Analysis of Archived Dried Blood Spots by Mass Spectrometry for Vitamin D and Real-time PCR for Its Enzymes and Receptor

Amod N. Joshi

Wright State University

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ANALYSIS OF ARCHIVED DRIED BLOOD SPOTS BY MASS SPECTROMETRY FOR VITAMIN D AND REAL-TIME PCR FOR ITS ENZYMES AND RECEPTOR.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

By

Amod N. Joshi
Bachelor of Pharmacy, University of Mumbai, 2008

2011
Wright State University
I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Amod N. Joshi ENTITLED “Analysis of archived dried blood spots by mass spectrometry for vitamin D and real-time PCR for its enzymes and receptor” BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Joshi, Amod N. M.S., Department of Pharmacology and Toxicology, Wright State University, 2011. Analysis of archived dried blood spots by mass spectrometry for vitamin D and real-time PCR for its enzymes and receptor.

Vitamin D is an essential micronutrient required for maintenance of the skeletal system and its deficiency leads to diminished availability of calcium for maintaining bone mineralization. Vitamin D deficiency (VDD) during pregnancy and/or in early infancy can lead to rickets and a fragile bone state resulting in fractures with minimal forces. Such multiple unexplained fractures (MUF) present in infants at less than 6 months of age are often misdiagnosed as cases of child abuse. Therefore, it is important to evaluate the status of vitamin D in early infancy. The analysis of dried blood spots (DBS) provides a unique screening method for early and late identification of diseases.

The objective of this study was to develop a mass spectrometry (MS) method to analyze vitamin D levels in newborn DBS. We used a methanol-hexane solvent system for the extraction of vitamin D analytes from filter paper spots. The use of methanol-hexane solvent system was evaluated using desipramine HCl as a positive control. We were able to extract desipramine HCl from standard spots spiked with concentrations 8 - 5000 ng/ml. We applied this extraction method to control and test DBS with slight modification involving preincubation of DBS in methanol and sodium hydroxide. Our MS results have shown qualitative separation of 25 (OH) D$_2$ (m/z - 413.2) and 25 (OH) D$_3$ (m/z - 401.2) with SNR > 3. The identity of vitamin D analytes was confirmed using MS/MS analysis showing the presence of respective fragment peaks (m/z - 336.2 and
365.2). For quantitative estimation i.e. SNR > 10, we derivatized vitamin D analytes in DBS using 4-Phenyl-1,2,4- triazoline-3,5-dione (PTAD) in a Diels-Alder conjugation reaction. However, both 25 (OH) D₂ and 25 (OH) D₃ show the formation of identical product ion (m/z 298.0) making it difficult to quantitate them separately.

In addition to MS method development, we also tried to evaluate genetic mutations in vitamin D metabolizing enzymes and vitamin D receptor as a possible cause of VDD in these infants. We extracted genomic DNA and RNA from DBS for mutation scanning using melt curve analysis. However, the outcome of such analysis was not successful owing to lack of integrity in genetic material extracted from DBS. In summary we developed a MS method for qualitative detection of vitamin D and also attempted to test genetic mutations as a cause of VDD in infants. Further experiments are necessary to evaluate the use of alternative options of liquid chromatography/gas chromatography (LC/GC) coupled with MS and/or use of atmospheric pressure chemical ionization (APCI) as source of ionization for quantitative assessment of vitamin D.
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CHAPTER 1

DEVELOPMENT OF A NOVEL MASS SPECTROMETRY METHOD FOR THE ANALYSIS OF VITAMIN D FROM ARCHIVED DRIED BLOOD SPOTS.
Chapter 1 - INTRODUCTION

Background:
Vitamin D collectively refers to a pair of fat soluble micronutrients which include vitamin D₂ (ergocalciferol) and vitamin D₃ (cholecalciferol). Vitamin D₃ is synthesized in the skin by high energy UV-B rays (290-315 nm) of the sun by photolysis of 7-dehydrocholesterol to cholecalciferol involving a series of biochemical transformations (Holick, 1987). Vitamin D₂ on the other hand is predominantly available from food resources such as vitamin D fortified milk, sea food including fish, cod liver oil and oak mushrooms (Lee et al., 2009).

Vitamin D - chemistry:
Both forms of vitamin D share a general structural backbone similar to that of the classical steroid hormones. However, presence of extra methyl group at carbon-24 and double bond between carbon-22 and carbon-23 in vitamin D₂ distinguishes it from vitamin D₃ (figure 1).
Figure 1: Structures of vitamin D3 and D2.

**Vitamin D - biotransformation:**

Vitamin D2 and D3 are as such biologically inactive. They are biotransformed in the body using the same metabolic pathways into active metabolites (Blunt et al., 1968; Suda et al., 1969). After biosynthesis or ingestion, protein bound vitamin D is transported to the liver through circulation where it is hydroxylated at carbon-25 position by cytochrome P450 dependent hydroxylase - 25-hydroxylase. 25 (OH) D thus formed is considered as the major circulating form of vitamin D (Holick and DeLuca, 1974). Circulating vitamin D is further hydroxylated at the 1α-carbon position by 1α-hydroxylase in the kidney to form 1α, 25-dihydroxy-vitamin D (1α, 25(OH)2 D), the most active form of vitamin D (Omdahl et al., 1971). The production of 1α, 25(OH)2 D from kidney is influenced by negative feedback mechanism of dihydroxy vitamin D itself and parathyroid hormone (PTH) in relation with calcium levels (Holick, 1987). Although liver and kidney are
major contributors of vitamin D bioactivation, these enzymes are also present in extra hepatic and extra renal sites and are responsible for the same function but to limited extent (Henry, 2011). Activated vitamin D is further inactivated at the target site by hydroxylation at carbon-24 position (Ishizuka et al., 2000). Vitamin D biotransformation is summarized in figure 2.

Figure 2: Biotransformation of vitamin D (Deeb et al., 2007)

Vitamin D - causes of deficiency

Most experts consider the normal vitamin D levels to be > 32 ng/ml or 80 nmol/L. Vitamin D deficiency is defined as levels of vitamin D in the blood below 20 ng/ml or 50
nmol/L. Alternatively, a person is said to be vitamin D insufficient when the levels of vitamin D are below 32 ng/ml but above 20 ng/ml (Johnson et al., 2011). Vitamin D levels are summarized in table 1.

**Table 1: Vitamin D levels**

<table>
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<th>Level of vitamin D</th>
<th>Concentration</th>
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<td>Normal</td>
<td>&gt; 32 ng/ml (80 nmol/L)</td>
</tr>
<tr>
<td>Deficiency</td>
<td>&lt; 20 ng/ml (50 nmol/L)</td>
</tr>
<tr>
<td>Insufficiency</td>
<td>20 ng/ml - 32 ng/ml</td>
</tr>
</tbody>
</table>

Lack of sufficient exposure to sunlight is a major risk factor responsible for vitamin D deficiency. This might be due to seasonal variation and/or living above 37 degrees north. In addition, skin pigmentation (Clemens et al., 1982) and excessive use of sunscreens (Matsuoka et al., 1987) presents a strong barrier to sun rays for penetration into the epidermis. Insufficient vitamin D supplements through diet in adults or through breastfed milk in infants is another reason for its deficiency. Elderly people are more at a risk of vitamin D deficiency because as the age of the person increases, the efficiency of vitamin D production in the skin from its precursor 7-dehydrocholesterol decreases (Lips, 2001). Recent studies have demonstrated that, deficiency complications are also caused by mutations in the genes responsible for synthesis of the hydroxylases involved in vitamin D biotransformation (Kim et al., 2007). Also, point mutations in the human vitamin D receptor gene lead to target organ resistance to active form of vitamin D (Hughes et al., 1988) resulting in hypocalcemic rickets.
**Vitamin D - deficiency complications:**

Physiological and pharmacological actions of active forms of vitamin D are executed through vitamin D receptors (VDR), controlling transcriptional activities in many cells (DeLuca, 1967). Vitamin D is not only involved in maintenance of healthy teeth and bones but also plays a crucial role in immune response, cell proliferation, differentiation and cardiovascular health (Holick, 2004).

Vitamin D is associated with calcium and phosphorus homeostasis for development of the skeletal system of the body (Norman, 1979). In the absence of vitamin D, calcium cannot be absorbed from the intestine for bone mineralization (Holick, 2007). This results in bone deformities such as rickets in infants and osteomalacia in adults often characterized by weak or fractured bones (Wick, 2009). Vitamin D is involved in innate and adaptive immunity since VDR are also expressed in macrophages, dendritic cells and T- cells. The net effect is that vitamin D helps in the improvement of innate immunity and is also involved in complex regulation of adaptive immunity. Recent studies have demonstrated that vitamin D deficiency is related to increased incidences of auto-immune diseases, encouraging research for development of VDR agonist in regulation and treatment of such diseases (Adorini and Penna, 2008). Type I diabetes pathogenesis is considered to be modulated by vitamin D via repression of type I cytokines, inhibition of dendritic cell maturation and upregulation of regulatory T-cells (Zella and DeLuca, 2003). Clinical studies have shown an inverse relationship between vitamin D levels and the occurrence of hypertension. The active form of vitamin D is effective in downregulating renin and angiotensinogen expression and thereby decreasing blood pressure (Li et al., 2002). Activation of VDR present in the tumor cells (breast,
prostate, colon) by vitamin D inhibits growth of tumor cells and promotes apoptosis (Chen and Holick, 2003) by interfering with the cell cycle. Vitamin D also induces cell differentiation. The presence of VDR in metastatic cells and tumor suppressive action of vitamin D are collectively helpful in the strategic development of anticancer medication (Thorne and Campbell, 2008).

