dispensed to subjects randomized within each respective group
d) Subjects with normal 25OHD and PTH at this stage will be dispensed multi-vitamin bottles
e) Only subjects dispensed multi-vitamins will be followed-up

I.2.2. Blood Collection Protocol

Label Preparation

1. Each subject will have a three (3) digit number assigned to them (ex. 067)
2. a. For samples to be stored in freezer: Add "UPG" before the 3 digit number followed by the tube id for each tube (ex. UPG055W18Gold3). W18 refers to week 18 sample and Gold to tube

   b. For samples to be taken to Central Processing Area: Label as Last Name: Upgrade, First Name: 3 digit numbers followed by week (ex: Upgrade, 055W18)

Sample Aliquoting and Storing

1. For each participant an UPGRADE Study Sample Checklist must be completed (See Section I.2.3). These are found in print form in the UPGRADE Study folder. Once completed these will be stored in the patient's section in that same folder for database entry.
2. Prepare labels for the days participants
   a. For week 1 patients, labels are printed on the spot using the Dymo LabelWriter
   b. Patients past week 1 have their labels printed out and ready in their designated section in the brown folder
3. *For weeks 1,18, 30, and 36:* Gold (G) and (if research consented) Purple (P) tubes will be delivered directly to research area of special chemistry. If no one is

available at time of receipt, samples will be stored in CPA freezer and a voicemail will be recorded at 47003 to inform of receipt.

For weeks 6, 12 and 24: G tubes must be located in freezer after receiving identifiable information provided by nurses.

4. G and P tubes will be centrifuged at 2000 x g for 10 min.
5. Remove the appropriate number of cryogenic vials depending on whether or not patient is research consented and week # (See checklist and form)
6. Arrange tubes by color code in 12 x 6 small racks (Regular cryotube for aliquots from Gold tubes and purple coded cryotubes for aliquots from Purple tube).
7. Uncap only one color at a time and aliquot appropriate amount into each tube listed in the checklist then recap tubes.
    a. For week 1 patients, if extra tests are needed, tubes W1Gold2 and W1Gold3 are aliquoted into 75 x 12 mm tubes (available at CPA) labeled appropriately (e.g."Upgrade, 001W1Gold2") and requested tests are checked in the list (Check email to see which tests should be ordered). These tubes are then taken to CPA along with a copy of the checklist, which serves as a requisition form. If no extra tests required, store aliquots in cryotubes and label as W1Gold2 and W1Gold3.
    b. For weeks 18,30 or 36, Gold1-3 are aliquoted in 75 x 12 mm tubes and labeled appropriately. These tubes are then taken to CPA along with a copy of the checklist, which serves as a requisition form.
    c. All other tubes are stored in cryogenic vials and placed in a labeled freezer box.

8. Each box should contain 5 NON research consented patients OR only 1 research consented patient. If box is being used for a research consented subject, then place tag on box (cover and inside) and also mark as "Research Consented".
9. Place samples in freezer #49012, currently used for Reference Range Study in the front area of L1-140.
10. Samples go to the fourth shelf from the top, starting from first rack (left to right) in the first available space (front to back).
11. Retrieve results from Sunquest using Last and First Name provided on tube
12. Report results using coded reference number to the following email list:
    a. brienzr@ccf.org
    b. garciam1@ccf.org
    c. sweene1@ccf.org
    d. seifert@ccf.org
    e. simonj2@ccf.org
13. Store original checklist along with instruction form in brown folder. Each patient is stored in 1 slot.

I.2.3. Sample Checklists

A total of 6 sample checklists are used in this study, which vary based on agreement of patient to additional research procedures and the number of weeks the patient has been in the study. These checklists are listed in the Appendix.

I.2.4. 25OHD, PTH, Calcium and Phosphorus Analysis

Serum samples were analyzed for 25OHD using a chemiluminescence immunoassay (Liaison®, Diasorin, Stillwater, MN). According to the manufacturer's insert (US310600; 37085) the assay is linear from 4.0 to 150 ng/mL with %CV less than 12.6% across different concentrations, lots and sites (following CLSI EP5-A2 guidelines). Intact PTH was analyzed on the ADVIA Centaur Assay (Siemens, Tarrytown, NY). According to the manufacturer's insert (129461 Rev E. 2004-05) the assay is linear from 2.5 to 1900 pg/mL with a total %CV less than 7.8 at three different concentrations tested over a 4 day period (n=144 for each sample). Serum/plasma calcium and phosphorus were both analyzed on the Roche P Modular System (Indianapolis, IN). The method is linear from 0.2 to 20.0 mg/dL and 0.3 to 20.0 mg/dL for calcium and phosphorus, respectively.

I.3. Results and Discussion

I.3.1. Baseline Subject Characteristics

Twenty-two subjects were eligible on prescreening on the basis of eGFR, 25OHD, PTH, calcium and phosphorus levels, and gave consent. Two subjects were withdrawn from the study before beginning with supplementation because their repeat 25OHD test on day 1 returned as >31 ng/mL. Two subjects were withdrawn from the study because they were started on dialysis during treatment. Another subject was withdrawn from the study because subject did not take the dosage as prescribed. During the study, three subjects withdrew consent, two were lost during follow-up and the third dropped out right after baseline measurements were performed. Data for the two patients lost to follow-up are

also included in the analysis. Thus, a total of 16 subjects (8 in each treatment group) completed the treatment phase, of which 8 were eligible for the follow-up phase, but only 6 completed it (3 from each treatment group).

Subjects in the vitamin D_2 and vitamin D_3 treatment groups were similar with respect to age, ethnicity, sex, eGFR, 25OHD, PTH, calcium and phosphorus (Table I.3).

I.3.2. Primary Endpoint: Percent of Subjects Achieving Goal 25OHD Level

Changes in the primary endpoint are summarized in Table I.4. Differences in the number and percent of subjects achieving 25OHD levels > 31 ng/mL between the two treatment groups were significant at week 12 (0 for D2 versus 5 for D3). However, by the end of treatment (week 24), both treatment groups had the same number of patients achieve goal 25OHD levels. This suggests both treatments are equally effective in raising 25OHD, but may exhibit different pharmacokinetics. However, it is important to note that given the small number of study subjects included, this study does not have significant power and therefore the findings are only preliminary and should serve as a basis for larger well controlled clinical trials.

Table I.3: Baseline characteristics of patients with chronic kidney disease treated with vitamin D_2 or vitamin D_3

Characteristics	**Vitamin D2 Group**	**Vitamin D3 Group**
Total, n	8	8
Age, y	64.50 (7.29)	67.63 (12.63)
Ethnicity, n:		
A. African American	A. 4	A. 4
B. Nonhispanic White	B. 4	B. 4
Female, n	2	4
eGFR, mL/min/1.73 m^2	39.4 (14.1)	36.9 (12.8)
25OHD, ng/mL	17.1 (4.9)	20.3 (6.9)
PTH, pg/mL	137 (62)	126 (42)
Calcium, mg/dL	9.7 (0.6)	9.6 (0.3)
Phosphorus, mg/dL	3.5 (0.7)	3.7 (0.5)

Values in parenthesis represent standard deviations.

Table I.4: Absolute and percent of subjects achieving 25OHD levels > 31 ng/mL during the course of the treatment.

No. (%) of subjects with 25OHD > 31 ng/mL	**Baseline**	**Week 6**	**Week 12**	**Week 18**	**Week 24**
Vitamin D2 Group, n=8	0 (0)	2 (25%)	0 (0%)	2 (25%)	4 (50%)
Vitamin D3 Group, n=8	0 (0)	3 (38%)	5 (63%)	4 (50%)	4 (50%)

I.3.3. Secondary Endpoint: Serial 25OHD, PTH, Calcium and Phosphorus Levels

Changes in the secondary endpoints are summarized in Table I.5. Differences in 25OHD between the two treatment groups were not significant at baseline (P=0.30), but began to emerge at week 6 (P=0.07), week 12 (P=0.07) and week 18 (P=0.02). However, these differences were completely resolved by week 24 (P=0.57), suggesting that by the end of treatment, both vitamin D forms were equivalent. When the baseline values were subtracted for each group, the increase in 25OHD after vitamin D_2 or vitamin D_3 administration were not statistically different ($P > 0.38$ for all weeks measured). Both treatments showed elevations in 25OHD that were significantly different than baseline ($P < 0.05$) from week 6 and on. There was no significant difference in PTH, calcium or phosphorus between the two treatment groups over the duration of the treatment. In addition, 2 subjects in the D2 treated group versus 1 subject in the D3 treated group developed hyperphosphatemia (Phos > 4.5 mg/dL) during the course of the treatment, while no subject developed hypercalcemia (Ca > 10.5 mg/dL).

Figure I.4 shows the 25OHD response profiles of the two treatment groups during the 24-week study. Also, it was noted that two patients in each treatment group did not have a significant response to treatment (non-responders), defined by a change of 25OHD less than 5 ng/mL. Figure I.5 shows the same response profiles after eliminating the non-responders from the analysis.

I.3.4. Follow-up Phase: Multivitamins for Maintaining 25OHD Levels

By the end of week 24, 8 subjects out of 16 were eligible to participate in the follow-up phase, but only 6 (3 from each treatment group) completed this phase. Multivitamins containing 400 IU of vitamin D_3 were prescribed daily for 12 weeks, with the subjects assessed every 6 weeks. Of the 6 patients on multivitamins, 5 (83%) successfully maintained 25OHD levels > 31 ng/mL after 12 weeks (data not shown).

Table I.5: Serial measurements of 25OHD, PTH, calcium and phosphorus in chronic kidney disease patients treated with either vitamin D_2 or vitamin D_3

25OHD, ng/mL	**Baseline**	**Week 6**	**Week 12**	**Week 18**	**Week 24**
Vitamin D_2 Group, n=8	17.1 ± 4.9	25.0 ± 6.3	24.8 ± 6.8	25.2 ± 6.0	29.4 ± 9.5
Vitamin D_3 Group, n=8	20.3 ± 6.9	31.9 ± 7.7	31.3 ± 6.5	32.1 ± 4.8	32.1 ± 9.1
P-value	0.30	0.07	0.07	0.02	0.57
PTH, pg/mL					
Vitamin D_2 Group, n=8	137 ± 62	171 ± 126	174 ± 114	167 ± 115	183 ± 148
Vitamin D_3 Group, n=8	126 ± 42	105 ± 57	105 ± 55	145 ± 94	96 ± 43
P-value	0.71	0.20	0.16	0.67	0.15
Calcium					
Vitamin D_2 Group, n=8	9.7 ± 0.6	9.4 ± 0.6	9.3 ± 0.5	9.2 ± 0.6	9.3 ± 0.6
Vitamin D_3 Group, n=8	9.6 ± 0.3	9.4 ± 0.5	9.6 ± 0.2	9.1 ± 0.3	9.6 ± 0.4
P-value	0.64	0.93	0.10	0.65	0.27
Phosphorus					
Vitamin D_2 Group, n=8	3.5 ± 0.7	3.8 ± 0.8	3.4 ± 0.7	3.8 ± 0.8	3.2 ± 0.5
Vitamin D_3 Group, n=8	3.7 ± 0.5	3.4 ± 0.6	3.5 ± 0.5	3.5 ± 0.9	3.6 ± 0.8
P-value	0.64	0.25	0.67	0.56	0.28

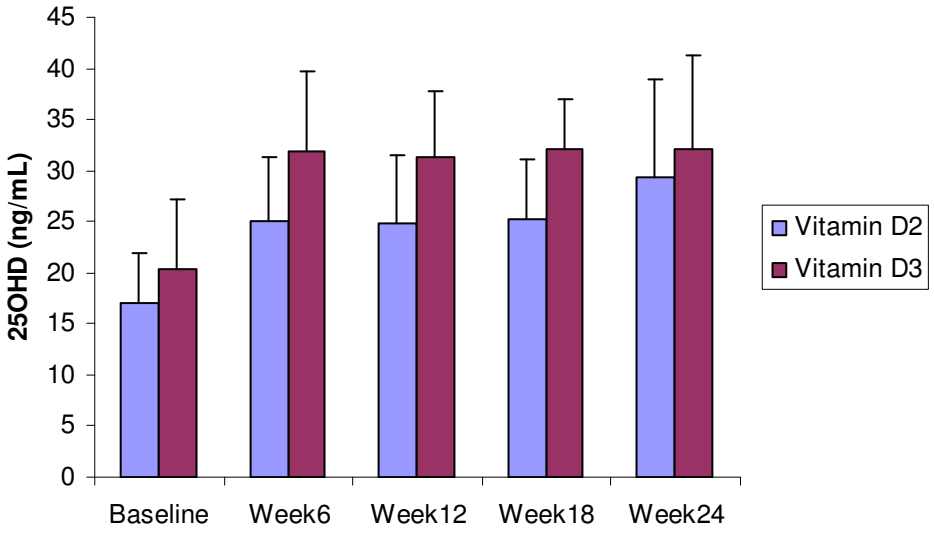

Figure I.4: Comparison of vitamin D2 (n=8) versus vitamin D3 (n=8) treatment per K/DOQI guidelines in raising 25OHD levels. Standard deviation bars are displayed.

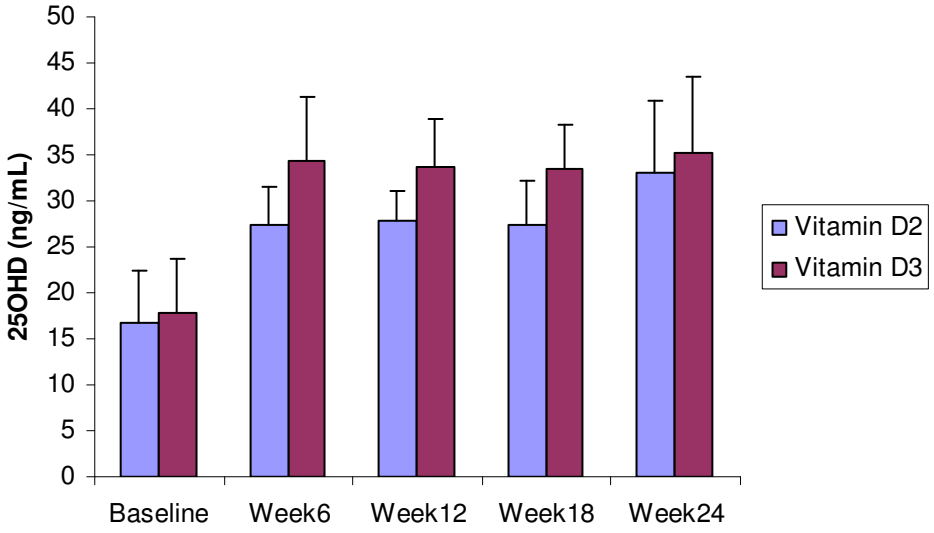

Figure I.5: Comparison of vitamin D2 (n=6) versus vitamin D3 (n=6) treatment per K/DOQI guidelines in raising 25OHD levels after eliminating non-responders to treatment (n=4), defined by Δ25OHD < 5 ng/mL. Standard deviation bars are displayed.

I.4. Conclusion

In conclusion, the findings from this pilot study show that both vitamin D_2 and vitamin D_3 are equally moderately effective (50%) when used per K/DOQI guidelines to treat vitamin D deficiency in stage 3 and 4 CKD patients. This implies that new treatment strategies should be designed to ensure that the maximum number of patients reach the 25OHD target level of 31 ng/mL. However, the data also showed that both forms of vitamin D exhibit different pharmacokinetic profiles, with vitamin D_3 causing a more rapid and sustained increase. In addition, daily mulitvitamins with 400 IU of vitamin D were found to be an effective strategy for maintaining 25OHD level > 31 ng/mL for the majority (83%) of CKD patients for at least 12 weeks. Aside from three cases of hyperphosphatemia, no signs of toxicity or side effects were observed from treatment with either form of vitamin D using K/DOQI guidelines.

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CHAPTER II

LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY METHOD DEVELOPMENT AND VALIDATION

II.1. Introduction to Liquid Chromatography-Tandem Mass Spectrometry

II.1.1. Liquid Chromatography-Tandem Mass Spectrometry in the Clinical Laboratory

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is a highly specialized analytical technique that is gaining widespread use in the clinical laboratory [1]. When first introduced, it was primarily used for the analysis of challenging low molecular weight compounds, such as vitamin D metabolites [2] and steroids [3]. These analytes required higher sensitivity and specificity than afforded by immunoassays (Figure II.1). However, LC-MS/MS has now expanded to include the analysis of proteins [4] and metabolic profiling [5]. For the purpose of this dissertation, the focus will be on the use of LC-MS/MS for the analysis of small molecules. The strengths, weaknesses, opportunities and threats (SWOT) of this technology are summarized in Table II.1.

There are several types of MS analyzers that can be coupled to LC systems and be used for the identification and quantification of small molecules, but triple quadrupole instruments represent the gold standard for LC-MS/MS analysis in clinical laboratories [6]. Although they have lower resolution when compared with other mass analyzers, triple quadrupole instruments provide a combination of superior sensitivity, selectivity and quantitative performance, making them ideal for quantitative analysis (Table II.2).

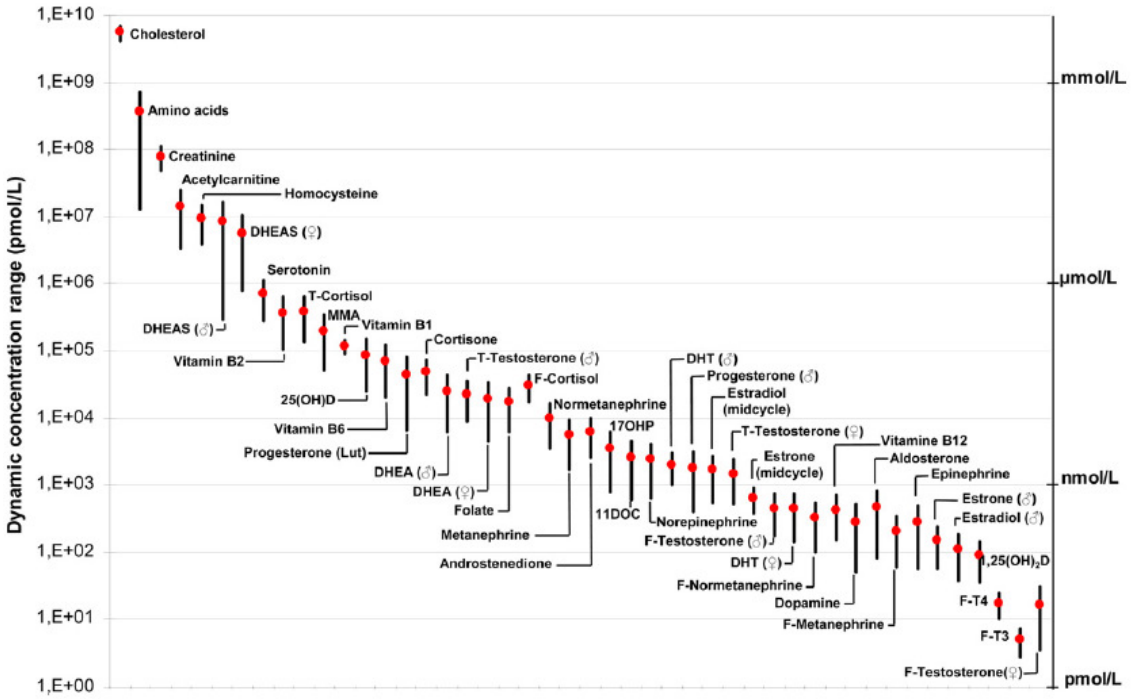

Figure II.1: Dynamic range of low molecular weight biomarkers in adult human serum.

Shown are mean and ranges. Reproduced from [1].

Table II.1: Analysis of LC-MS strength, weakness, opportunities, and threats (SWOT) in clinical diagnostics. Adapted from [1].

Strengths	**Weaknesses**
High Sensitivity High Selectivity High speed of development at low costs of new assays when compared to immunoassays by IVD companies Low costs per sample in terms of reagents Possibility to measure multiple analytes in the same sample simultaneously Versatility Near reference methodology in routine setting Matrix independency (saliva, CSF, urine, etc.) Compatible with automated sample handling configurations	High instrument costs Serial (batch-wise), non random-access operation Need for highly skilled personnel for method development, validation, operation and troubleshooting Lack of clearly defined quality regulations Limited sample throughput in conventional set–up Absence or limited availability of CE/IVD approved reagent-kits Limited experience of IVD requirements from MS vendors
Opportunities	**Threats**
Progress towards more user-friendly instruments (with integration of all components into a single system) Adoption of MS technology by major IVD companies Broader availability of CE/IVD approved kits for LC-MS/MS analysis Quantitative measurement of peptides and proteins Profiling of metabolically related metabolites (context)	Speed of development of new instruments > hard to keep up with Growing difficulty finding (skilled) technicians (and experience at an academic level) Lack of commitment from major IVD companies Regulatory bodies applying restrictions on using home-brew assays for diagnostic purposes Competition from innovations in immunoassays or from the introduction of new technologies

Table II.2: Comparison of the general features of single and triple quad instruments with quadrupole-time-of-flight (Q-TOF) and linear ion trap orbitrap (LTQ-orbitrap). Reproduced from [6].

	Single Quad	Triple Quad	Q-TOF	LTQ-Orbitrap
Sensitivity	+	+++	+++	+++
Selectivity	+	+++	+++	+++
Resolution	+	+	+++	+++
Performance for quantification	++	+++	++	++
Identification of target compounds	++	+++	+++	+++
Identification of unknown compounds	+	++	+++	+++

II.1.2. Liquid Chromatography-Triple Quadrupole Mass Spectrometry

Liquid chromatography-triple quadrupole mass spectrometry involves various stages of purification and separation that render it the gold standard for the analysis of low molecular weight analytes. The process involved is outlined in Figure II.2 and begins with obtaining a blood, serum or plasma tube, which is usually centrifuged to separate cells from plasma/serum (except in the case of intracellular analytes). The resulting supernatant (plasma/serum) is further purified by various sample preparation techniques to reduce the complexity of the biological matrix before introduction to the LC system. Purification by LC relies on the partition of the analyte between a stationary phase of choice (analytical column) and a mobile phase. The selection of the stationary phase depends on the properties of the analyte itself and creates an interaction with the analyte that is based on adsorption chromatography, ion-exchange chromatography, size-exclusion chromatography, or hydrophilic interaction chromatography (HILIC). The analytical column separates compounds into classes by having different affinities for them. The group of compounds that contains the analyte is then directed into the ion source of the mass spectrometer where gaseous ions are created by a combined process of evaporation and ionization. For most methods, this is achieved by either using atmospheric pressure chemical ionization (APCI) or electrospray ionization (ESI). APCI evaporates the compounds first by applying really high temperatures, and then ionizes the gaseous compounds via a charged corona needle before introducing them to the MS. On the other hand, ESI ionizes the surface of the solvent droplets first prior to evaporation and introduction into the MS. The two sources have differing applications, with ESI

being preferred for thermally unstable, larger molecular weight or very polar compounds. After the formation of gas phase ions, the beam of ions entering the MS is directed to the first quadrupole (Q1), where mass selection of the charged precursor ion is performed. This beam of ions then enters the collision cell (Q2), where the precursor ion is collided with an inert gas to induce the formation of product ions. The beam of product ions is then directed to the third quadrupole (Q3), where the product ion selection occurs, which is then sent to the electron multiplier to be detected. The end result is a chromatogram displaying relative ion intensity to retention time off the column for a particular reaction sequence that is monitored.

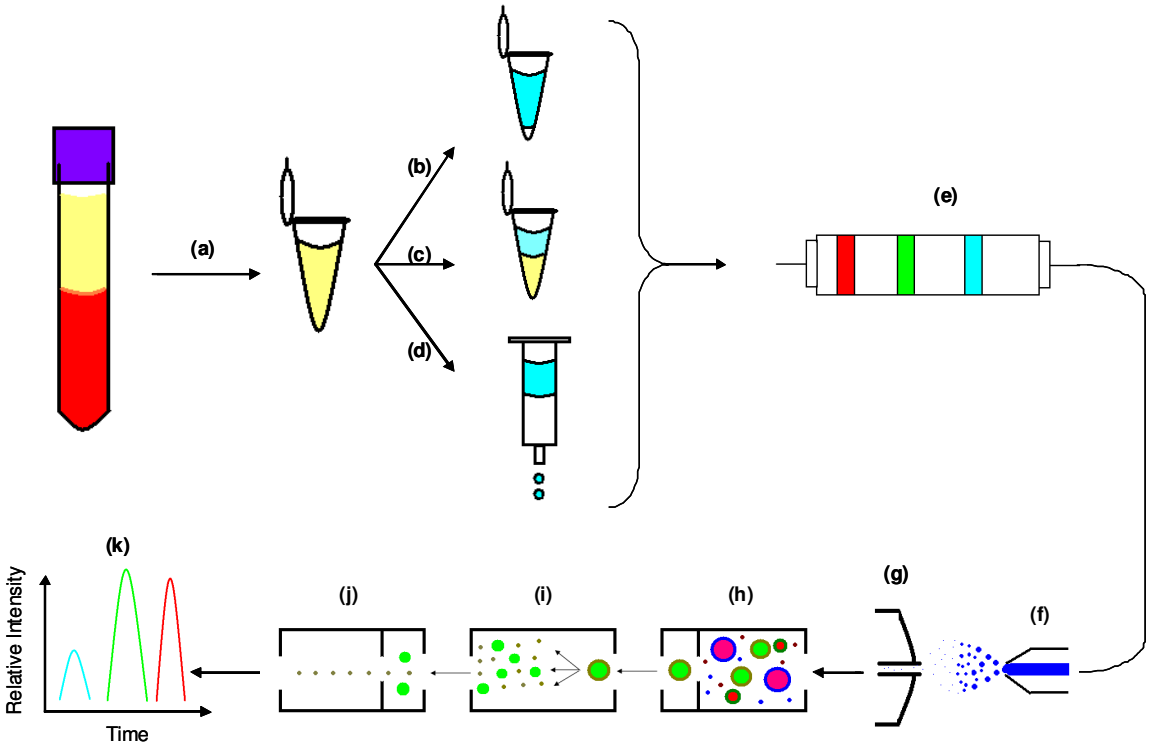

Figure II.2: Illustration of the steps involved in LC-MS/MS analysis. (a) Blood tubes are first centrifuged with the resulting supernatant (plasma or serum) transferred to a centrifuge tube. Plasma or serum is then further purified by (b) protein precipitation, (c) liquid-liquid extraction, (d) solid-phase extraction, derivatization, or online sample clean-up prior to injection onto the LC system. (e) Chromatographic separation is then performed on the analytical column before (f) evaporation and ionization of the analyte in the ion source and (g) being introduced through the ion tube into the MS. The (h) first quadrupole selects the precursor ion of the desired analyte, while the (i) second quadrupole fragments that ion by collision with an inert gas. The (j) third quadrupole then selects the product ion that is then (k) detected and quantified.

II.1.3. LC-MS/MS Method Development

The various steps involved in LC-MS/MS method development include optimization of the MS conditions, the LC conditions, and sample preparation procedures. They are followed in that order exactly because the MS parameters can be optimized independent of the LC parameters, which can be optimized independent of sample preparation procedures, but the reverse is not true. Optimization of the MS and LC parameters can be performed using solvent based solutions, and optimizing the MS first allows the detection of the analyte. This is then followed by the LC optimization which allows us to observe how well the analytical column retains our analyte and chromatographically resolves any potential interference. Then sample preparation is optimized to purify analyte from any interferences that could not be resolved by the LC-MS/MS platform, and to provide a cleaner extract for injection. The final composition of the extract is optimized to match the initial mobile phase conditions used for the LC separation.

II.1.3.1. Mass Spectrometry Optimization

This is generally the first step in LC-MS/MS method development and it involves preparation of a solvent based solution containing the analyte at a relatively high concentration (typically 1 µg/mL). This solution is then infused to the MS using a syringe pump. The MS is typically set to scan mode at start so that the expected peak is first identified. After locating the expected peak corresponding to the molecular weight of the analyte, the ion source conditions are optimized. Ion source conditions depend on the choice of ion source, ESI or APCI. In ESI, the ion source parameters that can be

optimized are spray voltage, capillary temperature, ion sweep gas, sheath gas, and auxiliary gas. In APCI, the only difference is instead of spray voltage there is corona needle voltage. The correct combination of parameters allows maximum number of analyte molecules to evaporate and ionize from the LC through the ion transfer tube and on to the MS, while reducing background noise.

After optimization of the ion source parameters, the MS mode is changed from scanning to product ion monitoring. In this mode, a selected mass/charge range is fragmented into several product ions using different collision energies. The purpose is to select a product ion in Q3 with the highest sensitivity to monitor for quantification, and possibly a second product ion for qualification.

After completion of this step, the MS mode is changed from product ion monitoring to single (SRM) or multiple reaction monitoring (MRM), depending on the number of analytes we are measuring. In this mode, the information gathered from the previous experiments is input together (Q1 m/z, collision energy, Q3 m/z) and a MS method is created.

II.1.3.2. Liquid Chromatography Optimization

Upon optimization of the MS conditions using solvent based solutions and creation of an MS method, development begins on the LC portion of the assay. In this step, there are three basic elements that need to be selected before optimization begins: a) analytical column, b) Mobile phase A, and c) Mobile phase B. As a general rule of thumb, it is

simpler and faster to test different analytical columns than to test different mobile phases. So, generic mobile phases spiked with additives (ammonium acetate, formic acid, trifluoroacetic acid…) are used at start, such as water with formic acid for mobile phase A and methanol for mobile phase B, and a variety of columns are tested using a general gradient. Whichever column provides the best retention, recovery of the analyte and peak shape is the one selected for further optimization. Then the mobile phases, flow rates and gradients are optimized using the solvent based solution. It would be wise at this stage to also prepare solvent based solutions of any potential isobaric (same molecular weight) interferences that we would like to chromatographically separate and inject those as well to make sure the LC method separates interferences from our compound.

II.1.3.3. Sample Preparation Optimization

Once LC optimization has been completed, it is time to begin optimizing sample preparation procedures using actual patient matrix, such as serum or plasma. In this step, we should be aware of any potential interference that was not separated by MS or the LC method and attempt to separate it in this stage. Besides separating interferences, the objective of the sample preparation is to further purify the sample from proteins, salts, phospholipids and other agents that cause ion suppression (explained in the next section), and to make sure the sample is compatible with LC-MS/MS analysis.

There are several options available for purifying the sample, with the most commonly used three being: a) protein precipitation (PPT), b) liquid-liquid extraction (LLE) or c) solid phase extraction (SPE), which can be performed online or off-line. There are

automated systems available that perform these procedures and can be coupled to the LC-MS/MS system to speed up the process and provide higher through-put [7]. PPT is the quick and easy option, but it is very non-selective and can require frequent maintenance of the instrument because of the introduction of unnecessary matrix components onto the MS. On the other hand, LLE and SPE are technically laborious but offer much cleaner extracts. Additional sample pre-treatment that can be performed to optimize separation from interferences or enhance the sensitivity of the analyte is derivatization. Once the sample preparation procedure has been finalized, it is essential to re-optimize both the MS and the LC methods and to make sure all stages of the LC-MS/MS method are compatible and synchronized.

II.2. LC-MS/MS Method Validation

II.2.1. Ion Suppression

Ion suppression, also known as matrix effect, is thought to occur mainly as a result of nonvolatile solutes originating from the sample matrix interfering with the ionization of the analyte of interest by altering the efficiency of droplet formation or droplet evaporation, which in turn affects the amount of charged ions in the gas phase reaching the detector and compromises the sensitivity of the LC-MS/MS assay [8]. Examples of materials shown to cause ion suppression include salts, ion-pairing agents, endogenous compounds, drugs, metabolites, and proteins. Importantly, the degree of ion suppression can be dependent on the concentration of analyte studied, which underscores the importance of using concentrations of analyte that reflect those that will be encountered under physiological conditions [9]. The post-column infusion method presented here

(Figure II.3) provides a qualitative assessment of matrix effects by identifying chromatographic regions where ion suppression/enhancement is most likely to occur. This test cannot be performed for endogenous compounds without an isotope-labeled internal standard. Concentration of analyte should be in the physiological range at the mass spectrometer source.

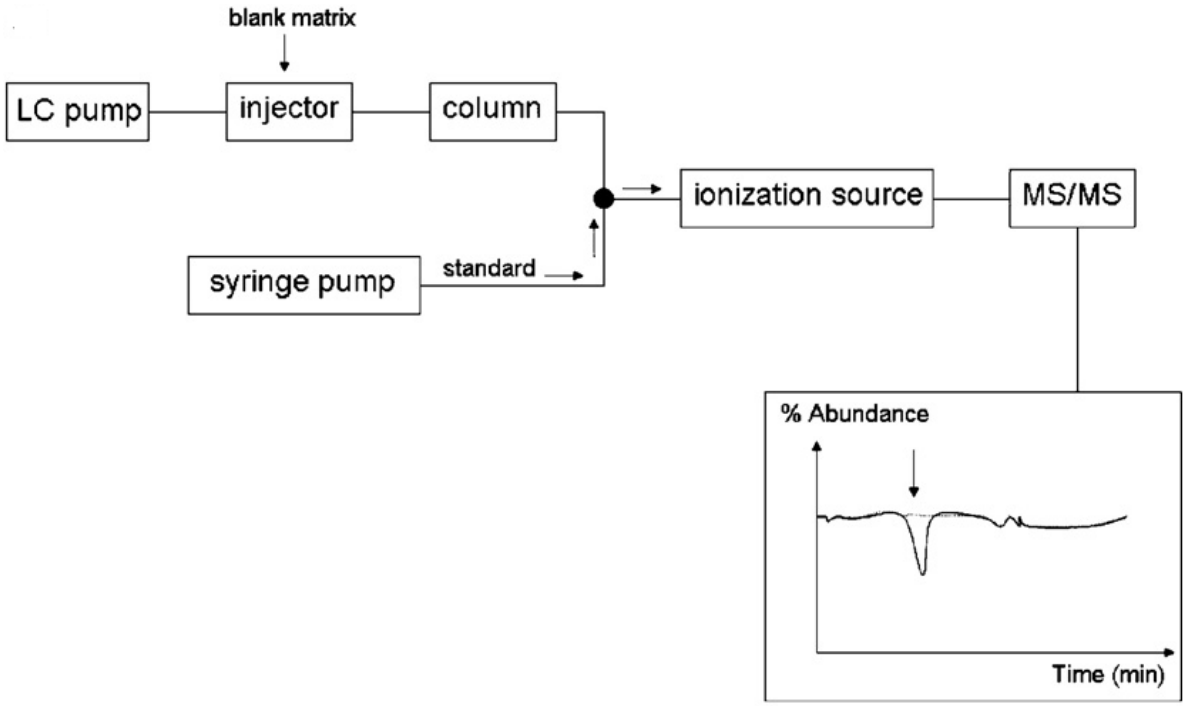

Figure II.3: Post-column infusion set-up used to evaluate the effect of absolute ion suppression. The dashed line represents the signal of the analyte in solvent matrix, while the full line is obtained when a blank matrix is injected. The arrow is pointing to a region of ion suppression. Reproduced from [10].

II.2.2. Mixing Study

The objectives of this experiment are three-fold: i) verify that the internal standard used accounts for ion suppression by behaving similar to the analyte in the matrix tested, ii) choose a matrix to be used as calibration matrix and diluting high samples, and iii) demonstrate reliability by testing various lots. The mixing study can be used for both exogenous and endogenous compounds. It is evaluated by extracting and injecting patient samples (n=6), a candidate matrix solution, and 1:1 mixtures of patient samples with the candidate matrix solution. The criteria for a passing test is the response ratio (analyte/IS) of each 1:1 mixture was within 20% of the theoretical response calculated from an average of the measured values of the patient and candidate matrix solution.

II.2.3. Method Interference (based on CLSI EP7-A2 guideline)

An interferent is a substance either exogenous or endogenous that affects or interferes with the measurement of a target analyte. An interferent study should be performed to assess the effects of common interferents on the target analytes. To determine if a substance would interfere under “worst case” conditions, the comprehensive interference screen should be conducted at the highest concentrations that a laboratory would expect to observe among patient specimens submitted for analysis. Since both positive and negative effects might occur from different mechanisms (e.g. hemoglobin has catalyst activity as well as strong absorbance in the visible spectrum) each substance should be tested at two different analyte concentrations to avoid the possibility that competing effects might cancel at the concentrations tested. Alternatively, appropriate low and high

controls or proficiency samples may be run containing multiple possible interfering substances. A minimum of triplicate sample preparations/extractions using two different analyte concentrations is necessary.

II.2.4. Analytical Measurement Range (AMR)/Calibration

The analytical measurement range (AMR) also known as linearity determines the values over which an accurate and repeatable value will be identified. Coefficient of variation (%CV)/relative standard deviation (%RSD) and recovery are used to determine the range. Functional sensitivity is determined with this assay. The AMR is determined in the linearity study with accuracy within 100 ± 20%, a total coefficient of variation (CV) within 20%, and a signal to noise greater than 10.The possible calibration range is determined by the AMR. A calibration/standard curve defines the relationship between a given analyte concentration and instrument response. A calibration curve should include five to eight-points covering the analytical measurement range and a zero calibration (matrix sample processed with internal standard), is necessary. After AMR is completed a full calibration should be performed with all assays.

II.2.5. Method Carryover

Carryover is defined as the amount of analyte not removed from an analytical system from a previous run. It is advisable to obtain a sample that is the highest possible concentration that could be received in a laboratory sample. All samples greater than the approved carryover must have the subsequent patient sample(s) repeated. Carryover is evaluated by 3 independent experiments each consisting of running two extractions in the

sequence of low_1-high-low_2, where low_2 is a re-injection of low_1. A passing test means low_1 is within 20% of low_2, and that low_2 is within 3 standard deviations of the low_1 value. The standard deviation is determined using low_1 values. High samples that are above assay linearity are diluted within the linear range and the values are back calculated. The dilution is used to determine actual concentration that action must be taken if value is higher.