**Vitamin D - analysis:**

It is clear from the information above that vitamin D deficiency is not only limited to bone deformities but also linked with auto-immune diseases, cardiovascular diseases and cancer. Therefore, a need for assay development was inspired for accurate estimation of vitamin D status. The lipophilic nature of vitamin D and its tight affinity for vitamin D binding protein are two major challenges in its assessment (Larkin et al., 2011). At present, 25 (OH)-vitamin D status can be measured by using available assay methods such as radio-immunoassay (RIA) (Glendenning et al., 2003; Terry et al., 2005), competitive protein binding assay (CPBA) and enzyme-immunoassay (EIA) (Seiden-Long and Vieth, 2007).

However, these methods have some shortcomings. CPBA is prone to a matrix effect and cross reactivity with other vitamin D metabolites and thus, may produce variable results (Kimball and Vieth, 2007). EIA has been automated for large scale processing to handle the increased demand of clinical testing but specificity and sensitivity issues of these assays (Wick, 2009) are a major concern. Fluorimetric methods have also been developed, but these methods are involved with complicated pretreatment steps (Shimizu et al., 1997). Chromatographic techniques such as High Performance Liquid
Chromatography (HPLC) for vitamin D analysis, demonstrates low sensitivity for target analytes at lower concentrations (pg/ml) (Masuda et al., 1997).

Over the years, mass spectrometry (MS) has received more attention due to its high sensitivity and specificity in analyzing vitamin D molecules. A typical MS analysis involves five steps; 1) sample introduction, 2) ionization, 3) mass analysis, 4) ion detection and 5) data analysis. The sample to be analyzed is either injected directly into MS or column effluents enters into MS after chromatographic separation using gas chromatography (GC) or liquid chromatography (LC). High temperatures maintained in the MS converts the sample into a gaseous phase. During ionization gaseous molecules are ionized to form positive, negative and neutral ions, however only one polarity is recorded at a time during MS analysis. Ionization is a very crucial step and, depending on the purpose of MS application, it can be achieved by different methods.

In Electron Ionization (EI) gaseous molecules are bombarded with a high energy electron beam. This method of ionization is useful for structural identification (Mark et al., 1985). Chemical Ionization (CI) is achieved by use of ionized gas (e.g. methane) for proton transfer. It is considered a ‘soft-ionization’ technique in comparison with EI (Fales et al., 1972) and is useful for structural determination based on more closely associated molecular ions. Electrospray Ionization (ESI) involves formation of charged droplets in the presence of electric field that, upon evaporation into gaseous phase, produces charged ions. ESI is generally used for qualitative assessment of biomolecules (Shuford and Muddiman, 2011). Atmospheric Pressure Chemical Ionization (APCI) is a gas phase ionization technique more useful for determination molecular weight of analytes (Hommerson et al., 2011). The primary function of a mass analyzer is to filter
ions. Magnetic and electric fields applied to most of the mass analyzers separate the ions based on their m/z ratios. After mass analysis, emitted ions strike on the ion detector surface and electrons are released. The current produced by these electrons is recorded by a detector. A typical mass spectrum is a plot of intensity versus m/z ratio which serves for qualitative and quantitative determination of the respective analyte.

**Use of dried blood spots (DBS) for newborn screening:**

Low fetal vitamin D levels is considered as a major risk factor for the development of various disorders which may develop in later stages of life (McGrath, 2001). Preliminary results obtained using banked maternal sera and other epidemiological studies suggest that low vitamin D levels at birth are associated with a risk of autoimmune disorders such as multiple sclerosis, diabetes (Hypponen et al., 2001) and schizophrenia (McGrath et al., 2003). There exists a strong correlation between 25 (OH) D levels of maternal sera and newborn cord blood sera which proves that the fetus is totally reliant on maternal vitamin D stores (Eyles et al., 2010). However, there are only limited such data available to verify this correlation due to shortage of stored maternal or cord blood samples because phlebotomy is not always feasible especially involving pediatric populations or patients from rural areas.

DBS are typically obtained 1-3 days after the birth using capillary blood from a heel prick (Olshan, 2007) and can be used for estimation of vitamin D levels in newborn infants. Although DBS measurements are lower than whole blood and serum measurements, these results are consistent across the group of patients (Larkin 2011). Other advantages of DBS include ease of sample availability for epidemiological
research and minimal biohazard since most infectious agents are inactivated by drying. Vitamin D analytes are stable in DBS and can be extracted (Newman et al., 2009).
Chapter 1 - HYPOTHESIS

Development of hypothesis

Vitamin D deficiency (VDD) during pregnancy and/or early infancy can lead to rickets or fragile bone state resulting in fractures with minimal forces. Presence of multiple unexplained fractures (MUF) in infants typically less than 6 months of age are often misdiagnosed as cases of child abuse (Keller and Barnes, 2008). Therefore, the measurement of vitamin D levels in archived DBS from young infants with MUF will help determine if deficiency of vitamin D could be a contributing factor in such cases.

Hypothesis:

A mass spectrometry method development will allow assessment of vitamin D in archived DBS that can be used to establish the types and levels of vitamin D from patients.

Specific Aims:

Aim 1 - To test the hypothesis with the development of suitable method of extraction for vitamin D analytes from archival dried blood spots and their qualitative determination using mass spectrometry.

Aim 2 - To test the hypothesis with quantitative determination of vitamin D analytes from archival dried blood spots using mass spectrometry.
Chapter 1 - MATERIALS AND METHODS

Reagents

4-Phenyl-1,2,4- triazoline-3,5-dione (PTAD), sodium acetate, vitamin D$_2$ and vitamin D$_3$ standards were purchased from Sigma-Aldrich. Cellulase was purchased from Spectrum Chemical Mfg. Corp. All solvents and glass tubes were obtained from Fisher. Control and test newborn blood spots were provided by Children’s Medical Center Dayton. Whatman filter paper was used for standard and control spot preparation.

Vitamin D standards

Standard stock solutions of vitamin D$_2$ and vitamin D$_3$ at a concentration of 1mg/ml were prepared in methanol (MeOH). Stock solutions were further subjected to serial dilutions in order to achieve target range of concentration (6.25ng/ml-100ng/ml).
Table 2: Serial dilution of standards

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<tr>
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<tr>
<td>100 µg/ml</td>
<td>9.0 ml</td>
<td>1.0 ml of 1000 µg/ml</td>
<td>10.0 ml</td>
</tr>
<tr>
<td>50 µg/ml</td>
<td>1.0 ml</td>
<td>1.0 ml of 100 µg/ml</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>25 µg/ml</td>
<td>1.0 ml</td>
<td>1.0 ml of 50 µg/ml</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>5 µg/ml</td>
<td>4.0 ml</td>
<td>1.0 ml of 25 µg/ml</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>1 µg/ml</td>
<td>4.0 ml</td>
<td>1.0 ml of 5 µg/ml</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>500 ng/ml</td>
<td>1.0 ml</td>
<td>1.0 ml of 1 µg/ml</td>
<td>2.0 ml</td>
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<tr>
<td>200 ng/ml</td>
<td>1.5 ml</td>
<td>1.0 ml of 500 ng/ml</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>100 ng/ml*</td>
<td>1.0 ml</td>
<td>1.0 ml of 200 ng/ml</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>50 ng/ml*</td>
<td>1.0 ml</td>
<td>1.0 ml of 100 ng/ml</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>25 ng/ml*</td>
<td>1.0 ml</td>
<td>1.0 ml of 50 ng/ml</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>12.5 ng/ml*</td>
<td>1.0 ml</td>
<td>1.0 ml of 25 ng/ml</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>6.25 ng/ml*</td>
<td>1.0 ml</td>
<td>1.0 ml of 12.5 ng/ml</td>
<td>2.0 ml</td>
</tr>
</tbody>
</table>

Preparation of standard and control filter paper spots

1. Filter paper spots with standards

1 ml standards of concentrations between 6.25 ng/ml-100 ng/ml (* in table 2), 1µg/ml, 5 µg/ml and 1mg/ml were dried by using speed vacuum concentrator and reconstituted in MeOH (50µl). Whatman filter paper was spotted with reconstituted standard (50 µl) making a spot of 10 mm in diameter. Spots were left to dry at room temperature for 2 hours and then were stored in zip lock plastic bags at -20˚ C for further analysis.

2. Filter paper spots with control blood sample

Human blood from healthy volunteers was collected in vacuum tubes containing heparin and gently inverted to allow mixing. Blood (50µl) was then used to make multiple spots of 10 mm diameter on Whatman filter paper. Sufficient care was taken to keep the
distance between two spots in order to prevent them from mixing. Spots were left to dry at room temperature for 2 hours and then stored in zip lock plastic bags at -20°C for further analysis.

**Analysis of fragmentation pattern of vitamin D$_2$ and D$_3$**

Standard solutions of vitamin D$_2$ and vitamin D$_3$ of 1mg/ml were prepared in MeOH. Solutions were dried using speed vacuum concentrator and reconstituted with MeOH (100µl) containing 1% formic acid. Reconstituted samples were directly injected into an ion trap MS and data was collected and analyzed to understand the fragmentation patterns of vitamin D$_2$ and D$_3$ separately.

**Method of Extraction:**

Spots were punched from filter paper, cut into fine pieces and soaked with ddH$_2$O (100 µl) in test tubes for 30 minutes. MeOH (500 µl) and 7M NaOH (60 µl) were then added to precipitate proteins. Samples were kept in a shaker for 3 hours before adding hexane (1000 µl) to extract vitamin D. Samples were vortexed for 1 minute followed by centrifugation at 5000 RPM for 5 minutes at 4°C. After centrifugation, the top hexane layer was removed and dried using speed vacuum. Samples were reconstituted in MeOH (100 µl) containing 1% formic acid. Reconstituted samples were subjected to ion trap MS analysis for presence or absence of target peaks of the vitamin D$_2$ and vitamin D$_3$.

**Extraction method modifications**

1. *Use of mineral oil:*
Vitamin D₂-D₃ standards of concentrations 8ng/ml, 40ng/ml, 200ng/ml, 1000ng/ml and 5000ng/ml in MeOH were selected for this experiment. Filter paper spots were prepared using these standards as described above. Dried filter paper spots (spots with standards and control blood spot) were cut into fine pieces, added with olive oil (1000 µl) and vortexed for 1 hour. Vitamin D₂ and D₃ were extracted using MeOH and hexane and analyzed as procedure described before. Unspiked filter paper spot treated in a similar manner served as negative control.

2. Use of sonication and higher temperature:

Filter paper spots spiked with 1µg/ml standard vitamin D₂ and D₃ were selected for this experiment. Before applying the above mentioned extraction protocol, spots were sonicated for 10 minutes and kept at 60°C for 3 hours to verify if sonication and higher temperature are useful in improving extraction of vitamin D₂ and D₃. Unspiked filter paper spot treated in a similar manner and MeOH (100 µl) containing 1% formic acid served as negative controls.

3. Use of Acetonitrile and hexane

Filter paper spots spiked with 1µg/ml standard vitamin D₂ and D₃ were used in this experiment. ACN is a good solvent for vitamin D₂ and D₃ and can be used along with hexane for the extraction. The method of extraction was same as mentioned before with the only difference of use of acetonitrile instead of MeOH. For negative control unspiked filter paper spot was treated in a similar manner. Unspiked filter paper spot treated in a similar manner and MeOH (100 µl) containing 1% formic acid served as negative controls.
4. **Use of Cellulase**

Cellulase solution 6 mg/ml was made in 50 mM sodium acetate (pH 5.0) 5 minutes before its use and kept at room temperature. Filter paper spots spiked with standard vitamin D₂-D₃ of concentrations 8ng/ml, 40ng/ml, 200ng/ml, 1000ng/ml and 5000ng/ml were selected for this experiment. Spots were punched from filter paper, cut into fine pieces, added with cellulase (1 ml) solution and kept at 40°C for 24 hours. Enzyme action was stopped by increasing the temperature to 90°C for 10 minutes. Spots were then extracted using MeOH and hexane and analyzed as described before. An unspiked spot treated with cellulase and extracted in a similar manner served as a negative control.

5. **Use of positive control- Desipramine HCL**

Standard solution of desipramine HCl of concentration 1mg/ml was made in MeOH and dried down by using speed vacuum concentrator. It was then reconstituted with MeOH (50 µl) containing 1% formic acid and injected directly in an ion trap MS to analyze respective m/z values. In another experiment standard filter paper spots spiked with the concentration 8ng/ml, 40ng/ml, 200ng/ml, 1000ng/ml and 5000ng/ml were used. Desipramine HCl was extracted from all spots using MeOH and hexane as mentioned before. Unspiked filter spot extracted in a similar manner was used as a negative control.

**Prolonged incubation before extraction:**

In an attempt to modify extraction procedure, five unspiked control blood spots were added with MeOH (1000 µl) and 7M NaOH (60 µl) and incubated for 72 hours at room temperature. All test tubes were sealed with paraffin films in order to avoid solvent evaporation. After 72 hours, samples were vortex-mixed for 60 seconds with hexane
(500 µl). Samples were then kept undisturbed in order to achieve phase separation and upper hexane layer was removed in fresh test tubes. Phase separated samples were then dried down using speed vacuum concentrator and stored at 4°C for further analysis.

**Newborn test DBS for qualitative analysis:**

Newborn test blood spots were extracted by applying prolonged incubation step as described before for control blood spots and analyzed through Ion trap. In addition to it, vitamin D$_2$ and D$_3$ present in newborn test blood spots were derivatized as the procedure described in PTAD derivative formation section before.

**Vitamin D derivative formation**

1. **Analysis of fragmentation pattern of 4-Phenyl-1,2,4-triazoline-3,5-dione (PTAD) for PTAD:**

   Standard solution of PTAD of concentration 1mg/ml was prepared in ACN and dried using speed vacuum concentrator. It was then reconstituted with MeOH (50 µl) containing 1% formic acid and injected directly in an ion trap MS for analysis.

2. **Deciding PTAD concentration for derivative formation:**

   In initial experiment, standard vitamin D$_3$ of concentration 100ng/ml was used in derivative formation using PTAD of concentration 500 ng/ml in ACN. Standard vitamin D$_3$ (1 ml) was dried using speed vacuum concentrator and reconstituted with MeOH (50 µl). It was then allowed to react with PTAD (1 ml) at room temperature for 1 hour to form corresponding Diels-Alder conjugate. At the end of 1 hour, reaction mixture was dried again using speed vacuum concentrator and kept at 4 °C for further analysis. This
experiment was repeated by changing the concentrations as standard vitamin D₃ (1µg/ml) and PTAD (500ng/ml). For ion trap MS analysis dried products were mixed with MeOH (100 µl) containing 1% formic acid and then injected directly into the trap. Standard PTAD (500ng/ml) alone, untreated with vitamin D₃ served as negative control for this experiment.

3. **PTAD derivative formation:**

Standard vitamin D₃ solutions of concentrations 6.25 ng/ml, 12.5ng/ml, 25ng/ml, 50ng/ml, and 100ng/ml were selected to react with PTAD of concentration 0.5mg/ml in ACN for this experiment. Derivative formation was carried similarly as protocol described before. Standard PTAD solution of concentration 0.5mg/ml untreated with vitamin D₃ solutions served as negative control for this experiment.

4. **Derivative formation at different time intervals**

Standard vitamin D₃ solution of concentration 12.5ng/ml was used for this experiment. Derivative formation using 0.5 mg/ml PTAD solution (1ml) was carried out as discussed before. Reaction aliquots were removed at 0, 1, 5, 30, 45 and 60 minutes, immediately vacuum dried and stored at 4°C for further analysis. Aliquots were analyzed through ion trap for the presence or absence of target peaks such as PTAD alone, PTAD derivatized product ion, PTAD derivatized precursor ion and unreacted vitamin D₃. MeOH (100 µl) containing 1% formic acid served as negative control for this experiment.

5. **PTAD derivative formation using control blood spots:**
Control blood spots were removed from -20°C and kept at room temperature for 15 minutes before using them for this experiment. Three blood spots were spiked with standard vitamin D₂-D₃ solutions of concentration 6.25ng/ml, 12.5ng/ml and 25ng/ml and one blood spot was kept unspiked. These standards were initially vacuum dried and reconstituted solutions (50 µl) were used for actual spiking of the control blood spots. All four blood spots were subjected to extraction using MeOH and hexane as described before. After extraction, vacuum dried samples were reconstituted with MeOH (100 µl) and used for derivative formation with 0.5mg/ml of PTAD (1ml) as per previously specified protocol. At the end samples were analyzed using ion trap. Standard PTAD solution alone served as negative control for this experiment.

**Ion trap MS analysis:**

Mass spectrometry was performed on a Bruker HCT ultra ion trap mass spectrometer using standard-enhanced mode with a range of 50-3000 m/z at a speed of 8100 (m/z)/sec. All standards and test samples were ionized by electrospray ionization (ESI). Flow rates of the nebulizer and dry gas were 10 psi and 5.0 L/minute respectively with a dry gas temperature 300°C. Positive polarity was used for data acquisition with Ion charge control (ICC) settings were 200000 with maximum accumulation time 200 ms. All fragmentation data was collected with manual MS(n) method. Sample to be analyzed was injected (100 µl) into the trap with the flow rate of 161 µl/hour for 10 minutes.
Chapter 1 - RESULTS

Fragmentation pattern of vitamin D$_2$ and Vitamin D$_3$

We have collected basic fragmentation pattern of standard vitamin D$_2$ and vitamin D$_3$ separately. This information is useful in identifying vitamin D$_2$ and D$_3$ from a mixture by comparing their fragmentation patterns.

**Figure 3 (A):** Fragmentation of vitamin D$_2$. Panel (a) MS of standard vitamin D$_2$; panel (b): isolated vitamin D$_2$ (M+H)$^+$ peak by manual MS(n); panel (c): MS/MS of Vitamin D$_2$ showing the presence of all fragments originating from parent peak 397.3.
Figure 3(B): Fragmentation of vitamin D₃. Panel (a): MS of standard D₃; panel (b): isolated D₃ (M+H)⁺ peak by manual MS(n); panel (c): MS/MS of Vitamin D₃ showing the presence of all fragments originating from parent peak 385.3.

Extraction method modification

Use of mineral oil

To verify if mineral oil can be used in extraction of fat soluble vitamin D, filter paper spots were treated with olive oil and further extracted with MeOH-hexane. However, use of olive oil seems to be ineffective for extraction. It resulted in polymer contamination which can be seen by repetitive pattern in MS of control and standards. Target peaks of
vitamin D$_2$ and D$_3$ (m/z 397.3 and 385.3) were absent in MS of standard spots. Target peaks of 25-hydroxy forms of vitamin D$_2$ and D$_3$ (m/z 413.3 and 401.2) were also absent in MS of unspiked control blood spot.