II.2.6. Assay Precision (based on CLSI EP10-A3 guideline)

Precision determines how repeatable a target analyte can be measured. Both total and within-run (intra-assay) precision must be evaluated using three different analyte concentrations. Precision is evaluated using a modified protocol based on the Clinical Laboratory and Standards Institute (CLSI) EP10-A3 guideline (Wayne, PA, USA) and includes running the sequence mid-hi-low-mid-mid-low-low-hi-hi-mid twice a day for 5 days using patient derived samples to determine both intra-assay and total CVs. The sequence used in the primary method was specifically designed to allow the nearly uncorrelated estimation of the effects of non-linearity, sample carryover, proportional and constant bias, and linear drift. This is also the time for assaying quality control (QC) materials. QC materials are made separately from precision samples but may be at the same analyte levels. Minimum of two QC levels need to be used. At least one QC sample is placed at the beginning and end of a batch and must be alternated.

II.2.7. Assay Comparisons (Comparative Accuracy)

Assay comparisons are a way of determining the accuracy of a method. This is also known as analytical method comparison (AMC).There are multiple ways that the AMC can be achieved. Comparisons that can be performed for this test are listed below in order of preference:

1. Reference Material: Assess accuracy by measuring certified reference materials if possible (e.g. National Institute of Standards and Technology).
2. Comparison with a Reference Method: Accuracy can also be estimated by measuring patient samples (~40 initially) by both the new method and a reference method.
3. Commonly Used Method: If neither a reference material nor comparison with a reference method is available, a comparison may be done using a commonly used method.

II.3. Conclusion

LC-MS/MS has become widely utilized in the clinical laboratory because it offers an unparalleled level of sensitivity and specificity for small molecule analysis. The process of developing and validating LC-MS/MS methods is rigorous and requires highly specialized training. However, the process can be standardized and procedures written down, greatly facilitating it and the lab can be confident that they are producing accurate, precise and reliable methods for clinical use.

II.4. References

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CHAPTER III

EVALUATION OF L-ARGININE, SYMMETRIC DIMETHYLARGININE, AND ASYMMETRIC DIMETHYLARGININE AS BIOMARKERS FOR CARDIOVASCULAR AND KIDNEY DISEASE

III.1. Introduction

Asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) are formed by hydrolysis of the proteins with post-translational methylation of arginine residues catalyzed by protein arginine methyltransferases [1]. ADMA is a potent endogenous inhibitor of nitric oxide synthase and an established biomarker for endothelial function [2,3]. SDMA, a structural isomer of ADMA, is an emerging biomarker for renal function that has been shown to outperform creatinine-based equations for determining estimated glomerular filtration rate, commonly referred to as eGFR [4,5]. ADMA and SDMA have been independently associated with increased cardiovascular and all-cause mortality, while ADMA has been established as a new independent cardiovascular risk factor [6,7]. ADMA is also elevated in people with

hypercholesterolemia, atherosclerosis, hypertension, chronic heart failure, diabetes mellitus and chronic renal failure [8].

In the past decade, increased interest in conducting clinical research investigating associations between ADMA, SDMA and various disease states has led to a surge in analytical method development. Initially, high-performance liquid chromatography (HPLC) with fluorescence detection, capillary electrophoresis or ion exchange chromatography with absorbance detection were applied to the measurement of these analytes in human blood or urine [9]. However, the need for derivatization and long chromatographic separation has promoted the need for faster and simpler methods. Hence, an ELISA assay was developed for the measurement of ADMA, however, it seemed to suffer from matrix effects producing concentration-dependent positive bias compared to a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method [10]. LC-MS/MS is considered the "gold standard" for the measurement of these analytes [9,11]. However, some of these methods do not measure SDMA [12,13], some require lengthy derivatization procedures [14-17], and the others have relatively long chromatography time [18-24].

In this study, our primary aim was to develop and validate a simple and fast LC-MS/MS assay for the measurement of ARG, SDMA and ADMA suitable for the demands of a high volume clinical research laboratory. Our secondary aim was to fully characterize these analytes including establishing reference intervals, investigating the impact of pre-

analytical variables, and investigating correlations with biomarkers of kidney function (creatinine, MDRD eGFR, and CKD-EPI eGFR) and cardiovascular disease (hsCRP).

III.2. Materials and Methods

III.2.1. Chemicals, Reagents and Solutions

Methanol and acetonitrile (Burdick and Jackson High Purity Solvent) were from VWR (West Chester, PA, USA). Type 1 water was from a Millipore Synergy System (Billerica, MA, USA). ARG (as L-arginine), SDMA [as N^G, $N^{G'}$-dimethyl-L-arginine di (p-hydroxyazobenzene-p'-sulfonate) salt], ADMA (as N^G, N^G-dimethylarginine dihydrochloride), formic acid (for mass spectrometry, ~98%) and ammonium formate (LC-MS grade) were purchased from Sigma (St. Louis, MO). The internal standards (IS), ARG-IS [as L-arginine:HCl (U-^{13}C6, 97-99%)] and ADMA-IS [as ADMA:HCl:H_2O (2,3,3,4,4,5,5-d_7, 98%)] were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). Saline (as 0.9% sodium chloride irrigation, USP) was obtained from Baxter Healthcare Corporation (Deerfield, IL). A silica column, Polaris Si-A 5 μm, 100 × 4.6 mm, was purchased from Varian, Inc. (Palo Alto, CA).

Two different stocks were prepared in water for ARG (56.54 mM and 28.24 mM) and ADMA (3.63 mM and 4.00 mM), and in 0.1N HCl for SDMA (266 μM and 314 μM). One stock was used for preparation of standard solutions, while the other was used for preparing quality controls and validation materials. The calibration standards were prepared in saline by serial dilution at 428.6, 214.3, 107.1, 53.6 and 10.7 μM for ARG, at 4.43, 2.22, 1.11, 0.55 and 0.11 μM for SDMA and at 4.43, 2.22, 1.11 and 0.55 μM for

ADMA. IS stocks were prepared in water at 2.32 mM for ARG-IS and 1.90 μM for ADMA-IS. An IS mix was prepared in water at 55.5 μM of ARG-IS and 0.76 μM of ADMA-IS. All solutions were stored at -70°C in Corning (Corning, NY) cryogenic vials until use.

III.2.2. Sample Preparation

Sample preparation consisted of adding 50 μL of the IS mix to 50 μL of plasma, calibrator, or quality control samples in polypropylene microcentrifuge tubes and vortexed for 5 seconds followed by protein precipitation with 300 μL of 1% ammonium acetate in methanol. The mixture was vortexed for 5 s then centrifuged for 10 min at 13,000 × g. The supernatant (100 μL) was mixed with 300 μL of 1% formic acid in acetonitrile in an LC-MS certified sample vial (Waters; Milford, MA, USA) with 25 μL injected for analysis.

III.2.3. LC-MS/MS Method

This method was developed on a Thermo Fisher TSQ Quantum Access with a TLX-4 HPLC system. Instrument software for this study consisted of Tune Master 1.5, Aria 1.6.1, and Xcalibur 2.0.7. The quadruplex HPLC system consisted of two robotic sampling arms and a refrigerated sampling compartment for six 96-well plates followed by four parallel and independent inline degassers, binary HPLC pumps, and quaternary HPLC pumps. Mobile phase A was 25 mM ammonium formate in water with 1% formic acid and mobile phase B was methanol. Samples were injected on the Polaris Si-A analytical column at a flow rate of 0.8 mL/min and a mobile phase composition of 15:85

A:B for 3 min. Then, the column was washed with 90:10 A:B at 0.7 mL/min for 0.5 min and re-equilibrated for 1.5 min with 15:85 A:B at 0.8 mL/min before the next injection. The total run time between injections for one channel is 5.0 min. The mass spectrometer was set to positive electrospray ionization. The spray voltage was set at 5000 V and the capillary temperature at 280°C. The sheath gas was 50 U, the ion sweep gas was 35.0 U, and the aux gas was 10 U. Multiple reaction monitoring was set to monitor the (M+1) transitions listed in Figure III.1. The collision energy was 23, 27, 12, 17, and 28 for ARG, ARG-IS, SDMA, ADMA, and ADMA-IS, respectively. The tube lens offset was set at 81 for all analytes.

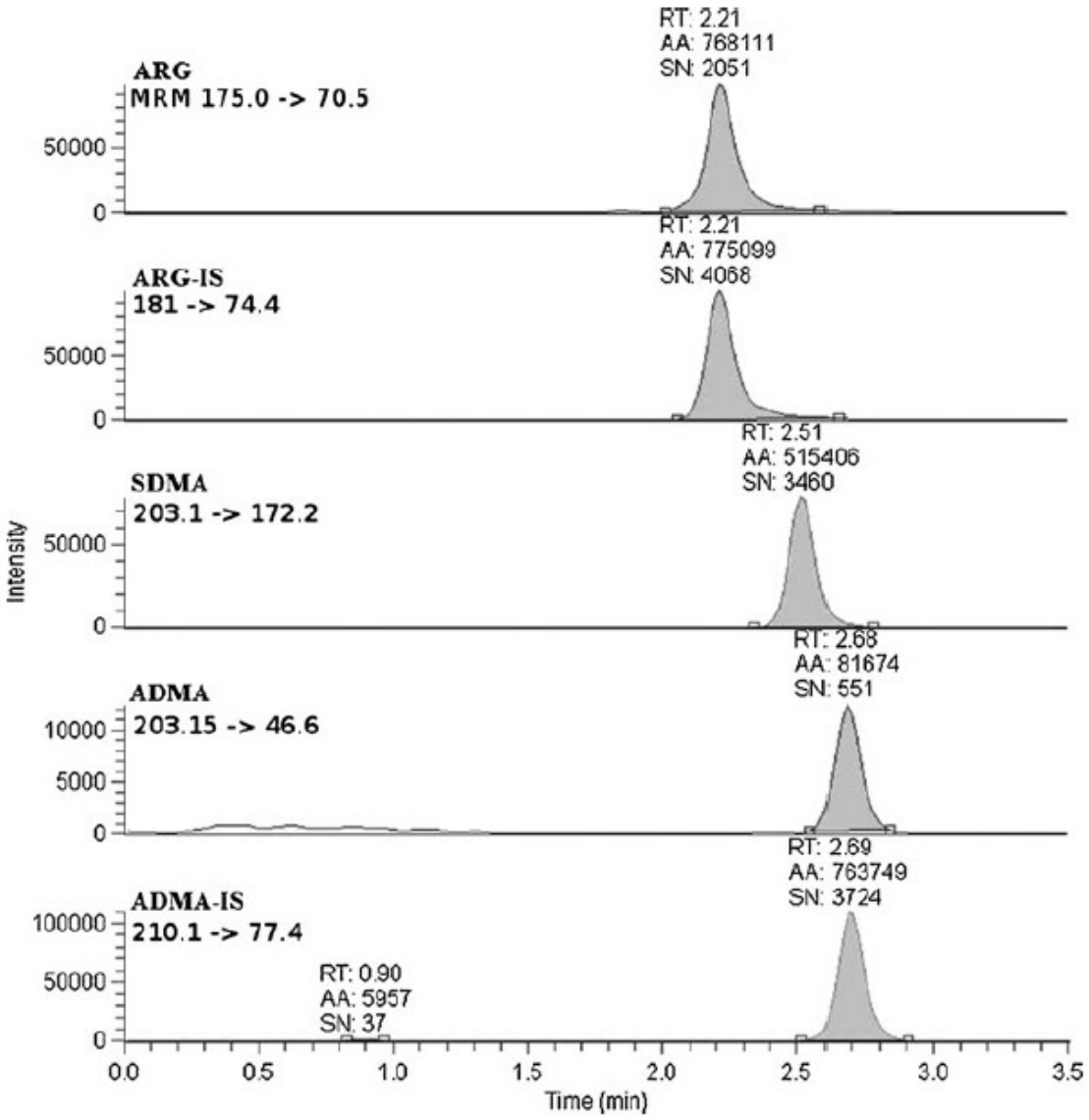

Figure III.1: Representative chromatograms of an extracted patient plasma sample showing multiple reaction monitoring transitions, retention times (RT), absolute areas (AA), and signal to noise ratios (SN)

III.2.4. Method Validation

Absolute ion suppression was evaluated by post-column infusion of a saline solution containing 5.52 μM of ARG-IS and 4.76 μM of ADMA-IS, while extracted patient samples (3 males and 3 females) without IS were injected into the system. Relative ion suppression was studied to test whether the IS accounted for ion suppression in the matrix for the analytes. It is evaluated by extracting and injecting a candidate matrix solution (saline spiked with ARG at 142.9 μM, and SDMA and ADMA at 1.47 μM), 6 patient samples (3 males and 3 females), and 1:1 mixtures of patient samples with the candidate matrix solution. The criteria for a passing test is the response ratio (analyte/IS) of each 1:1 mixture was within 20% of the theoretical response calculated from an average of the measured values of the patient and candidate matrix solution. Interference from lipemic, hemolytic, uremic and icteric plasma samples was investigated at two different analyte concentrations (low and high) by mixing each sample 1:1 with saline spiked with ARG at 42.8 μM, and SDMA and ADMA at 0.44 μM for low and ARG at 85.6 μM, and SDMA and ADMA at 0.88 μM for high. It was determined that there was no significant interference if the response ratio of each 1:1 mixture was within 20% of the theoretical response calculated from the average of interference containing sample and the spiked saline. Linearity was examined in triplicate by serially diluting with saline an EDTA plasma pool spiked with high concentration of each analyte. The endogenous concentrations of these analytes in the patient pool were determined by analyzing unspiked aliquots and were accounted for in the calculation. The analytical measurable

range was determined for each analyte in the linearity study with accuracy within 100 ± 20%, a total coefficient of variation (CV) within 20%, and a signal to noise greater than 10. Carryover was evaluated by 3 independent experiments each consisted of running two extractions in the sequence of low_1-high-low_2, where low_2 is a re-injection of low_1. A passing test meant low_1 is within 20% of low_2, and that low_2 is within 3 standard deviations of the low_1 value. The standard deviation was determined using low_1 values. High samples that were above assay linearity were diluted within the linear range and the values were back calculated. Precision was evaluated using a modified protocol based on the Clinical Laboratory and Standards Institute (CLSI) EP10-A3 guideline (Wayne, PA, USA) and included running the sequence mid-hi-low-mid-mid-low-low-hi-hi-mid twice a day for 5 days using patient derived samples to determine both intra-assay and total CVs. Statistics were calculated using Excel (Microsoft, Redmond, WA, USA) or EP Evaluator Release 9 (Data Innovations, South Burlington, VT, USA).

III.2.5. EDTA Plasma Tube and Serum Separator Tube (SST) Comparisons

Left-over EDTA plasma and SST serum samples that were drawn from the same patients (n = 20) via a single venipuncture were extracted within 24 h after blood draw along with calibrators and QCs and analyzed in a single batch. Significant difference was defined as over 20% between the two tube types.

III.2.6. Temperature Stability

Left-over patient EDTA whole blood (n = 10) was centrifuged at 2000 g for 10 min. With the plasma still sitting on the packed cells, one aliquot from each tube was

frozen at -70°C for baseline measurement, while the original five tubes were stored at either 2-8°C or room temperature (RT). One aliquot from each tube was moved to the -70°C freezer after 2 h, 6 h, 96 h, and 192 h at the specific storage conditions, respectively. Significant change was defined as a concentration change over the baseline by >20%.

III.2.7. Freeze/Thaw Stability

Left-over patient EDTA whole blood (n = 6) was centrifuged at 2000 g for 10 min. Three plasma aliquots (1 mL) spiked with 20 µL of combined sub-stock (7.14 mM ARG and 73.8 µM of SDMA and ADMA) along with 3 unspiked plasma samples were frozen at -70°C. Tubes were thawed and an aliquot per tube (150 µL) was transferred to a separate vial and refrozen at -70°C along with the original specimen tubes. This process was repeated with the original tubes for 5 freeze/thaw cycles. All samples were then thawed and analyzed in one batch.

III.2.8. Sample Collection for Reference Range Determination

Collection of blood samples for reference range determination was approved by the Cleveland Clinic Institutional Review Board. In brief, EDTA whole blood samples (n = 51) were collected from healthy adults (12 males, 39 females), aged 19-64 y (38.8 ± 12.6), after over 8 h fasting. Exclusion criteria were: body mass index (BMI) below 15 or above 30, had a cold, flu, virus or an infection in the past two weeks, diagnosed with diabetes, malabsorption syndrome or Crohn's disease, gastric or intestinal surgery, or frequent diarrhea, had received chemotherapy in the past year, on immunosuppressant

drugs, or pregnancy. Blood samples were centrifuged within 2 hours of collection at 2000 g for 10 min and the plasma was aliquoted into cryogenic vials and stored at -70°C until analysis. All the reference range samples were analyzed with QCs and calibrators in a single batch.

III.2.9. Sample Collection for Correlation with Biomarkers of Kidney Function

Collection of blood samples for correlation with biomarkers of kidney function was approved by the Cleveland Clinic Institutional Review Board. Left-over EDTA whole blood samples (n=132) were selected to cover a wide range of creatinine concentrations that ranged from 0.30 to 12.54 ng/dL (2.83 ± 2.45 ng/dL). Samples collected were not stored more than 72 hours refrigerated before being centrifuged at 2000 g for 10 min, aliquoted and frozen at -70°C until analysis. All samples were thawed and analyzed with QCs and calibrators on the same day in separate batches. A linear regression model was applied to each pair of variables and the slope coefficient was calculated. Creatinine and the two eGFR calculations (MDRD and CKD-EPI) had skewed distributions, and log-transformations were performed before correlation. A P-value <0.05 indicated significance.

III.2.10. Sample Collection for Correlation with High Sensitivity-C Reactive Protein

Collection of blood samples for correlation with high sensitivity C Reactive Protein (hsCRP) was approved by the Cleveland Clinic Institutional Review Board. Left-over EDTA whole blood samples (n=102) were selected to cover three categories of hsCRP, low relative risk for cardiovascular disease (CVD) (hsCRP < 1.0 mg/L, n=27),

average relative risk for CVD (hsCRP: 1.0 – 3.0 mg/dL, n=50), and high relative risk for CVD (hsCRP: 3.0 – 10.0 mg/dL, n=25). Samples collected were not stored for more than 72 hours refrigerated before being centrifuged at 2000 g for 10 min, aliquoted and frozen at -70°C until analysis. All samples were thawed and analyzed with QCs and calibrators on the same day in separate batches. Means and standard deviations were summarized by each hsCRP category. ANOVA was used to examine the association between each variable and hsCRP category. The Pearson correlation was also calculated by treating hsCRP as a continuous variable.

III.3. Results and Discussion

III.3.1. Method Development

Methanol with 1% ammonium acetate was selected as our extraction solvent because visually it gave the largest pellet after centrifugation compared to methanol, acetonitrile, or acetonitrile with 1% formic acid. In addition, it showed the least absolute ion suppression among the 4 tested solvents. Furthermore, addition of acetonitrile with 1 % formic acid to the methanol with 1% ammonium acetate extract (3/1) significantly improved the peak shape of the analytes. The use of a silica column with high organic mobile phase was selected because it offered the shortest chromatography time among methods published for underivatized ARG and methylated derivatives [24]. At the time of this work, isotope replaced SDMA was not commercially available. Therefore ADMA-IS was used as the internal standard for SDMA and extensive evaluation of matrix effect and accuracy was performed to confirm the validity of this approach. In comparison with published methods, some do not measure SDMA [12,13], some require

lengthy derivatization procedures [14-17], and the others have longer chromatography time [18-24].