**Figure 4:** Panel (a) MS of negative control spot; panel (b), (c), (d), (e) and (f): MS of filter paper spots spiked with 8ng/ml, 40ng/ml, 200ng/ml, 1000ng/ml and 5000ng/ml of vitamin D$_2$-D$_3$ standards respectively; panel (g) MS of unspiked control blood spot.

**Use of sonication and heating as extraction method modification**
Our intension was to use physical parameters such as sonication and heating to help release vitamin D from the filter paper spots during extraction. However, there was no effect of those physical parameters on extraction of vitamin D which is evident from the absence of target peaks of vitamin D$_2$ and D$_3$ (m/z 397.3 and 385.3) in MS of standard spot spiked with 1µg/ml. Targeted peaks were absent in MS of negative controls panel (a) and panel (b).

Figure 5: Panel (a) MS of negative control of MeOH containing 1% formic acid; panel (b) and (d) MS of unspiked filter spot as a negative control for sonication and heating
experiment respectively; panel (c) and (e) MS of filter spot spiked with 1µg/ml of standard vitamin D$_2$-D$_3$ and sonicated and heated respectively before extraction.

Use of ACN and hexane

ACN can be used as a solvent for vitamin D$_2$ and D$_3$. In extraction method development, ACN-hexane solvent system was used instead of using MeOH-hexane. There was no evidence of target peaks of vitamin D$_2$ and D$_3$ (m/z 397.3 and 385.3) in MS of standard spot spiked with 1µg/ml. Target peaks were absent in MS of negative controls.
Figure 6: panel (a) MS of negative control MeOH containing 1% formic acid; Panel (b) MS of negative control unspiked filter spot; Panel (c) MS of filter spot spiked with 1µg/ml standard vitamin D₂ and D₃ extracted with ACN-hexane solvent system.

Use of Cellulase:

Our aim was to find out if cellulase action in degrading filter paper is helpful in breaking any strong or weak bonding of vitamin D molecules with the filter paper (cellulose). Thereby it would be possible to get vitamin D molecules more easily in solvent system. However, target peaks of vitamin D₂ and D₃ (m/z 397.3 and 385.3) were absent in MS of spiked spots after cellulase treatment.
Figure 7: Panel (a) MS of negative control unspiked spot; Panel (b)-(f) MS of filter paper spots spiked with 8ng/ml, 40ng/ml, 200ng/ml, 1000ng/ml, 5000ng/ml of standard respectively, treated with cellulase and extracted with MeOH-hexane solvent system.

Use of positive control-Desipramine HCl:
In order to evaluate whether MeOH-hexane solvent system is suitable for vitamin D extraction from spots, we employed this method for extraction of desipramine HCl from spots spiked with standard solutions. Desipramine HCl has a cyclic ring structure and was used as a positive control in our lab for MS method development. Desipramine HCl peak was observed at m/z 267.2 in MS of standard solution before extraction. The same peak (m/z 267.2) was seen in MS of all spiked spots after extraction confirming the efficiency of MeOH-hexane solvent system.
Figure 8: Panel (a) MS of 1mg/ml standard solution of desipramine HCl; panel (b) MS of negative control of unspiked spot; panel (c)-(g) MS of filter paper spots spiked with 8ng/ml, 40ng/ml, 200ng/ml, 1000ng/ml, 5000ng/ml standard desipramine HCl.

Prolonged incubation before extraction:

1. Control DBS
Prolonged incubation of unspiked control blood spots for 72 hours helped in extracting 25 (OH) vitamin D₂ and D₃. MS of all five spots have shown the presence of 25 (OH) D₂ and 25 (OH) D₃ peaks (m/z 413.2 and 401.2). Presence of fragment peaks at m/z 336.2 and 365.2 confirms their identity. MS of the remaining four control spots are included in an appendix figure 27 (A) and (B).

**Figure 9:** Panel (a) MS showing presence of 25 (OH) D₂ at m/z 413.2 and 25 (OH) D₃ at m/z 401.2; fragments of 25 (OH) D₂ and 25 (OH) D₃ are shown by an arrow at m/z 336.2 and 365.2 respectively in panel (b).

2. **Newborn test DBS:**

Same method of extraction was applied to the newborn test blood spots. All 10 test blood spots have shown the presence of 25 (OH) D₂ and D₃ peaks at m/z 413.2 and 401.2 respectively. Also, characteristic fragments of 25 (OH) D₂ and 25 (OH) D₃ (m/z 336.2
and 365.2) were present in MS. MS data of remaining 9 test spots is included in appendix figure 28 (A) and (B).

**Figure 10:** Panel (a) MS showing presence of both 25 (OH) D₂ and 25 (OH) D₃ peaks in newborn test blood spots; Panel (b) MS showing presence of fragments of 25 (OH) D₂ and 25 (OH) D₃ indicated by an arrow.

**Manual MS(n) data analysis of test DBS**

Presence of respective peaks was further confirmed by manual MS(n) method for data acquisition shown in following figure 11. Manual MS(n) data of the remaining 9 test spots is shown in an appendix figure 29 (A)-(D).
Figure 11: Panel (a) Manual MS of 25 (OH) D₃ showing isolated peak of m/z 401.2; Panel (a-1) showing fragment of 25 (OH) D₃ at m/z 365.2; Panel (b) Manual MS of 25 (OH) D₂ showing isolated peak of m/z 413.1; Panel (b-1) showing fragment of 25 (OH) D₂ at m/z 336.3.
Vitamin D derivative formation

Analysis of PTAD fragmentation pattern

MS of pure solution of PTAD showed a peak at m/z 174.1. Compound MS of PTAD shows different fragments of PTAD.

![Figure 12](image)

**Figure 12:** Panel (a) MS of 1mg/ml solution of PTAD; panel (b) Compound mass spectra showing fragments of PTAD.

Deciding PTAD concentration for derivative formation:

To find out the concentration of PTAD required for vitamin D derivative formation, two experiments were carried out. In both experiments PTAD concentration was kept constant (500ng/ml), altering vit.D₃ concentrations (100ng/ml and 1µg/ml respectively). However, vitamin D₃-PTAD conjugate did not form in both experiments, which can be concluded from presence of separate peaks vitamin D₃ and PTAD (m/z 385.3 and 74.1)
in MS. Panel (a) represents negative control of standard PTAD. Square region of (a) is magnified to show the presence of PTAD peak (m/z 174.1) in panel (b).

**Figure 13:** Panel (a) and (b) MS of negative control PTAD alone; (c) MS of unreacted vitamin D3 in an experiment with vitamin D3 (100ng/ml) and PTAD (500ng/ml); (e) MS unreacted PTAD in the same experiment; (d) MS of unreacted vitamin D3 in an
experiment with vitamin D₃ (1µg/ml) and PTAD (500ng/ml); (f) MS of unreacted PTAD in the same experiment.

PTAD derivative formation:

Derivative formation of vitamin D₃ has occurred with PTAD concentration 0.5mg/ml. All the five concentrations (6.25ng/ml-100ng/ml) of standard solution of vitamin D₃ have reacted with PTAD which can be seen by presence of product ion (m/z 298.0) respective MS. Product ion peak was absent in MS of negative control.
**Figure 14:** Panel (a) MS of PTAD alone as a negative control; panel (b)-(f) MS of derivatized product ion. Panel (b), (c), (d), (e) and (f) were associated with standard vitamin D₃ concentrations 6.25ng/ml, 12.5ng/ml, 25ng/ml, 50ng/ml and 100ng/ml respectively.

**Derivative formation at different time intervals**

We studied PTAD derivative formation reaction at different time intervals over 1 hour. Results are arranged as presence (√) or absence (-) of respective peak in MS shown in
PTAD-vitamin D₃ conjugate (m/z 560) formed in the early stages of the reaction was converted into the product ion (m/z 298) towards the completion of 1 hour. Absence of vitamin D₃ peaks (m/z- 385.3) after 0 minute of reaction suggests that it was reacted with PTAD. Presence of PTAD peak (m/z 174.1) till the end of the reaction was due to excess PTAD added in the reaction. MS showing presence of respective peaks at specific time points are included in an appendix figure 26.

Table 3: Analysis of derivative formation-Time Course.

<table>
<thead>
<tr>
<th>Time interval</th>
<th>Target peaks</th>
<th>PTAD (m/z-174.1)</th>
<th>PTAD derivative Product (m/z -298.0)</th>
<th>PTAD Conjugation precursor (m/z-560)</th>
<th>Vitamin D₃ (m/z- 385.3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min</td>
<td>√</td>
<td>-</td>
<td>-</td>
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<td>60 min</td>
<td>√</td>
<td>√</td>
<td>-</td>
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</tr>
</tbody>
</table>

PTAD derivative formation using control DBS

PTAD derivative formation was also seen with vitamin D extracted from unspiked and spiked control blood spots. Product ion was seen at m/z 297.0.
Figure 15: Panel (a) MS of PTAD alone as a negative control; panel (b) MS of unspiked control spot with the presence of product ion (m/z~297.0); panel (c), (d) and (e) MS of control blood spots spiked with 6.25ng/ml, 12.5ng/ml and 25ng/ml of vitamin D₂ and D₃ showing the presence of product ion (m/z~297.0).
PTAD derivative formation using test DBS:

All test spots were treated with PTAD for derivative formation. MS of all test samples shows the presence of product ion at m/z 298.0. Following figure shows presence of product ion at m/z 298.0 from only one test sample. MS of remaining samples are included in an appendix figure 30.