III.3.2. Chromatography

Representative chromatograms for the analytes and internal standards from an extracted patient sample are shown in Figure III.1. Total analytical cycle time, including column re-equilibration, was 5 min using a single LC channel. Chromatographic resolution of SDMA and ADMA was not necessary because each was monitored using a unique mass transition. However, monitoring a second transition was not possible because there were no other unique fragments with significant intensity. Therefore extensive validation including interference study was performed to ensure a robust performance. The retention time (mean ± standard deviation) for ARG and ARG-IS was 2.20 ± 0.01 min, for SDMA was 2.50 ± 0.02 min, and for ADMA and ADMA-IS was 2.67 ± 0.03 min (n = 121) in plasma samples.

III.3.3. Assay Validation

Significant absolute ion suppression was observed only for ARG, however, all analytes passed the relative ion suppression test, with mean difference between the measured concentrations in the mixtures and the theoretical concentrations (n = 6) of 1.36%, 2.32%, and 0.78% for ARG, SDMA and ADMA, respectively. This indicates that ARG-IS sufficiently compensated the ion suppression for ARG, and that ADMA-IS was an acceptable internal standard for quantifying both SDMA and ADMA. No interference was observed from lipemic, hemolytic, uremic or icteric plasma samples for all analytes.

Linearity was determined to be 5.7-489.7 μM for ARG, 0.06-5.15 μM for SDMA, and 0.34-5.65 μM for ADMA with the accuracy ranging from 99 to 120% (Table III.1). The high recovery values seen for the lower end of ADMA may be due to the background signals. However the current confirmed sensitivity is sufficient to measure ADMA at the human plasma levels. No significant carryover was observed up to a tested concentration of 776 μM for ARG, 9.06 μM for SDMA and 9.08 μM for ADMA. The intra-assay and total CVs were all within 7.7% for all levels tested (Table III.2).

Table III.1: Precision and recovery data from the linearity study

Analyte	Mean, µM	Analytical recovery	%CV
ARG	5.7	105%	4.2%
	28.7	107%	1.8%
	54.8	102%	1.7%
	108.2	101%	3.9%
	213.5	99%	1.4%
	335.9	105%	7.2%
	489.7	101%	8.8%
SDMA	0.06	117%	1.6%
	0.33	118%	2.5%
	0.65	118%	1.8%
	1.26	112%	1.6%
	2.4	107%	2.9%
	3.51	104%	10.2%
	5.15	101%	2.9%
ADMA	0.08	155%	12.2%
	0.34	120%	4.8%
	0.65	114%	4.0%
	1.22	106%	2.9%
	2.52	111%	2.4%
	3.72	109%	5.2%
	5.65	110%	2.2%

Table III.2: Precision data based on CLSI EP10-A3 protocol

	ARG			SDMA			ADMA		
	Low	Mid	High	Low	Mid	High	Low	Mid	High
n	30	30	30	30	30	30	30	30	30
Mean, µM	24.3	85.9	146	0.4	0.96	1.51	0.41	1.14	1.89
Total %CV	3.1	2.7	3.9	4.2	5.9	7	7.7	3.7	5.2
Intra-assay %CV	1.8	2.6	3.9	3.9	3.8	4.7	4.5	3.2	5.3

III.3.4. Preanalytical Variables

Although EDTA plasma is the most commonly used sample type for the measurement of these analytes, SSTs are more commonly used in the clinical lab. Using left-over paired SST and EDTA specimens from 20 patients, we observed that SST serum showed a large positive bias for ARG (Figure III.2) while matched closely with EDTA plasma for SDMA, which was consistent with what has been reported in the literature [25,15]. However, the comparison result for ADMA was inconsistent with other reports which showed an insignificant difference between SST and EDTA plasma [15]. Our data showed only 75% (n = 15/20) of the samples matched between the two tube types for ADMA measurements. The discrepancy in results may be explained by the smaller number of samples used in the other study (n=4). As a result, we concluded that only EDTA plasma was the acceptable specimen type for all the analytes in this assay.

Di Gangi et al. found that ARG, SDMA, and ADMA are all stable for up to 120 h in plasma at RT , 4°C, and -20°C [15]. However, there has been no report regarding the stability of these analytes in EDTA plasma in contact with packed blood cells after centrifugation. Our data (Table III.3) showed that ARG, SDMA, and ADMA kept on the blood cells after centrifugation were stable for 6 h at RT while at 2-8°C ARG was stable for 6 h, SDMA for 192 h, and ADMA for 96 h. All the analytes were found stable up to 5 freeze/thaw cycles in this study, in agreement with previous reports showing that all the analytes where stable up to at least 4 freeze/thaw cycles [26].

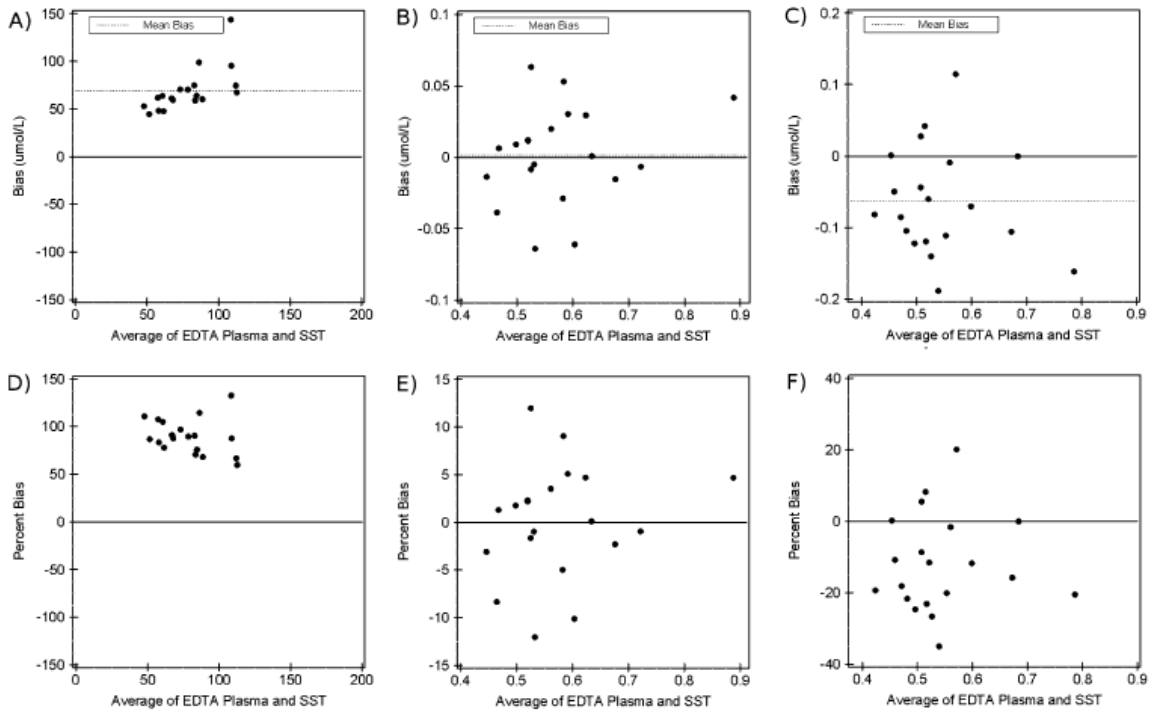

Figure III.2: Bland-Altman (A, B, C) and percent Bland-Altman (D, E, F) plots comparing SST with EDTA plasma for ARG (A and D), SDMA (B and E) and ADMA (C and F)

Table III.3: Stability of ARG, SDMA and ADMA in EDTA plasma

		ARG					SDMA					ADMA				
		Conc. (µM)	**% Recovery of t = 0h**				**Conc. (µM)**	**% Recovery of t = 0h**				**Conc. (µM)**	**% Recovery of t = 0h**			
Time	Sample	0h*	2h	6h	96h	192h	0h*	2h	6h	96h	192h	0h*	2h	6h	96h	192h
RT	1	32.3	97.2	94.0	88.5	84.6	1.0	107.6	102.8	129.4	115.4	0.6	87.9	94.8	134.5	117.0
	2	23.9	97.1	98.8	81.1	54.4	1.2	100.2	98.9	100.2	109.3	0.9	104.1	99.7	117.5	126.0
	3	47.5	101.7	95.3	68.1	52.6	0.6	102.3	100.8	108.3	119.1	0.6	98.6	100.2	130.8	143.3
	4	39.3	101.3	98.3	107.4	116.2	1.1	101.0	96.6	110.7	125.7	0.6	115.9	109.8	163.9	202.7
	5	31.0	97.4	94.3	67.8	60.6	0.4	100.6	100.5	109.3	130.5	0.4	106.2	102.0	151.4	183.6
	mean	**34.8**	**98.9**	**96.1**	**82.6**	**73.7**	**0.8**	**102.3**	**99.9**	**111.6**	**120.0**	**0.6**	**102.5**	**101.3**	**139.6**	**154.5**
2-8°C	6	20.9	98.4	97.8	80.3	63.7	0.9	100.6	95.6	94.7	105.8	0.8	95.2	95.8	98.3	107.7
	7	20.6	99.4	96.7	84.2	51.5	0.7	102.7	100.8	102.9	109.3	0.8	89.0	99.1	102.5	132.7
	8	40.1	103.4	98.3	75.2	55.1	0.4	102.6	97.1	103.9	103.1	0.5	93.4	94.6	106.3	127.1
	9	33.3	96.4	92.8	79.0	55.7	0.7	104.0	103.3	104.8	107.0	0.7	101.9	103.3	111.5	128.0
	10	25.5	101.3	96.9	76.9	62.3	0.8	96.5	93.5	97.6	98.4	0.6	94.2	91.6	102.0	124.9
	mean	**28.1**	**99.8**	**96.5**	**79.1**	**57.7**	**0.7**	**101.3**	**98.1**	**100.8**	**104.7**	**0.7**	**94.7**	**96.9**	**104.1**	**124.1**

*Denotes time of freezing first baseline aliquot and NOT time of draw.

III.3.5. Reference Intervals

Data analysis revealed a near Gaussian distribution of the reference population (n = 51, 12 males and 39 females, age 19-64) for ADMA and SDMA while Box-Cox data transformation was required for ARG to have a Gaussian distribution (Figure 3). Using a parametric method the reference intervals for SDMA and ADMA were determined to be 0.32 (0.29-0.36, 90% CI) to 0.65 (0.62-0.69, 90% CI) μM and 0.36 (0.33-0.39, 90% CI) to 0.67 (0.64-0.71, 90% CI) μM, respectively. Using a transformed parametric method the reference interval for ARG was found to be 53.1 (48.5-58.1, 90% CI) to 129.7 (118.5-142.0, 90% CI) μM.

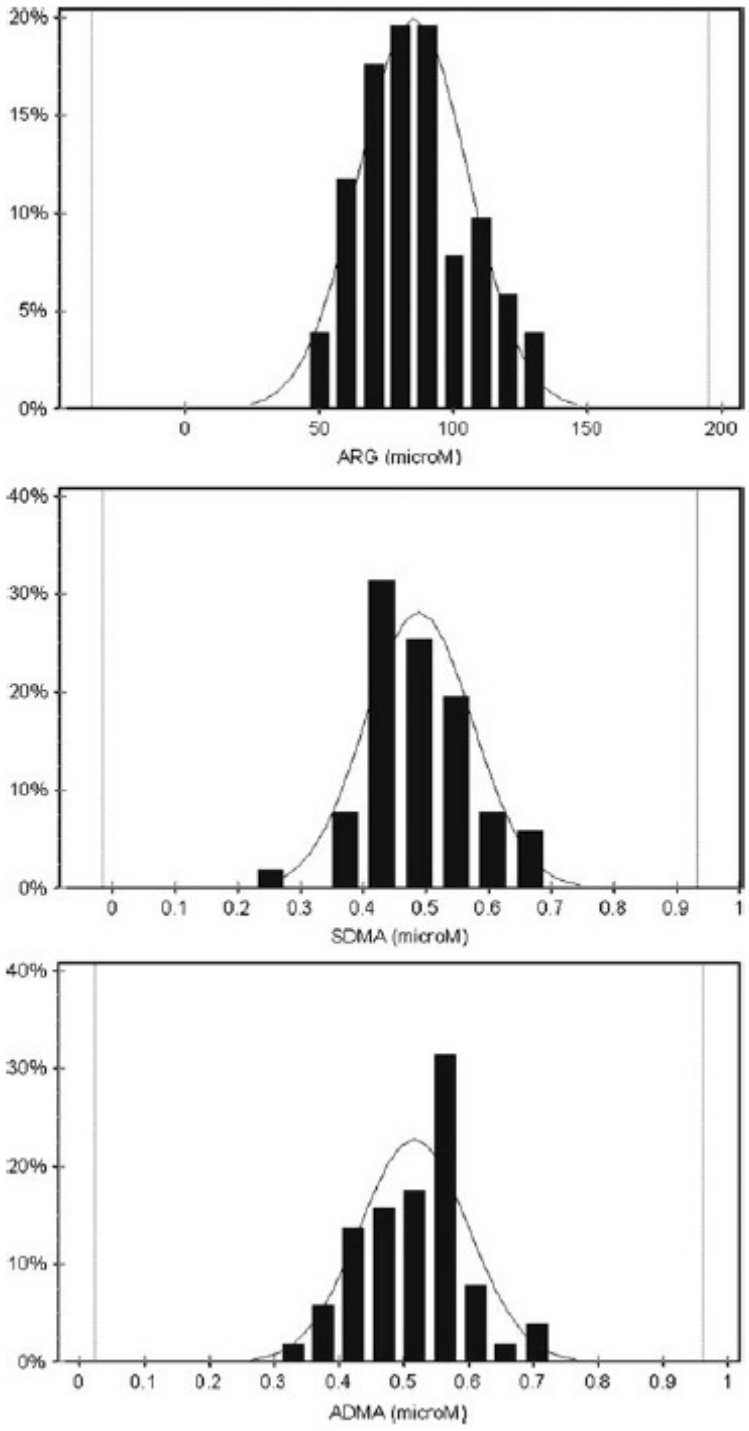

Figure III.3: Histograms showing reference population distribution for ARG (Top), SDMA (middle) and ADMA (bottom)

The reference intervals calculated in this study were in agreement with the existing literature (Table III.4). The reference range for ARG in adults is well established (21-138 μM) [27], while reference intervals for ADMA and SDMA are not well defined though it is generally agreed that average values are 0.4 μM for ADMA and 0.5 μM for SDMA [11]. Recent studies using large populations revealed a range of 0.47-0.85 μM for ADMA in 980 healthy older adults (60-72 y), a range of 0.36-1.17 μM using 500 non-smoking healthy adults, and a range of 0.22-0.69 μM in 150 adults (18-65 y) of Bulgarian nationality [28-30]. However, an ELISA assay was used to measure ADMA in these studies, which may have positive bias at higher concentrations of ADMA [10]. This might explain why reference range intervals for ADMA from these studies are generally higher than the one established here. Teerlink et al. measured ADMA and SDMA in 2311 older adults (50-75) by an HPLC method and the reference intervals, which more closely resembled our own, were 0.39-0.63 μM and 0.38-0.73 μM, respectively [31]. However, this study was exclusively conducted in older adults and ADMA is known to increase with age from 0.43 μM at 20-30 y to 0.54 μM at 70-80 y [32]. In addition, the reference intervals for ARG, SDMA and ADMA from another study conducted using 238 blood donors (112 F and 126 M) aged 19-69 y, also resembled those presented here and were determined to be 42-130 μM for ARG, 0.31-0.55 μM for SDMA, and 0.40-0.77 μM for ADMA using an HPLC method with fluorescence detection [33]. Similar data was reported in other studies that determined reference intervals for ARG, SDMA and ADMA using HPLC coupled to fluorescence detection [34,35]. Schwedhelm et al. reported 0.311 and 0.732 μM (2.5^{th} and 97.5^{th} percentile) as reference limits for ADMA in 1126 non-smoking individuals measured by an LC-MS/MS method [36]. Though the

number of subjects included in our study was small (n = 51) they covered a wide age range (19-64) with well defined criteria using a thoroughly validated LC-MS/MS method, the “gold standard” for analysis.

Table III.4: Summary of large scale studies investigating reference ranges for SDMA and ADMA

Population	n	Age group (y)	Method of analysis	SDMA (µM)	ADMA (µM)	Limitations	Reference
Healthy San Francisco Bay residents	980	60-72	ELISA[a]	ND[b]	0.47-0.85	Old population and ELISA use	[28]
Healthy Caucasians	500	19-75	ELISA	ND	0.36-1.17	Caucasians only and ELISA use	[29]
Healthy Bulgarians	150	18-65	ELISA	ND	0.22-0.69	ELISA use	[30]
Cohort study in general population (Hoorn Study)	2311	50-75	HPLC[c]	0.38-0.73	0.39-0.63	Old population and general population	[31]
Caucasian blood donors	238	19-69	HPLC + fluorescence detection	0.31-0.55	0.40-0.77	Caucasians only	[33]
Healthy Caucasian males	292	20-75	HPLC + fluorescence detection	0.25-0.81[e] 0.27-0.88[f] 0.30-0.84[g] 0.33-0.88[h]	0.43-0.69[e] 0.45-0.73[f] 0.46-0.78[g] 0.54-0.79[h]	Caucasian males only	[34]
Fasting Caucasian blood donors	225	18-65	HPLC + fluorescence detection	0.29-0.58	0.36-0.63	Caucasians only	[35]
White, middle-aged community (Framingham Offspring Study)	1126	56(9)[i]	LC-MS/MS[d]	ND	0.311-0.732	White only and middle-age population	[36]

III.3.6. Correlations with Biomarkers of Kidney Function

Data shown for creatinine and eGFR was adjusted for age, gender and race (Table III.5). As shown in Table III.5, the variables ARG, SDMA, ARG/SDMA, ARG/ADMA and SDMA/ADMA were significantly associated with creatinine and the two eGFR formulae ($p<0.05$), while ADMA was not. However, the strongest correlation with both creatinine and eGFR were SDMA, ARG/SDMA and SDMA/ADMA ($p<0.001$). This data strongly suggests that SDMA and its ratios with ARG and ADMA may be useful as endogenous biomarkers for kidney function. Further research is needed to compare the performance of these endogenous analytes versus creatinine when using actual measurement of GFR.

III.3.7. Correlations with hsCRP

Summary and associations of each variable (ARG, SDMA, ADMA, ARG/SDMA, ARG/ADMA, and SDMA/ADMA) with hsCRP category are summarized in Table III.6. As shown in Table III.6, it appears that ARG, ADMA, and the ratios of ARG/SDMA and ARG/ADMA have significant association with hsCRP categories. ARG, ARG/SDMA and ARG/ADMA have negative correlation with hsCRP, while ADMA has a positive correlation with hsCRP. On the other hand, SDMA and SDMA/ADMA are not significantly associated with hsCRP. This data suggests that measurement of ARG, ADMA, ARG/ADMA and ARG/SDMA may also be useful as a predictor of CVD events. However, further research is needed to establish the utility and proper use of this analyte for this purpose.