Figure 16: Panel (a) MS of negative control-PTAD alone. An arrow indicates presence of unreacted PTAD at m/z 174.1. Panel (b) MS of test sample showing presence of product ion at m/z 298.0 indicated by an arrow.
Chapter 1 - DISCUSSION

The objective of this study was to develop a mass spectrometry (MS) method to determine the types and levels of vitamin D in newborn dried blood spots (DBS). This will help in determining vitamin D deficiency (VDD) as a cause of multiple unexplained fractures (MUF) in infants, and thus preventing misdiagnosis of these cases as child abuse. The use of DBS is an innovative approach as compared to most of the MS based assays which involve vitamin D estimation in serum samples. The vitamin D analytes are found to be stable in DBS and also have strong correlation with the newborn cord blood levels (Eyles et al., 2009). Therefore, DBS can be used as a potential tool for MS based assay development.

Our first goal towards MS method development was to extract vitamin D from the DBS. Vitamin D is bound to vitamin D binding protein (DBP) in the circulation (Eyles 2009) and thus levels of vitamin D cannot be accurately measured until it is released from this binding protein (Wallace et al., 2010). Therefore, it is necessary that the extraction step should separate vitamin D analytes alone from DBP and any other artifacts present in the blood. We used organic solvents for extraction of vitamin D from DBS as it is simple and inexpensive (Vogeser, 2010). One of the challenging aspects of organic solvent extraction is the selection of solvents. Different solvents such as acetonitrile (ACN), hexane, methanol, chloroform and diethyl ether are useful for extraction of the fat soluble vitamin D (Musteata and Musteata, 2011). In order to evaluate best suitable solvent system for extraction, initial extractions were carried out with filter paper spots spiked with standard vitamin D solution of concentrations higher than normal levels of vitamin D in the blood. Initially, we tried to extract vitamin D using mineral oil-hexane and
ACN-hexane solvent systems; however, we were not able to detect vitamin D peaks in the mass spectra of these extractions.

Previous studies have used the methanol-hexane solvent system for vitamin D extractions with minimum loss of the target analytes (Adamec et al., 2011). Methanol and hexane are immiscible solvents and the combined use of this biphasic solvent system allows phase separation of lipophilic vitamin D in the hexane portion while precipitating cellular proteins in methanol (Maunsell et al., 2005). Initially, we tested the efficacy of this methanol-hexane system by extracting desipramine HCl from the standard filter spots. Desipramine HCl was used as a positive control in our lab for MS analysis due to its similar size to vitamin D (m/z 267.2) and its ring structure (figure 17). MS analysis showed the presence of target peak (m/z- 267.2) in all the extracts of the filter paper spiked with standard desipramine HCl solution of concentrations in the range of 8- 5000 ng/ml.

![Figure 17: Structure of desipramine. The structure of tricyclic antidepressant is shown.](image)
m/z for this structure is 267.2 d.

Next, we tested methanol-hexane solvent system by extracting standard vitamin D spots and control DBS. The resulting mass spectra of such extractions showed the respective
peaks of vitamin D analytes [25 (OH) D₂ - 413.2 and 25 (OH) D₃ - 401.2]. These extractions were coupled with slight modification of extended incubation in presence of methanol and sodium hydroxide.

To confirm the identity of vitamin D analytes we performed MS/MS analysis. In a typical MS/MS analysis for compound identification, parent ion of a target m/z value is subjected to fragmentation. The fragmentation of the parent compound is then compared with fragmentation of the test compounds for qualitative determination. A study by Adamec et al. suggested that MS/MS analysis of 25 (OH) D₂ and 25 (OH) D₃ generate two most intense fragments at m/z of 355.1, 337.2 and m/z 365.2, 257.2 respectively (Adamec 2011). Similar to these findings, our results showed that 25 (OH) D₂ and 25 (OH) D₃ generate fragments at m/z of 336.2 and 365.2 respectively. The difference in the fragmentation pattern (2 fragments vs. 1 fragment per analyte) may be a result of differences in experimental conditions. The MS/MS analysis of 25 (OH) D₂ has resulted in fragment ion at m/z 336.2 in contrast to 337.2 in the previous study. This 1 Dalton difference may be due to the loss of one hydrogen ion or structural rearrangement that may occur at high temperature and vacuum conditions in the ion trap MS. Fragmentation of 25 (OH) D₂ and 25 (OH) D₃ is described in figure 18.
Figure 18: Fragmentation of 25 (OH) D₃ and 25 (OH) D₂. The site of fragmentation is shown by dotted line in the structures of 25 (OH) D₃ and 25 (OH) D₂.

In MS analysis, detection and quantitation limit of an analyte is expressed in terms of signal to noise ratio (SNR). The SNR is a measure of signal strength with respect to background noise. For qualitative determination SNR should be at least 3, whereas for quantitative determination it should be at least 10 (Lamshoft et al., 2011). Intensity of an analyte signal (S) mainly depends upon the amount of sample and ease of ionization whereas various sources of contaminants such as mechanical parts of the MS system,
sample injector and sample preparation contribute to the ‘noise’ (N). Our results showed that the target vitamin D peaks from control and test DBS have the SNR of 5, confirming that they are within the minimum requirement of qualitative assessment.

Another goal of MS method development is to quantitate vitamin D levels from DBS. Quantitation of vitamin D is often considered to be challenging because of numerous reasons including lipophilic nature of vitamin D, low physiological levels, instability in presence of high temperature and loss of analyte during extraction (Luque et al., 1999). In addition, Eyles et al. has suggested that some degradation of vitamin D may also result from drying of blood spots (Eyles 2010). The lipophilic nature of vitamin D makes it difficult to ionize in the MS. We employed electrospray ionization (ESI) to ionize vitamin D analytes since it is considered to be a sensitive method which produces reproducible results with lipophilic molecules (Brugger et al., 1997).

Evidence suggests that poor ionization of vitamin D molecules can be overcome by derivative formation method using 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) in a cycloaddition reaction (Yeung et al., 1993). The net effect of derivative formation is the significant increase in the ionization due to addition of high proton affinity compound i.e. PTAD (Higashi et al., 2002). However, the end product of derivative formation i.e. vitamin D-PTAD conjugated product further undergoes cleavage at carbon 6-7 maybe due to high-energy environment in the MS. Since PTAD conjugated vitamin D2 and D3 both generates the same product ion after Carbon 6-Carbon 7 cleavage (Eyles 2009), it is imperative to detect this transition in the MS analysis to distinguish between vitamin D2 and D3. The fragmentation of derivatized vitamin D is summarized in figure 19.
Figure 19: Summary of vitamin D derivative formation.

Our results of initial derivative formation experiments with standard vitamin D₃ (6.25ng/ml-100ng/ml) showed the formation of product ion (m/z~298.0) with enhanced intensity. However, the results did not show the mass transition (m/z ~560→m/z ~298.0) in the MS analysis. In an attempt to monitor derivative formation over time we observed very small peaks of vitamin D₃-PTAD conjugated product ion at 1 and 5 minute but not
30,45 or 60 minutes. However, at the end of derivative formation, mass spectra showed only the presence of product ion (m/z 298.0) and unused PTAD (m/z 174.1). The PTAD derivative formation with control and test DBS showed similar results with the presence of only the product ion (m/z 298.0). It can be concluded from these results that PTAD-vitamin D conjugated product might be unstable in the MS conditions employed here and thus readily generates product ion (m/z 298.0). The quantitative analysis of vitamin D analytes without derivative formation has a potential problem of ‘interferences’. The term ‘interferences’ includes all naturally occurring and pharmaceutical compounds of sterols and fatty acid derivatives. The possibility of extraction of such compounds along with vitamin D cannot be rejected. The presence of such compounds certainly has a detrimental effect on the overall ionization of target analytes which eventually affects their quantitation. The coupling of MS analysis with liquid chromatography (LC) or gas chromatography (GC) will help to overcome this problem. The use of LC/GC will allow chromatographic separation of target analytes which will enter alone in MS for further analysis. Thus, the absence of interferences in MS will not have any effect on ionization of target analytes. The efficiency of ionization can also be improved by changing the source of ionization in MS. Recent advances in MS method development for vitamin D assessment have demonstrated the use of atmospheric pressure chemical ionization (APCI) as an alternative to ESI. The use of these alternative approaches will defiantly help in improving SNR ration and thus quantifying vitamin D levels from the DBS.
CHAPTER 2

REAL TIME PCR ANALYSIS FOR VITAMIN D ENZYMES AND RECEPTOR

USING DRIED BLOOD SPOTS
Chapter 2 - INTRODUCTION

Background:

Vitamin D is biotransformed by the liver and kidneys into active metabolites which then bind to the VDR to cause a particular biological activity. The liver is the major site for initial biotransformation of vitamin D, forming 25 (OH) vitamin D. Hydroxylation is achieved by 25-hydroxylase which is a cytochrome P450 (CYP) dependent enzyme. Six different CYP’s have been identified which are capable of catalyzing this reaction, of which, liver 25-hydroxylase or CYP2R1 is found to be the most active form (Aiba et al., 2006; Gupta et al., 2004). Although CYP2R1 is called as liver 25-hydroxylase, it is also actively present in extra-hepatic sites including testis and dermal fibroblasts (Foresta et al., 2011). The second step of bioactivation involves formation of 1α, 25-(OH)₂ D primarily in the kidney. Renal synthesis of the dihydroxy form of vitamin D is the rate limiting step involving another cytochrome P450 dependant enzyme, 1α-hydroxylase (CYP27B1). The presence of active CYP27B1 in extra renal sites such as bone, brain, colon, dendritic cells, macrophages, keratinocytes, endothelial cells and parathyroid has also been documented (Henry, 2011). The active form of vitamin D [1α, 25 (OH)₂ D] executes its physiological actions by binding to VDR which involves complex molecular mechanism. VDR are nuclear receptors that are found to be actively present in the heart, brain, skin and T-cells (Cantorna, 2011) as well as in certain cancers such as breast, colon, and prostrate (Thorne and Campbell, 2008). Like other nuclear transcription factors, VDR consists of a ligand binding domain (LBD) and a DNA binding domain (DBD). Upon binding to its ligand such as 1α, 25 (OH)₂ D, there is characteristic change in its conformation followed by heterodimerization with retinoid X receptor (RXR). The
entire complex then translocates to the vitamin D response element (VDRE) present in the promoter region of the target genes. Once the VDR-RXR heterodimer complex binds to the DNA, it recruits other co-activators to initiate gene transcription. Depending on the type of protein synthesized, different physiological actions are executed (Malloy et al., 1999).