Table III.5: Summary and association (adjusted for age, gender and race) of each variable with GFR and mortality information

	eGFR variable	Pearson correlation	Estimate	P for association
ARG	Creatinine	-0.04 (-0.21, 0.13)	-0.008 (-0.017, 0.002)	0.109
	eGFR MDRD	0.21 (0.04, 0.37)	0.275 (0.085, 0.464)	0.005
	eGFR CKD epi	0.25 (0.08, 0.40)	0.224 (0.096, 0.352)	0.001
	Log Creatinine	-0.14 (-0.30, 0.03)	-0.004 (-0.007, -0.001)	0.006
	Log eGFR MDRD	0.18 (0.01, 0.34)	0.005 (0.002, 0.009)	0.005
	Log eGFR CKD epi	0.17 (0.00, 0.34)	0.005 (0.001, 0.008)	0.007
SDMA	Creatinine	0.50 (0.36, 0.62)	1.03 (0.722, 1.337)	<0.001
	eGFR MDRD	-0.54 (-0.65, -0.40)	-22.827 (-29.165, -16.489)	<0.001
	eGFR CKD epi	-0.65 (-0.74, -0.53)	-18.869 (-22.787, -14.951)	<0.001
	Log Creatinine	0.64 (0.53, 0.73)	0.441 (0.35, 0.531)	<0.001
	Log eGFR MDRD	-0.66 (-0.74, -0.55)	-0.511 (-0.616, -0.406)	<0.001
	Log eGFR CKD epi	-0.66 (-0.75, -0.55)	-0.496 (-0.597, -0.396)	<0.001
ADMA	Creatinine	-0.04 (-0.21, 0.13)	-0.238 (-1.536, 1.06)	0.72
	eGFR MDRD	-0.09 (-0.26, 0.08)	-19.751 (-46.842, 7.34)	0.156
	eGFR CKD epi	-0.10 (-0.27, 0.07)	-14.896 (-33.396, 3.604)	0.117
	Log Creatinine	0.03 (-0.14, 0.20)	0.171 (-0.262, 0.603)	0.44
	Log eGFR MDRD	-0.05 (-0.22, 0.12)	-0.179 (-0.681, 0.322)	0.485
	Log eGFR CKD epi	-0.04 (-0.21, 0.13)	-0.16 (-0.644, 0.324)	0.518
ARG/SDMA	Creatinine	-0.45 (-0.57, -0.30)	-0.012 (-0.015, -0.008)	<0.001
	eGFR MDRD	0.70 (0.60, 0.78)	0.348 (0.284, 0.413)	<0.001
	eGFR CKD epi	0.78 (0.70, 0.84)	0.268 (0.229, 0.307)	<0.001
	Log Creatinine	-0.66 (-0.75, -0.55)	-0.006 (-0.007, -0.005)	<0.001
	Log eGFR MDRD	0.68 (0.58, 0.77)	0.006 (0.005, 0.008)	<0.001
	Log eGFR CKD epi	0.66 (0.56, 0.75)	0.006 (0.005, 0.007)	<0.001
ARG/ADMA	Creatinine	-0.003 (-0.17, 0.17)	-0.003 (-0.008, 0.003)	0.338
	eGFR MDRD	0.22 (0.05, 0.38)	0.171 (0.061, 0.281)	0.003
	eGFR CKD epi	0.27 (0.10, 0.42)	0.142 (0.068, 0.216)	<0.001
	Log Creatinine	-0.14 (-0.30, 0.03)	-0.002 (-0.004, -0.001)	0.006
	Log eGFR MDRD	0.19 (0.02, 0.35)	0.003 (0.001, 0.005)	0.006
	Log eGFR CKD epi	0.19 (0.01, 0.35)	0.003 (0.001, 0.005)	0.008
SDMA/ADMA	Creatinine	0.57 (0.44, 0.68)	0.888 (0.671, 1.106)	<0.001
	eGFR MDRD	-0.52 (-0.64, -0.39)	-16.427 (-21.259, -11.594)	<0.001
	eGFR CKD epi	-0.63 (-0.72, -0.51)	-13.654 (-16.667, -10.64)	<0.001
	Log Creatinine	0.67 (0.56, 0.75)	0.336 (0.268, 0.403)	<0.001
	Log eGFR MDRD	-0.67 (-0.75, -0.56)	-0.392 (-0.469, -0.314)	<0.001
	Log eGFR CKD epi	-0.67 (-0.75, -0.56)	-0.381 (-0.455, -0.307)	<0.001

Table III.6: Summary and association of each variable (ARG, SDMA, ADMA, ARG/SDMA, ARG/ADMA, and SDMA/ADMA) with hsCRP category.

	All (n=102)	Low (n=27)	Middle (n=50)	High (n=25)	P for association with hsCRP category	Correlation with continuous hsCRP (95% CI)
ARG, ng/mL	48.36 (19.70)	54.44 (23.49)	49.06 (17.54)	40.38 (17.26)	0.033	-0.244 (-0.418, -0.052)
SDMA, ng/mL	0.75 (0.53)	0.63 (0.09)	0.75 (0.55)	0.86 (0.73)	0.298	0.243 (0.051, 0.418)
ADMA, ng/mL	0.56 (0.09)	0.52 (0.05)	0.56 (0.08)	0.59 (0.12)	0.014	0.339 (0.154, 0.500)
ARG/SDMA	75.23 (36.05)	89.40 (42.14)	75.58 (31.52)	59.22 (32.00)	0.009	-0.322 (-0.486, -0.136)
ARG/ADMA	88.46 (37.15)	106.45 (45.38)	87.73 (30.42)	70.47 (31.39)	0.002	-0.337 (-0.499, -0.153)
SDMA/ADMA	1.31 (0.71)	1.22 (0.18)	1.30 (0.75)	1.44 (0.97)	0.533	0.202 (0.008, 0.382)

III.4. Conclusion

In conclusion, we have developed an LC-MS/MS assay for measuring plasma ARG, SDMA, and ADMA that is simple and fast. This method has been fully characterized for clinical research with reference ranges established. Preliminary clinical associations suggest that SDMA and its ratios may be used as endogenous markers for kidney function, while ARG, ADMA, ARG/ADMA and ARG/SDMA may be useful for prediction of cardiovascular events.

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CHAPTER IV

DEVELOPMENT AND VALIDATION OF A SIMPLE AND FAST LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY METHOD FOR THE QUANTITATION OF NON-RADIOACTIVE IOTHALAMATE IN SERUM AND URINE FOR THE DETERMINATION OF GLOMERULAR FILTRATION RATE

IV.1. Background

Assessment of kidney function for the evaluation and management of kidney disease is of daily practical use. Glomerular filtration rate (GFR) is a measure of the number of functional nephrons in the kidney and it can either be directly measured by monitoring the urinary excretion of a marker, or estimated (eGFR) using developed equations that include several markers as well as patient demographics [1]. The ideal marker, which could be exogenous or endogenous, is freely filtered at the glomerulus, not secreted nor reabsorbed [2]. Serum creatinine is commonly used and various mathematical equations to estimate GFR derived from serum creatinine are available. However, in several clinical

situations either serum creatinine alone or estimated GFR by the MDRD, Cockcroft-Gault and CKD-EPI equations are not sufficient due to poor precision and accuracy [3]. In those instances, as well as in situations where a direct measurement is needed, the gold standard approach of GFR measurement is needed. GFR measurement is clinically useful to assess the amount of functional renal tissue or mass. The GFR value gives three types of information: (1) the absolute filtration rate at that moment, (2) the relative filtration rate in comparison with similar individuals in a group, and (3) the percentage filtration rate change in an individual, when measured over time.

Historically, GFR was first measured by the renal clearance of an exogenous filtration molecule, inulin (first gold standard) [4]. This methodology proved accurate and precise when strict research quality procedures, including patient catheterization for urine collections, were followed. Such rigorous procedures were difficult to use in clinical situations, therefore the renal clearance of endogenous creatinine soon gained widespread favor in clinical medicine [5]. Although practical, GFR estimation through the measurement of creatinine has very well known limitations rendering it unreliable in patients were accurate GFR determination is required [6]. It is actively secreted and reabsorbed by renal tubules and eliminated extrarenally in severe CKD [7]. As a result, GFR methodology evolved both with regard to GFR marker molecules and instrumentation to measure them. Gamma emitter labeled GFR molecules gained popularity in the 1960's; gamma counter analysis proved to be highly sensitive, accurate and precise. Today, ^{125}iodine, ^{51}chromium and ^{99}technetium tracers are in use in larger medical centers where accurate GFR measurements are used to support transplant, cancer

chemotherapy, geriatric/pediatric, and other patients for whom GFR estimates can be inadequate [8,9]. The Renal Function Laboratory at the Cleveland Clinic uses radiolabeled iothalamate as a tracer (^{125}iodine).

Patient dosimetry for gamma tracer GFR measurements is on the order of a chest x-ray; this is a factor of a hundred less than CAT scans and less than many routine nuclear medicine scans. The regulatory requirements associated with labeled tracers have limited their widespread use, however, and GFR procedures employing non-radioactive ("cold") GFR molecules have become more attractive as the analytical instrumentation has become more sensitive. Improvements in HPLC, capillary electrophoresis and mass spec instrumental procedures now make it possible to analyze cold, exogenous GFR markers in the clinical chemistry lab, not nuclear medicine [10-13]. These new analytical methods are much more complex compared to the pipet-and-count gamma procedures, but they do eliminate the tracer-related regulatory concerns, i.e. dosimetry, radiation safety training and documentation, radioactive waste disposal, etc. However, these suffer from long run times and UV based methods are susceptible to interferences. There is currently only two published LC-MS/MS based method which measures unlabeled iothalamate in plasma and urine [14,15], however the performance of these methods were not compared against the gold standard radioactive measurement, they involved lengthy sample preparation and chromatography, and no qualifier transition was monitored for greater selectivity. In this study, our goal was to develop and validate a simple, fast and highly selective LC-MS/MS method, and compare its performance to radioactive measurement of iothalamate.

IV.2. Materials and Methods

IV.2.1. Chemicals, Reagents and Solutions

Methanol (Burdick and Jackson High Purity Solvent) was from VWR (West Chester, PA, USA). Type 1 water was from a Millipore Synergy System (Billerica, MA, USA). Ammonium acetate (98.7%) and sodium hydroxide (NaOH) (98.6%) were purchased from Fisher Scientific (Fairlawn, NJ). Formic acid (for mass spectrometry, ~98%) was purchased from Sigma (St. Louis, MO). Iothalamic acid (IA) was purchased as powder (98-102%) from U.S. Pharmacopeia (Rockville, MD). The internal standard (IS), sodium iothalamate labeled ^{13}C, ^{13}C, d_3 was purchased from Chemtos (Austin, TX). An ultra biphenyl column, Ultra Biphenyl 3 μm, 50 × 2.1 mm, was purchased from Restek (Bellefonte, PA).

Two different stocks were prepared in methanol:water:10M NaOH (69:29:2) for IA (4.22 mg/mL and 4.88 mg/mL). One stock was used for preparation of standard solutions, while the other was used for preparing quality controls and validation materials. The calibration standards were prepared in water by serial dilution at 60, 30, 15, 7.5, 3.25 and 1.87 μg/mL for IA. IS stock was prepared in methanol:water:10M NaOH (69:29:2) at 3.50 mg/mL. An IS precipitation solution was prepared in 1% ammonium acetate in methanol at 1.75 μg/mL. All solutions were stored at -70°C in Corning (Corning, NY) cryogenic vials until use.

IV.2.2. Sample Preparation

Sample preparation consisted of adding 150 µL of the precipitating solution to 25 µL of serum, urine, calibrator, or quality control samples in polypropylene microcentrifuge tubes and vortexed for 5 seconds followed by protein precipitation with 300 µL of 1% ammonium acetate in methanol. The mixture was vortexed for 5 s then centrifuged for 3 min at 13,000 × g. The supernatant (10 µL) was mixed with 1 mL of distilled water in LC-MS certified sample vial (Waters; Milford, MA, USA) with 2 µL injected for analysis.

IV.2.3. LC-MS/MS Method

This method was developed on an Applied Biosystems QTRAP 5500 system with a Shimadzu LC-30AD system. Instrument software for this study consisted of Analyst 1.5. Mobile phase A was water with 0.1% formic acid and mobile phase B was methanol with 0.1% formic acid. Samples were injected on the heated (T = 35°C) Ultra Biphenyl analytical column at a flow rate of 0.5 mL/min and a mobile phase composition of 99:1 A:B for 0.2 min. Then, the mobile phase composition was switched to 1:99 A:B for 0.3 min for washing, then back to 1:99 A:B for 1.5 min for re-equilibration before the next injection. The total run time is 2.1 min. The mass spectrometer was set to positive electrospray ionization. The spray voltage was set at 5500 V and the capillary temperature at 700°C. The ion source gas 1 was 50 U, the curtain gas was 40.0 U, and the ion source gas 2 was 50 U. Multiple reaction monitoring was set to monitor the (M+1) transitions listed in Table IV.1. A quantifier and a qualifier ion were monitored for IA

while only a single ion was monitored for the IS. The declustering, entrance and collision cell exit potentials were 125.0, 5.0, and 30.0, respectively.

Table IV.1: Multiple reaction monitoring parameters.

Q1 Mass (Da)	Q3 Mass (Da)	Time (msec)	ID	CE (volts)
614.700	486.800	50.0	IA-Qualifier Ion	24.000
614.700	360.900	50.0	IA-Quantifier Ion	33.000
619.700	365.900	50.0	IS	35.000

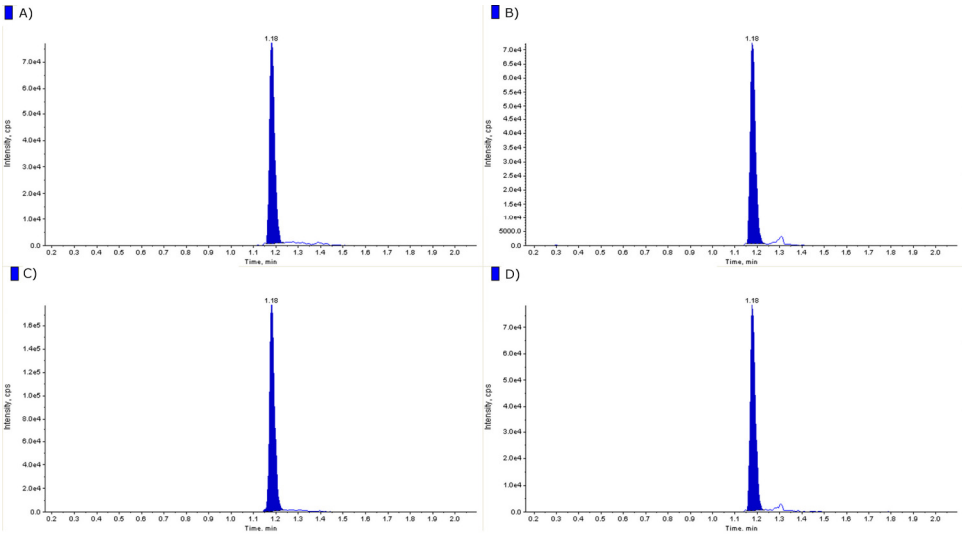

Figure IV.1:Chromatograms showing 10 ug/mL of iothalamate (A) and iothalamate-IS (B) in serum, and 20 ug/mL of iothalamate (C) and iothalamate-IS (D) in urine.

IV.2.4. Method Validation

Absolute ion suppression was evaluated by post-column infusion of a water solution containing 42.2 μg/mL of IA, while extracted patient urine and serum samples (3 males and 3 females each) were injected into the system. Relative ion suppression was studied to test whether the IS accounted for ion suppression in the matrix for the analytes. It is evaluated by extracting and injecting a candidate matrix solution (water spiked with IA at 60 μg/mL), 6 patient urine and serum samples (3 males and 3 females), and 1:1 mixtures of patient samples with the candidate matrix solution. The criteria for a passing test is the response ratio (analyte/IS) of each 1:1 mixture was within 20% of the theoretical response calculated from an average of the measured values of the patient and candidate matrix solution. Interference from lipemic, hemolytic, and icteric plasma samples was investigated at two different analyte concentrations (low and high) by mixing each sample 1:1 with water spiked with IA at 15 μg/mL and 60 μg/mL. It was determined that there was no significant interference if the response ratio of each 1:1 mixture was within 20% of the theoretical response calculated from the average of interference containing sample and the spiked water. Linearity was examined in triplicate by serially diluting serum or urine with spiked blank serum or urine, respectively. The endogenous concentrations of IA in the patient pools (serum and urine) was zero because the compound is exogenous. The analytical measurable range was determined in the linearity study with accuracy within 100 ± 20%, a total coefficient of variation (CV) within 20%, and a signal to noise greater than 10. Carryover was evaluated by 3 independent experiments each consisted of running two extractions in the sequence of low_1-high-low_2,

where low_2 is a re-injection of low_1. A passing test meant low_1 is within 20% of low_2, and that low_2 is within 3 standard deviations of the low_1 value. The standard deviation was determined using low_1 values. High samples that were above assay linearity were diluted within the linear range and the values were back calculated. Precision was evaluated using a modified protocol based on the Clinical Laboratory and Standards Institute (CLSI) EP10-A3 guideline (Wayne, PA, USA) and included running the sequence mid-hi-low-mid-mid-low-low-hi-hi-mid twice a day for 5 days using patient urine and serum samples to determine both intra-assay and total CVs. Statistics were calculated using Excel (Microsoft, Redmond, WA, USA) or EP Evaluator Release 9 (Data Innovations, South Burlington, VT, USA).

IV.2.5. Sample Collection for Radioactive versus Non-radioactive Iothalamate Comparison

Patients (n=22) were consented from the entire body of patients arriving for physician-ordered GFR testing. Patients were recruited to represent the entire spectrum of GFR from health through renal failure, based upon their pre-test estimated GFRs, serum creatinine values, and other data. GFR was measured as the renal clearance of the exogenous GFR marker molecules, ^{125}I – sodium iothalamate (Glofil®, Isotex Diagnostics, Friendswood, TX) – this is the standard of care – and iothalamate meglumine injection USP 60% (Malinkrodt Inc., St. Louis, MO) – this is the tracer that we are interested in implementing clinically for patient care in the future. The markers were given to the patient as two subcutaneous doses, delivered into opposite arms. The GFR testing procedure was identical to the procedure currently used for GFR testing in the Cleveland Clinic Renal Laboratory. Urine samples were collected by voluntary

voiding and blood samples were drawn at the beginning and end of each urine collection period, i.e. bracketing blood samples. Analysis of marker concentration in all serum and urine samples was done by gamma counting (GAMC) of the ^{125}I-iothalamate marker for the clinically ordered GFR, and by LC-MS/MS of the non-labeled iothalamate for the study GFR. Details of the sample collection protocol are described below.