**Vitamin D – genetic disorders:**

Although lack of exposure to the sun or insufficient dietary supply of vitamin D are the major factors responsible for VDD disorders, they also exist due to abnormalities associated with metabolizing enzymes and/or with VDR.

Any defect in the genes involved in bioactivation of vitamin D may lead to a deficiency or complete absence of its active metabolites. Mutations in vitamin D 25-hydroxylase are less common and result in low plasma levels of 25-hydroxyvitamin D. Transition mutation (T→C) in the second nucleotide at 99th residue of the exon 2 result in loss of 25-hydroxylases activity (Cheng et al., 2004). Similarly, more than 40 different mutations have been discovered in 1α-hydroxylase to date (Kim 2007). Such mutations result in deficiency of 1α-hydroxylase commonly known as vitamin D dependent rickets type I (VDDR-I) or pseudo vitamin D deficiency rickets (PDDR). It is an autosomal recessive disorder characterized by extremely low levels of active forms of vitamin D (Malloy and Feldman, 2010). Clinical symptoms of VDDR-I are characterized by muscle weakness, hypotonia, growth failure and rickets (Fraser et al., 1973). Mutations in VDR leads to a genetic disorder commonly known as hereditary vitamin D resistant rickets (HVDRR) or vitamin D dependent rickets type II (VDDR-II). Currently, 34 mutations in
the VDR gene have been identified leading to partial or complete resistance to 1α,25 (OH)₂D (Malloy and Feldman, 2010). VDDR-II is also an autosomal recessive disorder, but, in contrast to VDDR-I, it is characterized by elevated levels of active forms of vitamin D. The exact outcome of VDDR-II depends upon whether the mutation exists in the DBD, LBD, RXR or the co activators. Ligand-VDR complex cannot interact with the DNA due to mutations in DBD (Liberman et al., 1986;Yagi et al., 1993) whereas mutations in LBD lowers binding affinity of VDR to 1α, 25(OH)₂D (Malloy et al., 1997). Mutations in RXR (Whitfield et al., 1996) and co activators (Malloy et al., 2002) results in abnormalities with respect to the gene transcription process. Consequences of VDDR-II are mainly due to inability to absorb calcium from intestine which includes hypocalcemia, rickets, hypophosphatemia, alopecia and secondary hyperparathyroidism. Symptoms of VDDR-II can be reversed by oral or iv calcium supplementation (al Aqeel et al., 1993).

**Melt curve analysis and mutation scanning**

Melt curve analysis was first introduced in 2002 by Dr. Wittwer and his team for the analysis of genetic mutations. Currently it is a method of interest because of its simplicity, high specificity and cost effectiveness over other techniques for finding mutations (McKinney et al., 2004;Reed and Wittwer, 2004). Melt curve analysis is a fast approach for mutation scanning and there are less chances of potential contamination since it does not require physical separation of DNA after PCR amplification (Taylor and Taylor, 2004). The working principle of melt curve analysis is based on variation in fluorescence intensity from the amplicons. Special thermostable dye such as SYBR green is included in the reaction mixture during PCR amplification which fluoresces maximum
only in the presence of double stranded DNA. After PCR, the product is heated and fluorescence is monitored across the raising temperature. As the temperature increases, DNA starts denaturing and there is a reduction in fluorescence due to separation of the two strands of DNA. After complete melting of amplicons, it produces a melt curve as shown in the figure 20.

![Melt Curve Diagram](image)

**Figure 20:** Concept of melt curve.

The melting temperature (Tm) on the melt curve is the temperature at which 50% of double stranded DNA is melted. Relatively high temperature is required to break three hydrogen bonds between guanine and cytosine than two hydrogen bonds between adenine and thymine. Therefore, even a single base alteration in the normal sequence due to a possible mutation results in relative shift in a melt curve profile which can be easily compared and detected (Taylor, 2009). Use of melt curve analysis in mutational scanning is illustrated in figure 21.
Melt curve analysis is not only limited to mutation scanning, but also has its applications in genotyping, sequence matching, methylation analysis, and single nucleotide polymorphism scanning (Montgomery et al., 2010; Reed and Wittwer, 2004). Although melt curve analysis has wide spread applications, its efficacy is entirely based on size of the amplicons and PCR optimization (Dobrowolski et al., 2007; Reed and Wittwer, 2004). It can be performed with amplicons of size between 38-1000 bp, however best results of melt curve analysis are observed with amplicons 100-300 bp since Tm changes are greater in smaller amplicons (Gundry et al., 2003; Taylor, 2009).
Chapter 2 - HYPOTHESIS

Development of hypothesis

Genetic mutations associated with vitamin D metabolizing enzymes result in failure to form active metabolites of vitamin D. Similarly, mutations in the VDR may prevent it from binding active metabolites of vitamin D resulting in a failure to produce any physiological effects. Thus, a patient with such genetic disorders may eventually suffer from VDD consequences similar to those due to dietary insufficiency or lack of sun exposure. Newborn infants with MUF may also have mutations associated with vitamin D metabolizing enzymes and/or VDR as a cause of VDD. Genetic mutations need to be diagnosed to explore more specific treatment options to overcome VDD. Among the different methods, melt curve analysis is a faster and less expensive approach for mutation scanning (Winkel et al., 2011).

Hypothesis

Melt curve analysis will be helpful in determining genetic mutation as a cause of VDD in infants suffering from multiple unexplained fractures.
Chapter 2 - MATERIALS AND METHODS

Extraction of genomic DNA

Genomic DNA from blood spots was isolated using QIAamp® DNA mini kit (Qiagen, Valencia, CA, USA). Blood spots were placed in 1.5 ml microcentrifuge tube and buffer ATL (180 µl) was added. Buffer solution was then incubated at 85˚ C for 10 minutes and briefly centrifuged to remove any drops from inside the lid. Proteinase K (20 µl) was added to the tube, followed by brief vortexing and incubating at 56˚ C for 1 hour. Buffer AL (200 µl) was added to the sample and incubated at 70˚ C for 10 minutes. Ethanol (200 µl) was added to the sample and mixed thoroughly by vortexing. The mixture was then carefully applied to QIAamp spin column with 2ml collection tube without wetting the rim. Column cap was closed and it was centrifuged at 8000 RPM for 1 minute. Filtrate was discarded and column was place in a new 2 ml collection tube. Buffer AW1 (500 µl) was added to the column and it was centrifuged at 8000 RPM for 1 minute. Filtrate was discarded again and column was placed in a new 2 ml collection tube. Buffer AW2 (500 µl) was added to the column and centrifuged at 14000 RPM for 3 minutes. Collection tube containing filtrate was discarded again and column was placed in a new 1.5 ml microcentrifuge tube. Buffer AE (100 µl) was added to the column, incubated at room temperature for 1 minute and centrifuged at 8000 RPM for 1 minute to elute DNA. Eluted DNA was frozen at -80˚ C until use. Quantification of DNA samples was performed using NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc; USA).

Temperature gradient PCR
In order to decide specific annealing temperature for a particular primer set, temperature gradient PCR’s were performed using genomic DNA and primers for VDR, 25-hydroxylase and 1α-hydroxylase. The reactions were carried out by using Bio-Rad IQ™ supermix buffer (1X, 25 µl), SYBR Green (1X, 0.5 µl), fluorescein (10nM, 0.5 µl), primers (800nM) and 5 µl DNA template. DEPC treated water was used as a negative control for the reaction. Bio-Rad IQ™ supermix buffer consists of 2X reaction buffer with dNTP’s, iTaq DNA polymerase, 6mM MgCl₂ and stabilizers. The reaction conditions for 1 cycle were initiation at 95˚ C for 6 minutes, denaturation at 95˚ C for 30 seconds, annealing at gradient temperature for 1 minute, extension at 72˚ C for 1 minute, final elongation at 72˚C for 5 minutes and final hold at 4˚ C for an infinite time. PCR was performed for total 50 cycles. PCR products were finally analyzed by gel electrophoresis using 1.5% agarose gel. PCR product size was compared with 1Kb plus DNA ladder (Invitrogen, USA).

### Table 4: Primer sets and their sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>PCR product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>VDR</td>
<td>5’-CCAgTTCcTgTgAATgATgg-3’</td>
<td>383</td>
</tr>
<tr>
<td></td>
<td>5’-gTcgTCCATgTgAAggA-3’</td>
<td></td>
</tr>
<tr>
<td>25-OHase</td>
<td>5’-ggCAAgTACCCAagTACgg-3’</td>
<td>291</td>
</tr>
<tr>
<td></td>
<td>5’-AgCAAATAgCTCCCAAgg-3’</td>
<td></td>
</tr>
<tr>
<td>1-OHase</td>
<td>5’-TgTTTgCATTTgCTCAgA-3’</td>
<td>226</td>
</tr>
<tr>
<td></td>
<td>5’-CCggAgAgCTCATACAg-3’</td>
<td></td>
</tr>
</tbody>
</table>

**Extraction of total RNA:**

Total RNA from blood spots was extracted using Trizol® reagent (Invitrogen, Carlsbad, CA, USA). Blood spots were treated with lysis buffer (500 µl) at 80˚ C for 30 minutes.
and centrifuged at 8000 RPM for 1 minute. Supernatant was separated and incubated with Trizol® (500 µl) at room temperature for 5 minutes. Chloroform (200 µl) was added and vortexed until solution was homogenous. Samples were centrifuged at 13,000 RPM for 15 minutes at 4°C. The upper aqueous phase was transferred into fresh tube and isopropyl alcohol (500 µl) was added to precipitate RNA at room temperature for 10 minutes. Samples were centrifuged at 13,000 RPM for 10 minutes at 4°C to form RNA pellet at the bottom of the tube. Supernatant was decanted and RNA pellet was added with 80% ethanol and centrifuged at 13,000 RPM for 5 minutes at 4°C. Supernatant was decanted and samples were dried under a sterile hood for 30 minutes at room temperature.

Dried RNA samples were redissolved in 100 µl DEPC RNase free water and incubated at 57°C for 10 minutes. Reaction buffer (12 µl) and DNase I (4 µl) were added and mixture was incubated for 1 hour at 37°C with slight shaking. Acid Phenol Chloroform, 5:1; pH 4.5 (100 µl) (Ambion) was added and samples were placed on ice for 10 minutes. Samples were gently mixed by hand and centrifuged at 11,300 RPM for 5 minutes at 4°C. The upper aqueous phase was transferred to fresh tube. RNA was precipitated with 3M sodium acetate (12 µl, pH 5.5) and ice cold ethanol (560 µl, 100%) overnight at -80°C. After precipitation step, samples were centrifuged at 13,000 RPM for 5 minutes. The supernatant was decanted and samples were dried under a sterile hood for 30 minutes at room temperature. After drying, RNA samples were redissolved in DEPC RNase free water (100 µl) and stored at -80°C until use. Quantification of RNA samples was performed using NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc; USA).
Two step RT-PCR:

In the first step of RT-PCR, cDNA was synthesized from the extracted RNA using iScript™ cDNA synthesis kit (Bio-Rad, CA, USA). The reaction was carried out by using 5X reaction mix (4 µl), iScript Reverse Transcriptase (1 µl), RNA template (5 µl) and Nuclease free water (10 µl). Reaction conditions were 25°C for 5 minutes, 42°C for 30 minutes, 85°C for 5 minutes and 4°C infinite hold at the completion of the reaction. Second step of the RT-PCR was carried out using cDNA and primers for VDR as described before.

Analysis of RNA Integrity:

Integrity of RNA samples extracted from dried blood spots was checked using Experion™ (Bio-Rad, USA). Experion system was included with automated electrophoresis station, priming station, vortex station for RNA analysis and RNA std sens analysis kit which included with chips and reagents for standard-sensitivity RNA. Following procedure performed for RNA analysis using the Experion system.

In order to avoid any contamination during RNA integrity analysis, electrodes of the Experion system were cleaned using Experion electrode cleaner (800 µl) in the first step. After repeating this step for one more time, electrodes were rinsed with DEPC treated water (500 µl) for 5 minutes using electrode cleaning chip. At the end lid was kept open for 60 second to evaporate remaining water on electrodes. RNA stain, RNA loading buffer and RNA gel from the RNA std sens kit were removed from 4°C and equilibrated at room temperature for 20 minutes. RNA stain was wrapped in aluminum foil to avoid its light sensitive degradation. RNA gel was filtered from filter tube at 2000 RPM for 10
minutes. Filtered gel (65 µl) was taken into RNase-free microfuge tube and mixed with RNA stain (1 µl). RNA ladder was removed from -20°C and thawed it on ice for 10 minutes. RNA ladder (1 µl) and RNA samples (3 µl) was taken into RNase-free microfuge tube. RNA ladder and RNA samples were denatured at 70°C for 2 minutes. Ladder and samples were immediately placed on ice for 5 minutes, spun down for 2-5 seconds and stored on ice until needed. Gel-stain solution (9 µl) was taken in well labeled as GS on RNA std sens chip without forming any air bubble. Chip was primed by setting appropriate pressure for sufficient time on priming station. Chip was inspected for any air bubbles in micro channels and for incomplete priming. Gel-stain solution (9 µl) was taken other well labeled GS. Filtered gel (9 µl) was taken to well labeled as G. Loading buffer (5 µl) was taken to each sample well 1-12 including ladder well. RNA ladder (1 µl) was taken to the well labeled as L. RNA samples were taken to all wells numbered as 1-12. Chip was placed tightly and vortexed for 60 seconds on vortex station. Primed chip loaded with RNA samples and ladder was then kept on electrophoresis station for 5 minutes and electrophoresis run was started. After completion of the run, electrodes were cleaned using DEPC water (800 µl) filled in a cleaning chip. Electropherograms generated were analyzed by Experion software version 3.2.

**Melt curve**

Second step RT-PCR protocol was modified when Bio-Rad CFX96™ Real-Time PCR System (Bio-Rad, USA) was used with Ssofast™ EvaGreen® Supermix. The Ssofast™ EvaGreen® Supermix contains reaction buffer (2X) with dNTPs, Sso7d-fusion polymerase, MgCl₂, EvaGreen dye and stabilizers. Each reaction contained Ssofast EvaGreen supermix (10 µl), primers (500nM), cDNA template (2 µl), and nuclease free
water (7.2 µl). Reaction conditions for 1 cycle were as follows: enzyme activation 95˚ C for 30 seconds, denaturation at 95˚ C for 5 seconds, annealing and extension at 55-65˚ C for 5 seconds and melt curve 65-95˚C (in 0.5˚ C increment) for 5 sec/step. PCR was performed for total 40 cycles.
Chapter 2 - RESULTS

Temperature gradient PCR using genomic DNA:

In order to decide specific annealing temperature for a primer set, genomic DNA was subjected to PCR amplification using DNA samples extracted from DBS and VDR, 25-hydroxylase and 1α-hydroxylase primers sets at gradient temperatures.

(A) VDR:

(B) 25-OHase:

(C) 1-OHase:
Figure 22: Gel (A), (B) and (c) shows non specific PCR amplification at gradient temperatures for all three primer sets. From left to the right, first lane is a ladder lane and 2\textsuperscript{nd} lane is of negative control.

**Reverse-transcriptase-Polymerase Chain Reaction (RT-PCR):**

Temperature gradient 2\textsuperscript{nd} step PCR was carried out using VDR primer set and cDNA synthesized from RNA extracted from DBS.

(A)

(B)
Figure 23: Gel (A) and (B) showing PCR amplification product at gradient temperatures from 48.8-60°C. cDNA was synthesized from same RNA sample in both the cases. Both (A) and (B) shows irregularity in amplification which can be seen by presence of desired PCR product size band (384) at 58°C in (B) and absence of the same in (A). From left to the right, first lane is a ladder lane and 2nd lane is of negative control.

Analysis of RNA integrity:

Integrity of RNA samples was analyzed using Experion automated electrophoresis system. Electropherogram and gel view of RNA sample is shown in figure 23(C). Electropherograms and gel pictures of remaining RNA samples are included in appendix figure 30.
Figure 24: Electropherogram and gel view of (a) ladder showing distinct peaks and bands respectively; (b) positive control RNA sample showing presence of 5S, 18S and 28S ribosomal subunits indicated by arrows and respective bands; (c) Absence of ribosomal subunits in RNA sample and no distinct band.

Melt curve analysis:

Melt curve analysis was done on PCR product formed from RNA sample.
Figure 25: Panel (A-1), (B-1) and (C-1) represents PCR amplification profiles (Fluorescence vs. no. of cycles) at gradient temperature (55-65°C) of VDR, 25-OHase and 1-OHase respectively. From panel (C-1) it is evident that amplification was below threshold with 1-OHase. Panel (A-2), (B-2) and (C-2) represents melt curve (fluorescence vs. temperature) of VDR, 25-OHase and 1-OHase respectively. As temperature increases there is a decrease in the fluorescence since DNA strands separates from each other.
Panel (A-3), (B-3) and (C-3) represents melt peak (derivative function of fluorescence and temperature vs. temperature) of VDR, 25-OHase and 1-OHase respectively.
Chapter 2 – DISCUSSION

There is a worldwide epidemic of VDD that likely has its origin from two major factors. These include insufficient vitamin D supplementation and genetic disorders associated with vitamin D system. Genetic mutation in vitamin D metabolizing enzymes and/or VDR ultimately leads to deficiency of vitamin D and its associated comorbidities. The objective of this study was to determine if infants with MUF have such genetic mutations as a cause of possible VDD. It is possible that such mutations exists in a recessive manner in parents and they can be inherited in the progeny (Song et al., 2011). The use of melt curve is a rapid method for overall scanning and detection of genetic mutations (Wittwer, 2009). Winkel et. al. have previously shown that DBS can be used as a reliable source of genetic material (DNA/RNA) for mutational studies using melt curve analysis (Winkel, Hollegaard, Olesen, Svendsen, Haunso, Hougaard, and Tfelt-Hansen, 2011).