IV.2.5.1. Specimen type/tube; minimum volumes

a. Serum and urine samples are collected as described in detail in the PROCEDURES section under PRE-ANALYTICAL instructions.

b. The volume pipetted from each (serum or urine) sample for GAMC analysis is 0.5 mL (500 µL). The minimum volume of whole blood or urine sample required for gamma analysis is 2 mL; Renal Lab personnel may obtain and process smaller samples under exceptional circumstances.

c. The volume pipetted from each (serum or urine) sample for LC-MS/MS analysis is 50 µL. The minimum volume of whole blood or urine required for LC-MS/MS analysis is 2 mL; Renal Lab personnel may obtain and process smaller samples under exceptional circumstances.

d. Blood samples should be drawn only in Vacutainer SST yellow-top (serum separator) tubes. Urine collections are collected in clean containers with no additive.

IV.2.5.2. 1. Sample Acquisition and Handling

GFR test blood and urine sample acquisition from patients (primary samples) within the Cleveland Clinic will follow institutional guidelines regarding sample

collection. Specimen identification and safety related exposure and radiation guidelines are available in detail in Renal Lab MOPS policies: Specimen Labeling Policy and Procedure and Maintaining Specimen Identity and Integrity, and Renal Lab Safety Manual Sections: Exposure Control Plan and Radiation Safety Manual, Laboratory Use of Radioactive Material (section 8).

IV.2.5.3. Contraindications

a. The labeled/unlabeled iothalamate GFR by renal clearance test(s) should not be performed if the patient has a true allergy to iodine. If an iodine allergy is suspected the patient's doctor or the staff nephrologist should be consulted.

b. The test(s) should not be performed if the patient is pregnant.

c. The test cannot be performed if the patient has had a recent nuclear medicine procedure involving the administration of an interfering gamma-emitting isotope other than technetium.

d. The test cannot be performed if the patient cannot collect reliable, complete, voluntary voided urine.

e. The test cannot be performed if the patient cannot tolerate the hydration necessary to obtain acceptable urine flow rates.

IV.2.5.4. ^{125}iodine-Sodium Iothalamate (Glofil®) Dose Preparation

The Director of the Renal Lab, A5-403, is the approved user, and orders all patient doses of ^{125}iodine-sodium iothalamate (IOTH) from the radiopharmacy at Desk Jb3. Renal lab personnel, working under the Director's supervision, obtain the doses.

The vial of ^{125}iodine-sodium iothalamate is received and kept in the radiopharmacy ("hot lab") in the Department of Nuclear Medicine at Jb3.

For each adult patient (age > 18 years), order 15 + 5 microCuries of Glofil® drawn up in a 0.5 mL insulin syringe, from the radiopharmacy.

Pick up the dose at the radiopharmacy; have the radiopharmacist sign the DAILY ISOTOPE PICKUP LOG, verifying that (at the time of pickup) no product recalls or other warnings have been received at Jb3.

The dose is carried to Q7-261, and is stored in the Avanti I isotope refrigerator until use. After use, Renal Lab personnel transport the empty dose syringe back to the radiopharmacy for disposal.

IV.2.5.5. Non-labeled Sodium Iothalamate (Conray) Dose Preparation

a. The Director of the Renal Lab, Q7-261, will order doses of Conray iothalamate meglumine Injection USP 60% (CON) through the department of Nephrology as this marker is for research purposes only. Nephrology nursing personnel, working under the Directors supervision, will draw up the doses as specified.

b. Vials of Conray are received and kept in the medications refrigerator in the Q7 nursing station, room Q7-268.

c. For each adult patient, 0.5 mL (500 µL) of Conray will be drawn up in a 1 mL TB syringe. This initial dose will be diluted in the syringe by then drawing up 0.5 mL of 0.9% saline for injection to give a total volume of 1.0 mL. The unused portions of Conray

and saline will be discarded; in no case will the respective vials be used for multiple doses.

IV.2.5.6. Patient Preparation

a. Patients are given preparation instructions, by scheduling personnel, in advance of the study. The evening before the test, the patient is instructed to drink an additional one liter (one quart) of liquid between dinner and bedtime. The morning of the test, the patient must drink 500 mL (2 eight oz. glasses) of water or other beverage to initiate a diuresis. If the volume or time of the patient's pre-test hydration vary from this ideal, the test may be started if the patient can provide a background urine sample. If pre-test hydration is minimal, it is preferable to delay the study at least one-half hour and hydrate the patient with at least 500 mL of water. After the dose injections, adjust the hydration amount given to the patient as needed.

b. Patients routinely taking morning doses of a diuretic should be instructed to not take the diuretic first thing in the morning, but rather, to bring the diuretic with them to the GFR test. They will take the diuretic at the start of the test.

IV.2.5.7. Test Procedure

a. All samples will be labeled, at the time of collection, with the patient's name and date of birth identifiers (the patient's Cleveland Clinic number may also serve as an identifier). The official "patient" format label, printed from the patient's scheduling page in Epic, will be used first and foremost. In addition, the test date, sample collection time, and sample name/abbreviation will be written, in ink, on the label.

b. Examine the patient's medical chart and talk with them to determine if any contraindications to the test exist. If contraindications exist, contact the ordering physician, Director of the Renal Lab, or Nephrology Doctor of the Day to explain the situation and need to cancel testing. Record and file the contact information as per MOP policy. If contraindications do not exist, fill in the initial patient information on the Standardized glomerular filtration rate (GFR) Data Form, Version 02/10/2008.

c. Measure the patient's height (cm) and weight (kg) and record on the Standardized GFR Data Form. As the test continues, fill in test data as indicated on the form.

d. Have a Nephrology nurse give the patient, by mouth, 5 drops of super-saturated potassium iodide solution (SSKI) diluted in about 15 mL of water or other suitable beverage.

e. Immediately before the IOTH/CON dose injections, have the patient void completely and record the time at the moment of completion of voiding (Tbkg). This is the background urine sample. The sample name code for this sample is Ubkg. All urine collection times should be recorded to the nearest minute in the applicable space on the Standardized GFR Data Form.

f. Save an aliquot (5 mL minimum) of Ubkg. The total volume need not be recorded.

g. Since no interfering isotope test was found as a contraindication in step 4b, no background blood sample is needed. If there is any doubt or question about any background isotope interference, draw a blood sample in a 5 mL SST tube. Label the background blood sample Bbkg. This blood sample and all other blood samples should be

drawn as soon as possible after their respective urine collections. The times of the blood draws are not recorded.

h. Have the Nephrology nurse first administer the IOTH dose as a subcutaneous injection in the side of the upper arm and note the time (Tinj). Immediately following the IOTH injection, the CON dose should be given, in an identical fashion, in the opposite arm. The injections should always be above any site where a tourniquet could be applied.

i. Instruct the patient to drink 10 mL/kg of water or other beverage in the hour following the injection, to maintain hydration and insure adequate urine flow as the test progresses. Avoid refrigerated or iced beverages.

j. After a minimum of 60 minutes has passed since the Tinj, and when the patient has a voiding urge, instruct the patient to urinate completely and collect the entire specimen. Record the time that the urine collection is completed as T0. Label the urine collection U0.

k. Measure the volume, V0, of U0. All urine volumes must be recorded using a graduated cylinder; record volume to the nearest milliliter. If the volume, Vo, is>250 mL, record the volume and proceed to step l. If the volume is not >250 mL, wait an additional 30 minutes (minimum) and have the patient void again, record the new void time as T0 (disregard the earlier T0 time). Measure this additional urine volume, V02, and add the value to V0 to give a new volume for U0. If the total volume is >250 mL, record and go to step l. If the volume is still not >250 mL, continue to collect urine until U0 volume is sufficient; if this does not occur after 3 hours, the test may have to be discontinued. Call ordering physician to discuss discontinuation/re-ordering of test. Document any such decision as per MOP policy.

l. Draw a blood sample and label it B0.

m. Once an adequate flow rate has been established and B0 has been drawn, the urine collection U0 and any additional urine collected in step k. can be discarded.

n. Hydrate the patient from this point on at the rate of 200-400 mL/hour. At hydration equilibrium, this rate would provide a urine flow of approximately 3-6 ml/minute. Hydration volumes may be adjusted at the discretion of the testing personnel; patients should be questioned about their comfort and the presence of any symptoms (e.g. nausea, dizziness, shortness of breath, etc) regularly throughout the test. Discontinue hydration and/or consult with the Nephrology nurse or Doctor of the Day if any significant symptoms occur.

o. After a minimum wait of 30 minutes from the urine collection time, T0, when the patient has a voiding urge, have the patient urinate completely and collect the entire specimen. Record the time that the collection is completed,T1. Label the urine U1. Measure the volume of U1, record the volume, V1, and save the entire specimen.

p. Calculate the flow rate: V1 / (T1 – T0). The flow rate must be at least 1.0 ml/minute for collection U1 and all following collections. If the flow rate is sufficient, go to step q. If not, wait additional time, as in step k., and collect additional urine to mix with U1 until the flow rate is adequate. The technologist may accept lower urine flow rate collections, at their discretion, where exceptionally difficult circumstances exist. The urine must be physically mixed before any processing. Similarly, record the final T1 time as the collection time and V1, the collection volume.

q. Collect blood sample and label B1.

r. If more than one test period is desired, repeat steps o. through q.; for example, for a second gfr urine collection, record time T2; measure and record the volume, V2, of urine U2, and draw and label blood B2. This step may be repeated for additional collections if desired. Number them sequentially.

IV.2.5.8. Sample Processing and storage

a. Whole blood specimens should be centrifuged for 10 minutes at 3000 RPM in the PowerSpin MX centrifuge. Inspect specimens post-spin to insure silicone gel has sealed off cells. After centrifugation, GAMC of samples will proceed in a timely fashion for clinical purposes. Serum may be stored in primary tube, at room temperature, for up to one week after GAMC. Store GAMC serum samples for longer intervals at 2 – 8°C. LC-MS/MS analysis of serum samples will occur as batch runs at a later time. Aliquots of serum samples will be stored at -70°C until LC-MS/MS analysis.

b. GAMC and LC-MS/MS urine samples are processed with no further routine preliminary preparation. If the urine sample contains suspended debris, cells, etc., spin an aliquot in a 15 mL Falcon urine tube (BLUE MAXTM Jr. 15 ml Polypropylene Conical Tube, 17 X 120mm, Becton Dickinson Labware) at 2500 RPM for 15 minutes. Pipette/store the clear supernatant for analysis. GAMC of samples will proceed in a timely fashion for clinical purposes. Urine may be stored in primary containers at 2 – 8°C for up to one week after GAMC analysis. LC-MS/MS analysis of urine samples will occur as batch runs at a later time. Aliquots of urine samples will be stored at -70°C until LC-MS/MS analysis.

IV.3. Results and Discussion

IV.3.1. Method Development

During the early stages of development, several reverse-phase analytical columns were tested including C18 Kinetex (Phenomenex, Torrance, CA) and Hypersil Gold aQ (Thermo Scientific, Waltham, MA), but the Ultra Biphenyl column used gave the best peak shape. Similarly, various extraction solvents were tested, including acetonitrile, 1% formic acid in acetonitrile, methanol and 1% ammonium acetate in methanol at various dilution ratios. Acetonitrile was ruled out because IA was sparingly soluble in it and therefore a concentrated precipitation IS mixture could not be prepared using acetonitrile. Methanol with 1% ammonium acetate provided the cleanest extract and best sensitivity among the remaining options and was used to prepare the precipitation mixture. In addition, the final water dilution step was optimized at 1:100 (supernatant:water) and 2 uL injected because the method was very sensitive and the sample needed to be diluted much further to provide a wider dynamic range and to prevent detector saturation at high concentration of IA. In comparison with the existing method, the 2.1 min run time of this method makes it significantly faster. In addition, the same sample preparation is applied for both urine and serum here, which makes sample preparation very easy and fast for the technician. Furthermore, this method employs ion ratios (quantifier and qualifier ion) and is therefore highly selective in comparison with other existing methods. This combination of advantages makes this method highly adaptable to a clinical laboratory setting.

IV.3.2. Method Validation

Significant absolute ion suppression was not observed for IA in either urine and serum and it passed the relative ion suppression test, with mean difference between the measured concentrations in the mixtures and the theoretical concentrations (n = 6 for each) of -1.86%, and -0.76% for serum and urine, respectively. No interference was observed from lipemic, hemolytic or icteric plasma samples for all analytes. Linearity in serum and urine was determined to be 0.44-62.1 μg/mL and 1.73-423 μg/mL with the accuracy ranging from 80 to 97% and 100 to 111%, respectively (Table IV.2). No significant carryover was observed up to a tested concentration of 407 μg/mL. The intra-assay and total CVs were all within 7.2% for all levels tested (Table IV.3).

Table IV.2: Precision and recovery data from the linearity study

Iothalamic acid in	Mean, µg/mL	Analytical recovery	%CV
Serum	0.44	80%	13.3%
	1.03	94%	8.4%
	2.30	84%	4.3%
	4.63	84%	2.2%
	9.39	85%	5.6%
	26.8	97%	4.7%
	37.3	95%	2.1%
	53.9	96%	5.5%
	62.1	88%	12.1%
Urine	1.73	111%	5.0%
	3.18	102%	3.3%
	6.38	102%	4.7%
	12.6	101%	1.2%
	25.4	101%	2.8%
	50.1	100%	5.1%
	102	102%	1.0%
	213	106%	1.8%
	423	106%	4.3%

Table IV.3: Precision data based on CLSI EP10-A3 protocol

	Serum			**Urine**		
	Low	Mid	High	Low	Mid	High
n	30	30	30	30	30	30
Mean, µg/mL	3.88	24.7	44.4	9.0	96.7	188
Total %CV	7.2	6.8	6.9	6.2	3.6	3.9
Intra-assay %CV	6.7	2	2.2	3.0	0.9	0.7

IV.3.3. Radioactive versus Non-radioactive Method Comparison

Comparison of the GFR results obtained from measuring iothalamate using the LC-MS/MS method versus the radioactive method showed a mean difference of 2.656 mL/min/1.73m^2 and the Deming regression analysis showed a slope of 1.056 (95%CI: 1.002 to 1.111), intercept of -0.454 (95% CI: -9.827 to 2.920) and standard error of estimate of 3.572. Coefficient of correlation (R) was 0.9940. This data suggests that the two methods agree closely with a few exceptions, which required us to keep collecting patient samples and compare again with a larger number of patients. The scatter plot, Bland Altman plot and percent Bland Altman plot are displayed Figure IV.2, IV.3 and IV.4, respectively.

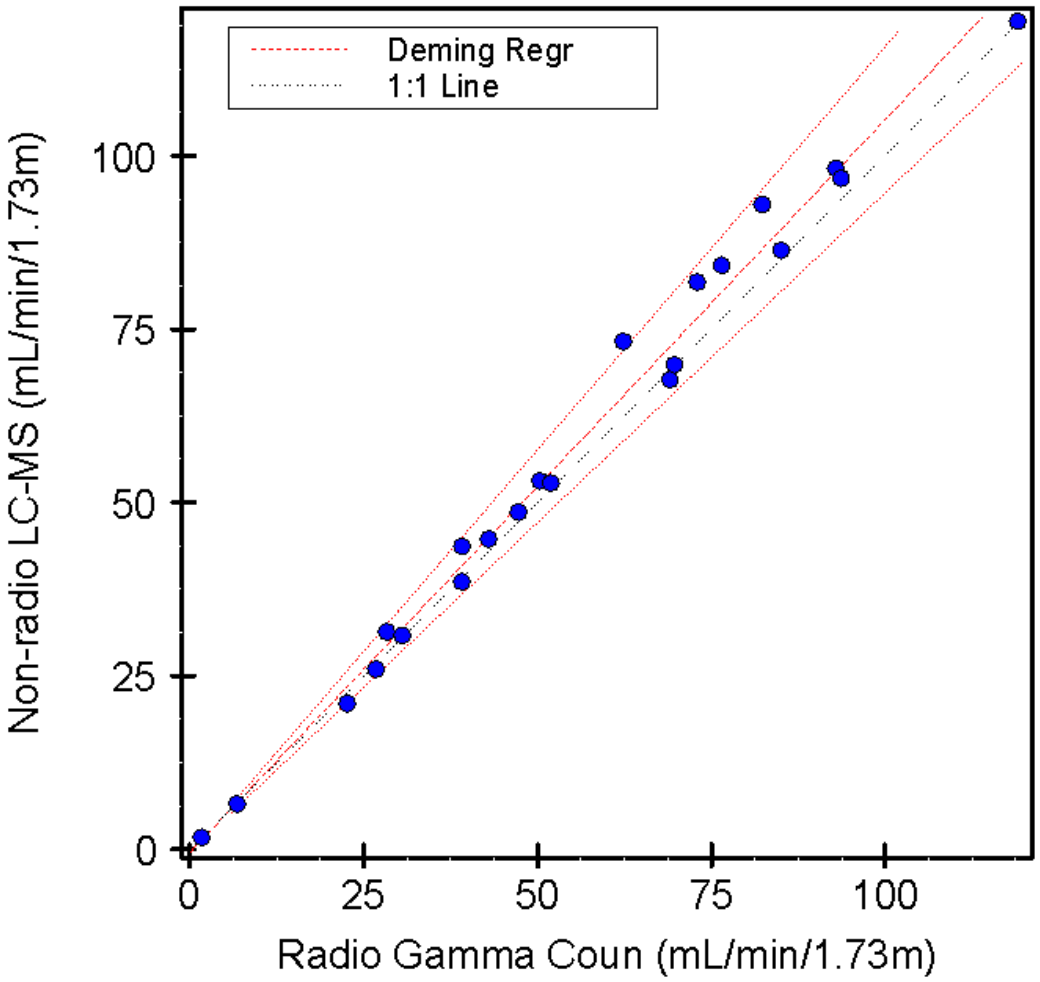

Figure IV.2: Scatter plot displaying GFR results from Non-radioactive LC-MS/MS versus Radioactive gamma counting.

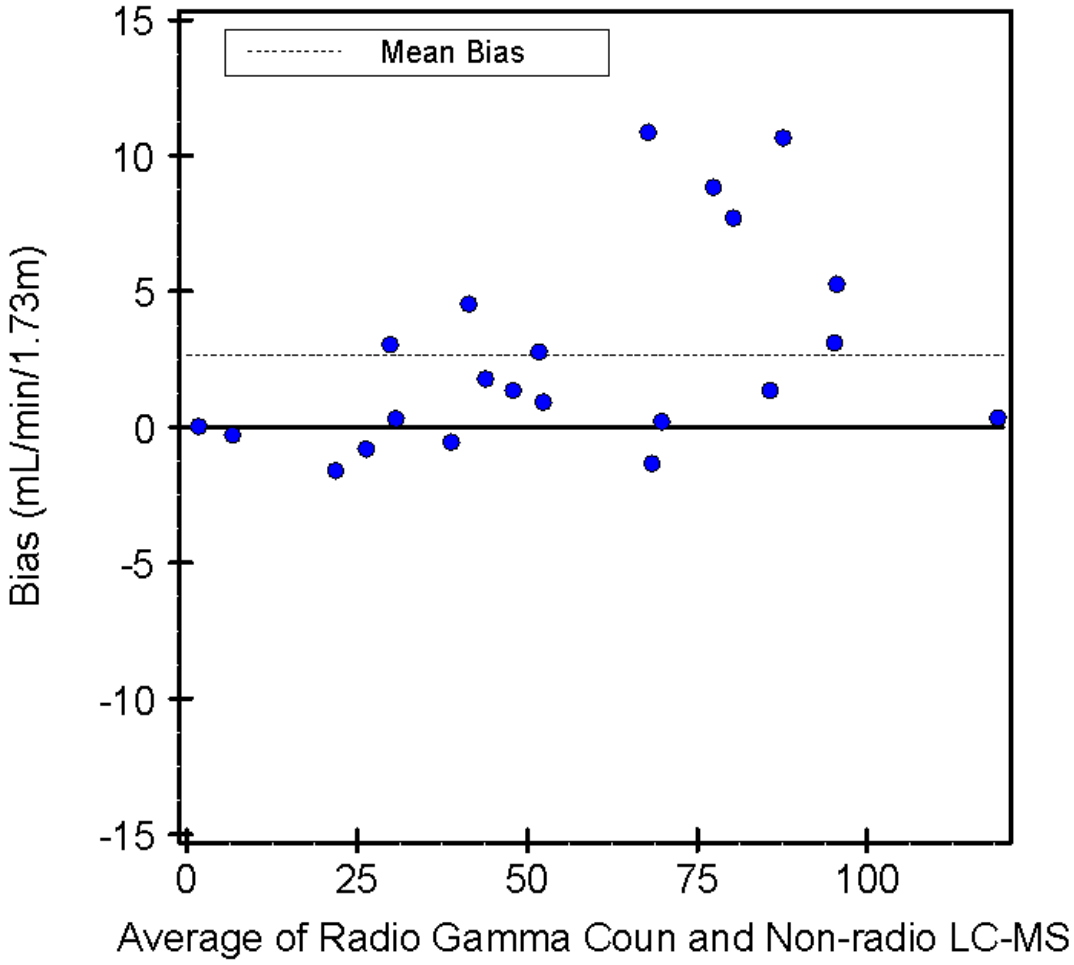

Figure IV.3: Bland Altman plot showing the difference in GFR results between the radioactive gamma counting method and the non-radioactive LC-MS/MS method (bias) versus the average of the two methods.

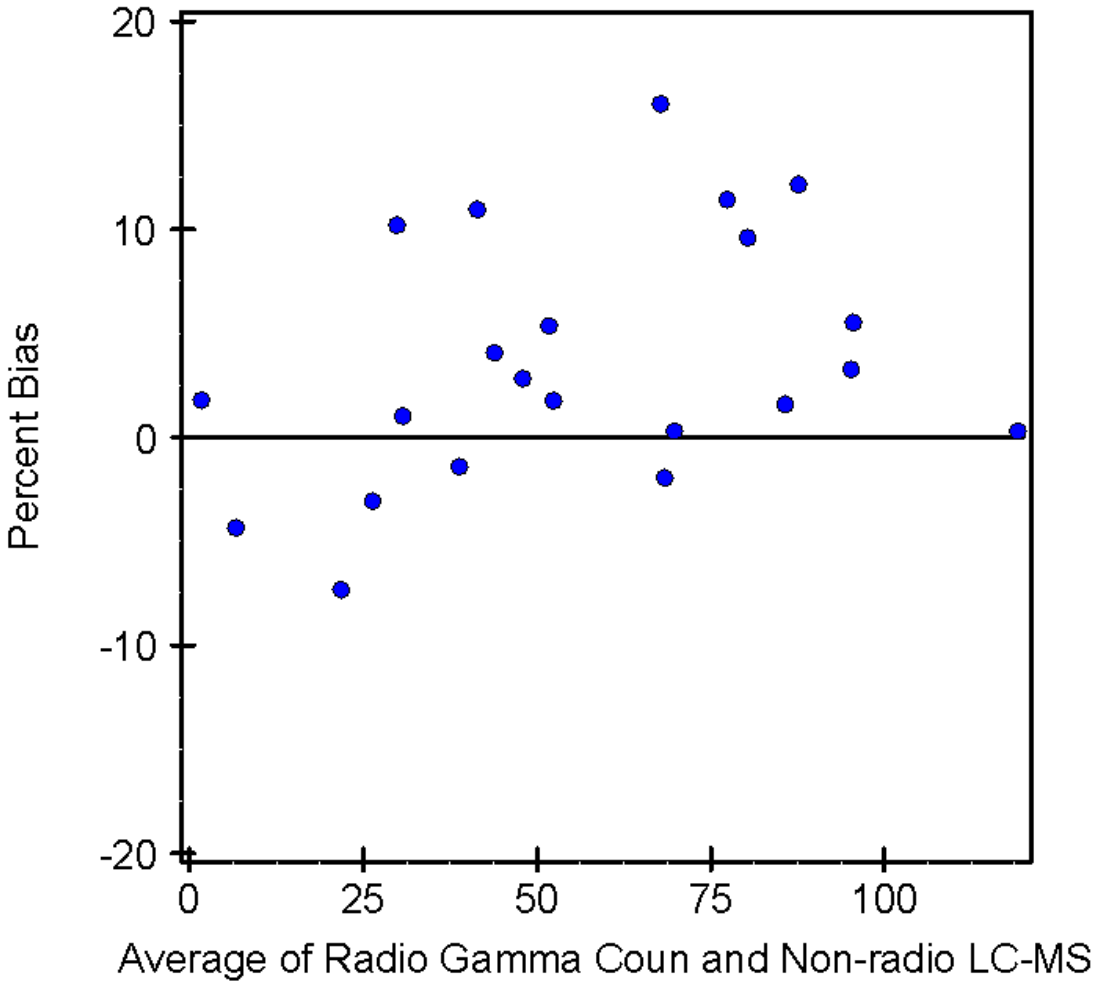

Figure IV.4: Percent Bland Altman plot showing the percent difference in GFR results between the radioactive gamma counting method and the non-radioactive LC-MS/MS method (bias) versus the average of the two methods.

IV.4. Conclusion

We have developed a simple and fast LC-MS/MS assay for the measurement of sodium iothalamate in serum and urine. The ability to perform the same sample preparation for urine and serum is very advantageous because it is simpler for the technician performing the test and both can be quantified off the same calibration curve. In addition, the rapid chromatography employed (2.1 min) allows for high throughput analysis, and the use of ion ratios provides enhanced selectivity for this assay.

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CHAPTER V

OVERALL CONCLUSIONS AND FUTURE DIRECTIONS

V.1. Chapter I

The results obtained from the pilot study comparing the effectiveness of vitamin D_2 versus vitamin D_3 in restoring normal 25OHD levels in pre-dialysis CKD patients were a surprise even in such a small patient sample population. The existing literature seemed to point to a direction that suggested that vitamin D_3 would clearly emerge as the more effective form; however this study demonstrates that the difference is not clear cut and easy to discern. Larger prospective studies must be performed to completely discern the differences between the two analogues, but we would recommend using different dosing patterns because this one is clearly ineffective as only 50% (n=8) in both treatment groups reached target levels. This work is being prepared for publication.

Furthermore, we have collected additional tubes from the patients enrolled in this study that will be used to measure associations of certain cardiovascular and kidney biomarkers, such as ADMA and SDMA, to treatment with vitamin D in CKD patients.

V.2. Chapter II

The method validation protocol presented in this Chapter has been completely developed in-house and is a useful resource for the validation of LC-MS/MS assays that will go into clinical use. A more detailed version of this protocol, which provides step-by-step instructions to validation, is stored in LabQMS at the Cleveland Clinic. The great advantage that this protocol provides over existing guidelines is that it gives detailed step-by-step procedures for method validation from data acquisition to data analysis. We have already used this protocol for most LC-MS/MS methods developed at the Cleveland Clinic, and several of these have already been published [1-6]. This protocol will certainly continue to be used and further refined as the technology evolves. We are currently submitting the detailed protocol for publication.

V.3. Chapter III

The reported method for the measurement of Arginine, SDMA and ADMA is a significant enhancement over existing methods in terms of throughput and simplicity of the sample preparation procedure. The determined reference range is in concordance with what has been reported in the literature using larger patient populations and LC-MS/MS. The developed method has already been published [1]. The clinical data obtained shows good correlation between SDMA and its ratios with markers of kidney function, and ADMA and its ratios with markers of cardiovascular disease.

Additional large cohort studies should be performed to demonstrate the clinical utility of these tests. SDMA should be evaluated against the gold standard way of determining GFR in clinical practice, iothalamate clearance, and compare its performance to creatinine and creatinine-based equations. Once comparison with actual GFR is available, this data will be prepared for publication.

V.4. Chapter IV

The newly developed method for the measurement of non-radiolabeled iothalamate is a significant enhancement over existing methods. The simple sample preparation which involves only PPT prior to injection is the same for both urine and serum and provides an added level of convenience for the technologist performing the test. In addition, the sample volume requirement is lower (25 µL), and chromatography time is significantly shorter (2.1 min), which greatly enhances the throughput of this method. Furthermore, the use of ion ratios (qualifier/quantifier) provides higher degree of selectivity over existing methods, and data showing the correlation of this method with the existing gold standard radioactive method prove that this method is accurate as well.

Further studies that need to be done include additional collection of patient samples to cover the entire spectrum of GFR values (ideally n = 40). Once all 40 patients have been measured, the procedure will be published. Furthermore, additional studies must be performed on the non-radioactive iothalamate to confirm it is safe to use in adults as well as children, such as degradation.

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APPENDIX

Appendix A

Patient Consent Form

The Cleveland Clinic Foundation

Consent to Participate in a Research Study

Study title: UPGRADE: A Randomized, Double-Blind Study to Evaluate the Effectiveness of Cholecalciferol versus Ergocalciferol following Kidney Disease Outcomes Quality Initiative (K/DOQI) Guidelines for Vitamin D Therapy in Stages Three and Four Chronic Kidney Disease (CKD) Patients.

Principal Investigator: James Simon, MD

Please carefully review this consent document. The purpose of a consent document is to provide you with information to help you decide whether you wish to participate in this research study. Your decision is completely voluntary and will not affect your medical care if you choose not to participate. It is important for you to ask questions and understand the research risks, benefits and alternatives.

- **You are being asked to participate in a research study**
- **Your participation is voluntary**
- **Carefully consider the risks, benefits and alternatives of the research**

Your health care provider may be an investigator in this research study, and as investigator, is interested in both your welfare and in the conduct of the study. Before entering this study or at any time during this research, you may ask for a second opinion about your care from another doctor who is in no way associated with the research study. You are not under any obligation to participate in any research project offered by your doctor.

1. INFORMATION ON THE RESEARCH

Why Are You Being Asked To Take Part In This Research?

You are being asked to take part in this study because you have decreased kidney function (chronic kidney disease or CKD), low vitamin D levels in your blood and an elevated level of a substance called parathyroid hormone (PTH) in your blood.

Why Is This Study Being Done?

It is very common to have low levels of vitamin D in your blood. This form of vitamin D is also called "inactivated" vitamin D because the body (mainly the kidneys) has to activate it before it provides most of its benefits. You get vitamin D from 2 main sources: sunlight and food (dairy product, certain fish). Despite drinking dairy products such as milk and going out in the sun, many people cannot maintain normal levels of vitamin D. Vitamin D deficiency is common in patients with kidney disease. Having low vitamin D levels has been associated with increased risk for falls, broken bones, diabetes, cancer, high blood pressure, heart disease and certain immune disorders called autoimmune disease. In addition, this problem can stimulate the overproduction of a hormone called parathyroid hormone, or PTH. This hormone can lead to problems with weakened bones or hardening of the arteries. There are treatment guidelines (K/DOQI guidelines)

published by the National Kidney Foundation that outline how to replace vitamin D levels in patients with kidney disease. However, how often vitamin D levels are fully replaced when following these guidelines have not been studied. In addition, there are different formulations of vitamin D that can be given. The guidelines recommend a form called ergocalciferol (Vitamin D2). Research data suggests that an alternate form, called cholecalciferol (Vitamin D3) may be more effective at building up the vitamin D levels in your blood. No one has compared these head-to-head to determine which is better in patients with kidney disease.

In addition, studies suggest that replacing vitamin D levels in kidney disease can help reduce the PTH levels back to normal ranges in a portion of patients. It is unclear how many people have this benefit or what dose is necessary to do this. If replacing vitamin D does not normalize PTH levels, your provider may prescribe a more expensive compound called an "activated" vitamin D medication. Therefore, it is important to establish whether the "inactivated" Vitamin D (D2 and D3) can normalize PTH levels when following the published guidelines.

This study will help determine which vitamin D compound, ergocalciferol or cholecalciferol, is more effective at replacing vitamin D in your blood when used according to the current published K/DOQI guidelines. It will also determine how well the K/DOQI guidelines work to replace low vitamin D levels and lowering the elevated PTH levels. The treatment of your low vitamin D levels will follow current standard of care, except that there is a 50/50 chance you will be assigned to use cholecalciferol, which is not currently part of the published guidelines.

The U.S. Food and Drug Administration (FDA) has approved vitamin D2 (ergocalciferol) for commercial use for the treatment of low Vitamin D levels. Vitamin D3 is available over-the-counter at lower doses and by prescription at higher doses equivalent to those approved for D2. It has been previously studied at the higher doses used in this study and deemed to be safe and effective.

The purpose of this study is to examine the effectiveness of vitamin D3 versus vitamin D2 in raising vitamin D and suppressing PTH levels of patients with kidney disease not on dialysis.

How Many People Will Take Part In The Study?

This study will include a total of about 86 subjects enrolled from the Cleveland Clinic Nephrology clinics at Main Campus and the Westlake Family Health Center.

What Is Involved In The Study?

If you are eligible for this study, you will be randomly assigned (flip of a coin) to one of the following treatment groups:

1. Vitamin D2 treatment group
2. Vitamin D3 treatment group

Initial Screening:

Once you give your consent to participate in the study, any required labwork that is not already available from within the last 45 days will be ordered. If this is the case, you will be asked to go to the lab and have about 2 teaspoonfuls of blood collected to check these missing labs. This may be part of your routine labwork. Since we have already determined that you meet the criteria to join the study, we will not wait for these extra

results before starting the study medication. If you are a woman of child-bearing potential you will also have a blood pregnancy test performed.

Information on your date of birth, gender and race will be recorded. In addition, your medical record will be reviewed and pertinent medical problems, medications, vital signs or exam findings will be recorded for the study.

We will dispense the study Vitamin D medication (either D2 or D3) to you today. The total duration of this study will be 38 weeks – the first 24 weeks are the Treatment Phase, the following 12 weeks are the Follow-Up Phase, and the final 2 weeks are for us to contact you at the end of the study.

Treatment Phase:

The Treatment Phase will last for 24 weeks. You will begin taking the vitamin by mouth today. How often you have to take the vitamin D supplement will depend on how low your vitamin D level is. This will be explained to you by the study investigator. You will be given stickers to place on your calendar to remind you when to take the medication. You will have labwork once every 6 weeks during this period. About 2 teaspoonfuls of blood will be drawn at weeks 6, 12, 18, and 24 to measure your PTH, vitamin D, calcitriol, and renal function panel. A study investigator or a research nurse will call you to remind you to have your blood drawn and to ask if you are experiencing any side effects that might be related to the medication. If we were unable to fully replace your vitamin D levels at the end of 24 weeks, you will not continue on to the Follow-Up Phase of the study.

Follow-Up Phase:

The Follow-Up Phase will last for 12 weeks. The purpose of this phase of the study is to see how well a daily multivitamin maintains your vitamin D levels once we have replaced them. If you are in either treatment group and your vitamin D level is normal at 24 weeks, you will stop taking the prescribed 50,000 IU capsules, and start taking daily multi-vitamin capsules which contain no more than 400 IU of vitamin D per capsule.

You will be asked to give blood 2 more times during this phase, at weeks 30 and 36. The same tests will be run as during the Treatment phase. The total amount of blood to be drawn over the course of the study will be about 14 teaspoonfuls over the course of about 9 months.

Before every scheduled lab draw, a study investigator will contact you to ask if you are having any medical problems that might be related to the study medications, to make sure you are still taking the medications as prescribed and to remind you about your upcoming labwork.

All labwork must be collected at either a Cleveland Clinic Main Campus laboratory or one of the Cleveland Clinic Family Health Center laboratories. Please do not go to a Cleveland Clinic-affiliated hospital lab or to a non-Cleveland Clinic lab for this study.

Telephone Contacts:

You will be contacted via telephone by a study participant 1-2 weeks before your scheduled labs are due to be drawn. During this phone interview, you will be asked about medication compliance, whether any outside vitamin D or other prohibited medication is being used, whether calcium supplementation is being used and any adverse side effects. You will be reminded to have your 6-week interval blood tests done. If blood tests are not

completed within the allotted time frame, you will be contacted again by telephone to remind them again to have their blood drawn. Phone calls will occur according to the following time-frame: week 4, 10, 16, 22 (and weeks 28 and 34 if you are included in the Follow-Up Phase).

Exit Interview

An exit telephone interview will take place between week 24 and 26 for those patients censored after the Treatment Phase and between weeks 36 and 38 for those included in the Follow-up Phase. Subjects will be asked all of the questions in the above Telephone Interview as well as be informed that the study has ended. If you decide to withdraw from the study, an exit interview will be performed at the time of withdrawal unless one was performed in the last 2 weeks.

How Long Will You Be In The Study?

If you complete the study, your time in the study will be approximately 38 weeks.

2. RISKS AND DISCOMFORTS

What Are The Risks Of The Study?

Vitamin D2 and D3 are generally well tolerated. All side effects are rare enough that percentages of patients who experience them are unpublished. Rare or previously unknown or unforeseeable side effects, which may be serious, may occur. You will be monitored closely for these side effects, and if your doctor thinks it is necessary, your study drug will be stopped. If you experience any side effects, you should notify your doctor or a study investigator immediately.

<u>Common risks</u>

Nausea, vomiting and diarrhea

In our clinical experience, while still rare, these are the most common side effects. You might experience stomach pains after taking the medication. Less often you may experience nausea, vomit or have loose stools.

High Vitamin D levels

If taken at too high of a dose for too long, Vitamin D levels may become too high. This rare event can lead to a problem called hypervitaminosis D, which can lead to high calcium levels in your blood, headache, nausea, vomiting, lethargy, confusion, abdominal pain, increased urination, increased thirst, muscle weakness, and in severe cases problems with your heart rhythm or deposition of calcium into other organs. To avoid this, your blood tests will be monitored frequently enough to identify if the Vitamin D or calcium levels become concerning. In this case, you will be called and asked to either stop the medication or decrease how often you are taking it.

Drawing blood

The risks of drawing blood from a vein includes discomfort at the site of the needle stick, possible bruising and swelling around the site of the needle stick, rarely an infection, and uncommonly feeling faint from the procedure.

Uncommon risks

Allergic Reaction

Allergic reactions to vitamin D compounds are very rare. However, there is always the potential for an allergic reaction to a medication. Symptoms of an allergic reaction could include a rash, itching, low blood pressure, breathing difficulties or swelling. If you develop signs and symptoms of an allergic response, you should contact your physician or

a study coordinator immediately to determine whether you should stop taking the study medication. If you experience difficulty swallowing or breathing, chest discomfort, the feeling that you are going to pass out or any other symptoms you might consider life-threatening, you should call 911 or go directly to an emergency room.