The initial temperature gradient PCR amplification experiments were carried out with genomic DNA to determine specific annealing temperature for 3 primer sets mainly, 25-OHase, 1α-OHase and VDR. However, the results of such PCR amplifications showed multiple bands across the temperature gradient. This could be due to multiple priming sites resulting in non-specific amplifications or incomplete primer binding at the intron-exon junctions. Although DBS are a good source of genetic material, DNA undergoes degradation in vitro over the time. To overcome the problems with degraded DNA and incomplete primer binding at the intron-exon junction, we used RNA as a starting material.

We carried out the RT-PCR using total RNA extracted from DBS. However, reverse transcribed cDNA used for temperature gradient PCR amplification showed the similar
results of multiple bands across the temperature gradient. This could be due to degradation of RNA by RNase. The RNA integrity is a measure of its quality i.e. whether it is intact or degraded. Although RNA is a stable molecule *in vitro*, it could be rapidly digested by ubiquitous RNase (Schroeder et al., 2006). Such small fragments of degraded RNA cannot be used for PCR as it may lead to either incomplete or non-specific amplification (Fleige and Pfaffl, 2006). The effectiveness of melt curve analysis in detecting genetic mutations entirely depends on efficient RT-PCR amplifications which in turn depend on integrity of the RNA. The RNA integrity is expressed in terms of a ratio of 28S and 18S ribosomal subunits, as they are considered to be vital structural constituents of RNA.

We evaluated the integrity of test RNA samples using Bio-Rad Experion™ electrophoresis system. The major advantage of using Experion™ system is the rapid and accurate analysis of RNA integrity. It is an automated electrophoresis system which is based on micro fluidic separation technology and fluorescent sample detection using laser. Our results of these experiments showed the absence of 18S and 28S peaks in the electropherogram of test RNA samples. Also, the test RNA samples showed smearing of bands in a virtual gel generated by Experion™ system. We also tested RNA sample from Bio-Rad (positive control) which showed distinct peaks of 18S and 28S in the electropherogram as well as separate bands in virtual gel of electrophoresis. This implies that Experion™ system gives an accurate analysis of RNA integrity. This also suggests that there might also be a problem with the test RNA samples. This could be due to a possible problem with the extraction protocol for RNA or due to degradation of the RNA.
We suspect that the presence of humidity may be responsible for degradation of RNA from the DBS. The World Health Organization (WHO) guidelines for the storage of DBS have recommended the use of desiccants for their long term use in genetic analysis. Although drying and storage of DBS at -80°C reduces the chances of RNA degradation, it is extremely prone to degradation in the presence of moisture.

We further performed temperature gradient PCR using Bio-Rad CFX96™ Real-Time PCR system coupled with melt curve analysis. The advantage of this method is that if a specific annealing temperature is established for a particular primer set, mutations could be identified based on comparison of normal and test melt curves. Unfortunately, we were not able to determine the specific annealing temperature because of the aberrant melt curve and amplification profiles.
CONCLUSION

The importance of vitamin D in maintenance of skeletal, cardiovascular and immune systems in all the stages of human life is well known. Vitamin D exists in two different active forms which are equally important. Their deficiency leads to serious health consequences, and therefore it is necessary to develop accurate assays to measure vitamin D levels in the body. The immunoassays developed so far; lack their ability to distinguish between the two forms of vitamin D. The MS based assays are more popular for being most sensitive and accurate in the measurement of the two forms of vitamin D. DBS are useful to analyze minute samples which provide a unique screening method for both early and late identification of diseases.

We used newborn DBS to assess the vitamin D levels in infants with MUF in which child abuse was suspected. We observed that the methanol-hexane solvent system was the best suitable option for the extraction of vitamin D from DBS. Under the optimized MS conditions we were able to distinguish between the two forms of vitamin D from the extracted control and test samples. The fragmentation analysis using MS/MS further confirmed their identity. The results of chemical derivatization for quantitative analysis showed that both forms of vitamin D generate the same product ion. Thus it became difficult to quantitate each form separately. The coupling of LC or GC with MS will help in selective chromatographic separation of target vitamin D analytes before they enter in MS. Also, the use of an alternative source of ionization such as APCI may also help in improving their ionization efficiency. The application of these approaches will definitely help in improving quantitative assessment of vitamin D from DBS.
The vitamin D metabolizing enzymes and VDR are essential to execute vitamin D functions in the body. Any genetic mutations in the genes for metabolizing enzymes or VDR may result in similar consequences of VDD as those that occur due to inadequate nutrition and/or exposure to the sun. Therefore it is necessary to evaluate if such genetic mutations are the cause of VDD in infants with MUF. We tried to use DBS for the analysis of genetic mutation using melt curve analysis. However, such an attempt was inconclusive because of possible degradation of genetic material. Our results suggest that the RNA extracted from DBS is degraded and is not useful for melt curve analysis. Therefore it is necessary to store DBS under appropriate conditions of zero humidity and temperature.
APPENDIX

Derivative formation at different time interval

Figure 26 represents presence of unique peaks at specific time interval of 1 hour derivative formation reaction.
Figure 26: Panel (a) and (b) MS showing presence of PTAD (m/z 174.1) and unreacted vitamin D₃ (m/z 385.3) at 0 minute; panel (c) and (d) MS showing presence of PTAD and PTAD-conjugation product (m/z 560.0) at 1 minute; panel (e) and (f) showing presence of PTAD and PTAD-conjugation product at 5 minutes; panel (g) and (h) showing presence of PTAD and PTAD-derivative product (m/z 298.0) at 30 minutes; panel (i) and (j) showing presence of PTAD and PTAD derivative product at 45 minutes and panel (k) and (l) showing presence of PTAD and PTAD-derivative product at the completion of 1 hour.

Control DBS with prolonged incubation before extraction:

Figure 27 (A) shows presence of 25 (OH) D₂ and D₃ peaks and (B) shows fragments of 25 (OH) D₂ and D₃ respectively in control blood spots.
Figure 27(A): Panel (a), (b), (c) and (d) MS showing presence of 25 (OH) D$_2$ and D$_3$ peaks (m/z 413.2 and 401.2 respectively) in control blood spots.
Figure 27(B): Panel (e), (f), (g) and (h) MS showing presence of fragments of 25 (OH) D$_2$ and D$_3$ peaks (m/z 336.2 and 365.2 respectively) from control blood spots.
Test DBS with prolonged incubation before extraction:

Figure 28 (A) shows presence of 25 (OH) D₂ and D₃ peaks in MS of test samples (2-10).
Figure 28(A): Panel (a)-(i) MS showing presence of 25 (OH) D$_2$ and D$_3$ peaks (m/z 413.2 and 401.2 respectively) in test samples 2-10.
Figure 28(B) shows presence of fragment peaks of 25 (OH) D$_2$ and D$_3$ in compound mass spectra of test samples. 25 (OH) D$_2$ fragment is present only in test 5 [panel (d)], indicated by arrow.
Figure 28(B): Panel (a)-(i) MS showing presence of fragments of 25 (OH) D₂ and D₃ peaks (m/z 336.2 and 365.2 respectively) in test samples.
Manual MS(n) data set of test DBS

Figure 29(A) shows the presence of 25 (OH) D₃ peak (m/z 401.2) isolated by manual MS(n) method of data acquisition.
Figure 29(A): Panel (a)-(i) Manual MS(n) with isolated peak of 25 (OH) D₃ at (m/z ~ 401)
Figure 29(B) below shows the presence of fragment peak of 25 (OH) D₃ (m/z 365.2) isolated by manual MS(n) method for data acquisition.
Figure 29(B): Panel (a)-(i) manual MS(n) with fragment peak at m/z 365.2 of 25 (OH) D₃ indicated by arrow.
Figure 29(C) below shows the presence of 25 (OH) D₂ peak (m/z ~ 413.2) isolated by manual MS(n) method of data acquisition.
Figure 29(C): Panel (a)-(i) Manual MS(n) with isolated peak of 25 (OH) D$_2$ at (m/z ~ 413).
Figure 29(D) below shows the presence of fragment peak of 25 (OH) D₂ (m/z 336.2) only in test 5 (panel d). Fragment peak is absent in remaining test spots.
Figure 29(D): Panel (a)-(i) except panel (d) with the absence of fragment peak of 25 (OH) D₂. Panel (d)-test 5 shows presence of fragment peak at (m/z 336.2) indicated by arrow.
PTAD derivative formation using test blood spots:

Figure 30 represents PTAD-derivative formation using test spots 2-10.
Figure 30: Panel (a)-(i) MS of test spots showing PTAD-derivatized product as indicated by arrow at (m/z 298.0)
Analysis of RNA integrity:

Electropherograms and gels pictures of RNA test samples (2-10) are shown in following figure 31.
Figure 31: Electropherograms of test RNA samples (a) to (i) showing absence of peaks belonging to ribosomal subunits (18S and 28S) of RNA. Also, there is no evidence of distinct band in a virtual gel of all test RNA samples. This result confirms degradation of test RNA samples from the dried blood spots.


a kindred with hereditary 1,25-dihydroxyvitamin D-resistant rickets. 