If you agree to participate, you or your family members should tell your nurse or doctor immediately if you have any unusual health experiences, injuries or side effects while you are in this study.

Unknown risks

Pregnancy

The risks of vitamin D supplementation to an unborn child are unknown. Studies in animals suggest that if vitamin D levels are too high (Hypervitaminosis D), there may be risks to the unborn child. Therefore, if you are capable of giving birth to a child, you and your sexual partner should use adequate birth control measures while you are in the study. These measures may include but are not limited to abstinence, oral contraceptives (birth control pills), IUD, diaphragm, Norplant, approved hormone injections, condoms, or documentation of medical sterilization. If you are unwilling to do this, we ask that you not participate in this study.

If you do become pregnant while taking part in this study you must notify the study investigator immediately. The study medication will be stopped and you will be withdrawn from the study. We may request initial pregnancy information and information on the pregnancy outcome for both the mother and child.

Breastfeeding

High levels of calcium have been detected in children who are breastfeeding from mothers taking large doses of vitamin D. Therefore if you are breastfeeding, you will not be able to participate in this study.

3. BENEFITS

Are There Benefits To Taking Part In The Study?

The study drug and supplement, vitamin D2 and D3, are expected to increase vitamin D and lower PTH levels in patients with chronic kidney disease not on dialysis. It has been suggested that this may have long-term benefits on your bone health and heart health, among others. However, no guarantee of any results or outcome can be made.

You do not have to take part in this study to be treated for your vitamin D deficiency, your kidney disease or any other condition you have. If you decide to be in this study, it is possible that you will receive no direct benefit.

4. ALTERNATIVES

What Other Options Are There?

If you do not participate in this study, it is likely that your doctor will recommend that your low vitamin D levels be treated with ergocalciferol, one of the study drugs, in exactly the same manner as is prescribed in the study. They may also prescribe cholecalciferol, the other study drug, at lower doses available over-the-counter. Alternatively, they may not recommend treatment. Other medical therapies for secondary hyperparathyroidism include the administration of phosphate binders, calcium, cinacalcet, and/or calcitriol. Phosphate binders can lower your serum phosphorus levels, and cinacalcet, calcium and calcitriol can decrease your PTH levels. However, no

alternatives other than vitamin D2 or vitamin D3 exist for treatment of low vitamin D levels.

5. PRIVACY AND CONFIDENTIALITY

Will Your Information Be Kept Private?

The medical and research information recorded about you will be used within the Cleveland Clinic as part of this research. The results of your bloodwork done solely for this research study may be placed in your medical record. Otherwise, a copy of the results will be forwarded to your nephrology provider. Upon completion of the study, you may have access to the research information if contained in the medical record. Your medical records may also be reviewed and copies made by members of either the institutional review board/independent ethics committee responsible for this trial site or a regulatory agency.

Your access to research information about you will be limited while the study is in progress. Preventing this access during the study keeps the knowledge of study results from affecting the reliability of the study. This information will be available should an emergency arise that would require your treating physician to know this information to treat you best.

Your research information may be disclosed to the Cleveland Clinic research review staff and the U.S. Food and Drug Administration. The Cleveland Clinic also may use and disclose this information for treatment and payment reasons. The Cleveland Clinic must comply with legal requirements that mandate disclosure in unusual situations. Otherwise, the information recorded about you as part of this research will be maintained in a

confidential manner. It is possible that information disclosed about you outside the Cleveland Clinic could be re-disclosed and no longer protected by federal privacy laws. Your research information may be used and disclosed indefinitely, but you may stop these uses and disclosures at any time by writing to Dr. James Simon, at The Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, Ohio 44195, 216-445-4891. If you do so, any information previously disclosed cannot be withdrawn. The Cleveland Clinic will not use or disclose the information collected in this study for another research purpose without your written permission; unless the Cleveland Clinic Institutional Review Board gives permission after ensuring that appropriate privacy safeguards are in place. The Institutional Review Board is a committee whose job is to protect the safety and privacy of research subjects.

Federal Regulations require that you authorize the release of any health information that may reveal your identity. The persons and entities that you are authorizing to use or disclose your individually identifiable health information may include the study doctor, the study staff, Cleveland Clinic monitors/auditors, and the IRB, the U.S. Food and Drug Administration and the Department of Health and Human Services (DHHS). Because of the need to release information to these parties absolute confidentiality cannot be guaranteed.

By signing this informed consent form, you are authorizing such access to your medical records.

If you choose not to sign this consent form, you will not be permitted to participate in this research study.

6. RESEARCH RELATED INJURIES

What Happens If An Injury Occurs?

If physical injury occurs due to your involvement in this research, medical treatment is available, but your medical insurance will be billed the cost of treatment.

The Cleveland Clinic will not voluntarily provide compensation for medical expenses or any other compensation for research-related injuries. Further information about research-related injuries is available by contacting the Institutional Review Board at (216) 444-2924.

7. COSTS

What Are The Costs?

The Cleveland Clinic will pay for the study drug and supplement, and extra study specific tests that are not routine and only being performed because you are participating in this study. You will not be charged for these specific tests. You will receive the study drug and supplement free of charge as long as you participate in this study. The Cleveland Clinic will not pay for the costs of procedures, tests, visits and hospitalizations not in connection with this study.

Because many of the lab tests required for this study would normally be performed even if you didn't participate in the study, some of the study lab tests will be billed to your insurance company.

You will receive by mail $10 dollars for each study related blood draw (7) and vouchers for free parking if the lab at main campus is used. There is no parking fee to park at the CCHS family health centers.

It is possible that through the use of your medical data and sample for exploratory research, a commercial pharmaceutical product may be developed from your medical data and/or samples. If you decide to sign this consent form you are releasing (giving) to Cleveland Clinic your blood sample, the by-products of your sample, and any products developed from the sample or use of the sample. Cleveland Clinic, other researchers, or research companies may patent or sell discoveries that result from this research. Neither Cleveland Clinic nor the principal investigator will compensate you if this happens.

8. VOLUNTARY PARTICIPATION

What Are Your Rights As A Participant?

Taking part in this study is voluntary. You will be told of any new, relevant information from the research that may affect your health, welfare, or willingness to stay in this study. You may choose not to take part or may leave the study at any time. Withdrawing from the study will not result in any penalty or loss of benefits to which you are entitled. If you decide to withdraw from the study you should discuss with your study doctor your decision to ensure a safe withdrawal.

You will be removed from the study if you receive a kidney transplant, start on dialysis or have your parathyroid glands removed. You will also be removed from the study if you become pregnant during the study.

9. QUESTIONS

Whom Do You Call With Questions Or Problems?

If you have any questions about the research, concerns or complaints about the research, or develop a research-related problem, you should contact James Simon MD at (216) 445-4891. During non-business hours, you should contact the page operator at (216)

444-2200; ask for the Nephrology fellow on-call. If you have questions about your rights as a research subject, you should contact the Institutional Review Board at (216) 444-2924. You can also contact the Institutional Review Board if you have concerns or complaints about the research, if you cannot reach the research team, or wish to talk to someone other than the research staff.

10. SIGNATURE

Statement of Participant

I have read and have had verbally explained to me the above information and have had all my questions answered to my satisfaction. I understand that my participation is voluntary and that I may stop my participation in the study at any time. Signing this form does not waive any of my legal rights. I understand that a copy of this consent will be provided to me. By signing below, I agree to take part in this research study.

You agree that Cleveland Clinic's research using your medical data/biologic materials may lead to the development of commercial pharmaceutical products. Cleveland Clinic and other researchers may use these data and may patent or commercialize discoveries or inventions that result from this research. Neither Cleveland Clinic nor other participants in this research will compensate you if this happens.

_____

Printed name of Participant

_____ _____

Participant Signature Date

Statement of Person Conducting Informed Consent Discussion

I have discussed the information contained in this document with the participant and it is my opinion that the participant understands the risks, benefits, alternatives and procedures involved with this research study.

_____

Printed name of person obtaining consent

_____ _____

Signature of person obtaining consent Date

ADDITIONAL BLOOD SAMPLES FOR STORAGE AND FUTURE USE:

In addition to the study labwork, you are being asked to provide 2 additional blood samples (2 teaspoonfuls) for each scheduled blood draw during the study for a total of 21 additional teaspoons over the course of approximately 9 months. If you agree, this blood will be saved for future analysis of additional markers that may be associated with CKD. These blood samples will be stored indefinitely at Cleveland Clinic Clinical Pathology Laboratory 9500 Euclid Ave. /L11, Cleveland, OH. You may request that your blood samples be destroyed at any time. The blood samples will be stored without identifying information about you (such as your name or Social Security number) but will be marked

instead with a coded ID. Your personal information and the ID will be kept in a secure computer system that will only be available to study personnel. The use of your blood will be under the supervision of the principal investigator Dr. James Simon. No information identifying you will be provided to any investigator requesting access to your blood samples. These samples will not be used for any genetic studies. The results of any testing done on these samples will not be reported to you or your health care provider.

I AGREE to have additional blood samples collected that will be used for future studies _____ _____

Initial Date

I DO NOT agree to have additional blood samples collected that will be used for future studies _____ _____

Initial Date

Appendix B

Case Report Form-Screening Sheet

CCF #: _____

STEP 1: INCLUSION CRITERIA

Is the patient over 18 years of age?	Y/N
Does the patient have stage 3 or 4 CKD (GFR 15-60cc/min)?	Y/N
Does the patient have a vitamin D level <30ng/mL?	Y/N

IF YES TO ALL OF THE ABOVE, THE PATIENT MEETS INCLUSION CRITERIA, PROCEED TO STEP 2.

STEP 2: EXCLUSION CRITERIA

Does the patient have:

History of primary hyperparathyroidism in EPIC?	Y/N
History of Liver failure?	Y/N
History of chronic diarrhea or malabsorption syndrome?	Y/N
Serum calcium >12.0mg/dL?	Y/N
Treatment with an activated vitamin D formulation (calcitriol, doxercalciferol or paracalcitol) within the past 6 months?	Y/N
Current or expected treatment with phenobarbital, phenytoin, rifampicin, sucralfate, steroids or digoxin	Y/N

Active malignancy other than squamous or basal cell skin cancer?	Y/N
Is the subject currently pregnant or want to become pregnant in the next year?	Y/N
Serum phosphorus level greater than 4.5 or treatment with an oral phosphate binder within the past 6 months	Y/N
Treatment with cinacalcet or other calcimimetic within the past 6 months	Y/N
Anticipated dialysis within 6 months after randomization	Y/N
Inability to swallow tablets	Y/N
Known sensitivity, intolerance, or other adverse response to the study drugs which would prevent compliance with study medication	Y/N
Have an unstable medical condition, defined as having been hospitalized within 30 days before screening, the expectation of recurrent hospital admissions or life expectancy of less than 6 months in the judgment of the investigator	Y/N
Currently enrollment in, or fewer than 30 days have passed since subject has completed another investigational device or drug	Y/N

study(s); or subject is receiving another investigational agent(s).

IF THE ANSWERS TO ANY OF THE ABOVE IN STEP 2 ARE YES, THEN THE PATIENT CANNOT BE ENROLLED IN THE STUDY.

IF ALL THE ANSWERS IN STEP 2 ARE "NO", THEN THE PATIENT IS ELLIGIBLE FOR ENROLLMENT

Appendix C

Phone Interview Form

Subject identified number:

Study personnel making the call:

1) Introduction – name, affiliation with the study

2) Have they been taking their study medicine as scheduled? Y/N

3) Are they taking any other vitamin D supplement? Y/N

4) Have they been prescribed paracalcitol, doxercalciferol, calcitriol or cinacalcet? Y/N

5) Have they become pregnant? Y/N

6) Have they been experiencing any side effects from the study medication? Y/N

If yes, ask if the following:

- Abdominal pain or cramps? Y/N
- Loose stools or diarrhea? Y/N
- Constipation? Y/N
- Nausea? Y/N
- Palpitations? Y/N
- Dizziness? Y/N
- Muscle cramping or stiffness? Y/N
- Twitching? Y/N

Other:_____

7) Did they have to stop the study medication as a result? Y/N

8) Reminder about the blood work due in the next 1-2 weeks.

9) Any other questions or concerns? Y/N

If yes: _____

Appendix D

Adverse Event Reporting Form

Subject identifier number:_____

Study Personnel recording the adverse event: _____

Adverse event:_____

Serious or life-threatening?	Y/N
Was study medication stopped as a result?	Y/N
Did the patient require:	
Emergency room visit	Y/N
Admission to the hospital	Y/N
Invasive procedure as a result of the event	Y/N
Did the event lead to death of the subject?	Y/N

Appendix E

Sample Checklists

UPGRADE Study Sample Checklist 1

NOT Consented for Research: Week 1

Last Name: Upgrade **First Name:**_____

Date & Time Collected_____**Time Received**_____ **Time Frozen**_____

Centrifuge Type Used_____ **Centrifuge Speed (xg)**_____

Dr. Sihe Wang: ____

Use Memo Account:_____

Vial Types (check)

- ○ Gold

Requested Tests (check):

- ○ HCGQT
- ○ PHOS
- ○ Other_____

For each checked requested test, an aliquot is transferred to a 75x12 mm tube instead of cryogenic vials, this form is copied and taken to CPA along with labeled tubes. In Actual Volume, write down "CPA" for these samples. Tubes are labeled "Last Name, First Name - TubeID" e.g. Upgrade, 001W1Gold1.

	Tube ID	Expected Volume (mL)		Actual Volume (mL)
○	W1Gold1	1.0	For 1,25D	_____
○	W1Gold2	0.5	For requested test	_____

○ W1Gold3 0.5 For requested test _____

Comments_____

_____

_____

_____

UPGRADE Study Sample Checklist 2

Consented for Research: Week 1

Last Name: Upgrade **First Name:**_____

Date & Time Collected_____**Time Received**_____ **Time Frozen**_____

Centrifuge Type Used_____ **Centrifuge Speed (xg)**_____

Dr. Sihe Wang: ____

Use Memo Account:_____

Vial Types (check)

- ○ Gold (x2)
- ○ Purple

Requested Tests (check):

- ○ HCGQT
- ○ PHOS
- ○ Other_____

For each checked requested test, an aliquot is transferred to a 75x12 mm tube instead of cryogenic vials, this form is copied and taken to CPA along with labeled tubes. In Actual

Volume, write down "CPA" for these samples. Tubes are labeled "Last Name, First Name - TubeID" e.g. Upgrade, 001W1Gold1.

	Tube ID	Expected Volume (mL)		Actual Volume (mL)
○	W1Gold1	1.0	For 1,25D	_____
○	W1Gold2	0.5	For requested test	_____
○	W1Gold3	0.5	For requested test	_____
○	W1Gold4	1.0	Research	_____
○	W1Gold5	1.0	Research	_____
○	W1Gold6	1.0	Research	_____
○	W1Purple1	0.5	Research	_____
○	W1Purple2	0.5	Research	_____
○	W1Purple3	0.5	Research	_____

Comments_____

_____

_____

UPGRADE Study Sample Checklist 3

NOT Consented for Research: Week 6,12, or 24

Last Name: Upgrade **First Name:**_____

Week_____

Date & Time Collected_____**Date & Time Retrieved**_____

Vial Types (check)

○ Tracked down Gold sample

Tube ID	Expected Volume (mL)		Actual Volume (mL)
○ W6/12/24Gold1	1.0	For 1,25D	_____
○ W6/12/24Gold2	0.5	For D2/D3	_____

Comments_____

_____

_____

_____

UPGRADE Study Sample Checklist 4

Consented for Research: Week 6, 12, or 24

Last Name: Upgrade **First Name:**_____

Week_____

Date & Time Collected_____**Time Received**_____**Time Frozen**_____

Date & Time Tracked Gold Tube is Retrieved_____

Centrifuge Type Used_____ **Centrifuge Speed (xg)**_____

Vial Types (check)

- ○ Tracked down Gold sample
- ○ Gold
- ○ Purple

Gold 1 and 2 are for samples tracked down and aliquoted, while 3, 4 and 5 is for Gold tube received for research.

Tube ID	Expected Volume (mL)		Actual Volume (mL)
○ W6/12/24Gold1	1.0	For 1,25D	_____
○ W6/12/24Gold2	0.5	For D2/D3	_____

- ○ W6/12/24Gold3 1.0 Research _____
- ○ W6/12/24Gold4 1.0 Research _____
- ○ W6/12/24Gold5 1.0 Research
- ○ W6/12/24Purple1 0.5 Research _____
- ○ W6/12/24Purple2 0.5 Research _____
- ○ W6/12/24Purple3 0.5 Research

Comments_____

_____

_____

_____

UPGRADE Study Sample Checklist 5

NOT Consented for Research: Week 18, 30, or 36

Last Name: Upgrade **First Name:**_____

Date & Time Collected_____**Time Received**_____ **Time Frozen**_____

Centrifuge Type Used_____ **Centrifuge Speed (xg)**_____

Dr. Sihe Wang: ____

Use Memo Account:_____

Vial Types (check)

- ○ Gold (x2)

For Gold1-3, fill in 75x12 mm tubes, label, copy this form and take to CPA along with samples. In Actual Volume, write down "CPA" for these samples. Tubes are labeled "Last Name, First Name - TubeID" e.g. Upgrade, 001W1Gold1. For Gold 4 and 5, store frozen.

Requested Tests (check):

- ○ PTHI
- ○ RFP
- ○ VITD

Tube ID	Expected Volume (mL)		Actual Volume (mL)
○ W18/30/36Gold1	1.0	For PTHi	
○ W18/30/36Gold2	0.5	For RFP	
○ W18/30/36Gold3	0.5	For 25OHD	
○ W18/30/36Gold4	1.0	For 1,25D	
○ W18/30/36Gold5	0.5	For D2/D3	

Comments_____

UPGRADE Study Sample Checklist 6

Consented for Research: Week 18/30/36

Last Name: Upgrade **First Name:**_____

Date & Time Collected_____ **Time Received**_____ **Time Frozen**_____

Centrifuge Type Used_____ **Centrifuge Speed (xg)**_____

Dr. Sihe Wang: ____

Use Memo Account:_____

Vial Types (check)

- ○ Gold (x3)
- ○ Purple

For Gold1-3, fill in 75x12 mm tubes, label, copy this form and take to CPA along with samples. In Actual Volume, write down "CPA" for these samples. Tubes are labeled "Last Name, First Name - TubeID" e.g. Upgrade, 001W1Gold1. For Gold 4-8 and Purple 1-3 store frozen.

Requested Tests (check):

- ○ PTHI
- ○ RFP
- ○ VITD

Tube ID	Expected Volume (mL)		Actual Volume (mL)
○ W18/30/36Gold1	1.0	For PTHi	
○ W18/30/36Gold2	0.5	For RFP	
○ W18/30/36Gold3	0.5	For 25OHD	
○ W18/30/36Gold4	1.0	For 1,25D	
○ W18/30/36Gold5	0.5	For D2/D3	
○ W18/30/36Gold6	1.0	Research	
○ W18/30/36Gold7	1.0	Research	
○ W18/30/36Gold8	1.0	Research	
○ W18/30/36Purple1	0.5	Research	
○ W18/30/36Purple2	0.5	Research	
○ W18/30/36Purple3	0.5	Research	

Comments