EVALUATION OF A NOVEL LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY BASED METHOD FOR VITAMIN D ANALYSIS IN BLOOD

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ABSTRACT

Objective: To evaluate a novel liquid chromatography tandem mass spectrometry based method for serum 25 hydroxy vitamin D (D2 and D3 metabolites) analysis.

Study Design: Cross sectional validation study.

Place and Duration of Study: Department of Chemical Pathology and Endocrinology, Armed Forces Institute of Pathology, Pakistan, from Mar 2019 to Mar 2020.

Methodology: Samples were extracted and 25 OH vitamin-D was separated by means of chromatography and finally quantified via mass spectrometer. A quadrupole- tandem mass spectrometer with Electron spray Ionization coupled to high performance liquid chromatography was adopted for detection and quantitation of 25-hydroxyvitamin D2 and D3 in serum.

Results: Limit of detection (LOD) was at the level of 2.49 ng/ml and limit of quantitation (LOQ) was estimated to be 3.9 ng/ml for both the metabolites. The correlation coefficient was 0.99. For D3 observed recovery was 98% and 97.5% respectively while for D2 the recovery was calculated to be 97% and 98%. Percentage relative standard deviation (RSD) was 0.8% and 1.3% respectively. This method has an advantage of less matrix effects, minimal cross-reactivity with 24, 25 hydroxy vit D and 25, 26 di-hydroxy vitamin D metabolite than the routinely used antibody-based assays.

Conclusion: This LC-MS/MS methodology is sensitive, specific and can quantitate Vitamin D2 and D3 quite efficiently. This method may be employed for vitamin D analysis in clinical laboratories.

Keywords: 25 Hydroxy vitamin d2, 25 Hydroxy vitamin d3, Liquid chromatography mass spectrometry (LCMS), Validation.

INTRODUCTION

The 25 Hydroxy vitamin D is a secosteroid pro-hormone which is essential for teeth, bone and development of muscle. The most accurate and reliable assessment of vitamin D status is quantification of 25-hydroxyvitamin D concentration. Vitamin D occurs in 2 major types; 25 Hydroxy vitamin D3 is derived from human skin which is the natural and predominate Vit D source in humans. Whereas 25 Hydroxy vitamin D2 also known as ergocalciferol, only occurs by supplementation & fortifying food products. It exerts significant effects local frequently. Low serum vitamin D levels increases the risk of osteomalacia and rickets. Recent literature review reports a global pandemic of 25OHD deficiency.

The clinical usage of these parameters is pertinent in diagnosis and treatment of the patients. There is an immense improvement in laboratory sciences technology in the field of vitamin D analysis. The methods available for estimation of Vitamin D include immuno-chemical methods which use radioactive, chemiluminescence and enzyme labels. Physical detection methods for example HPLC & LC-MS/MS are also used. Among these methods, Immunoassays are treated as common but have certain limitations. It is not as much specific as LC/MS and it is unable to differentiate between vitamin D3 and vitamin D2 forms and has therefore low selectivity.

There is a need to identify & quantify D2 & D3 fractions of 25 hydroxy-vitamin D for correct measurement of deficiency & subsequent management. Various analytical techniques are engaged for its authentic quantitation. Liquid chromatography tandemmass spectrometry (LC, MS/ MS) is the universally accepted reference method for vitamin D estimation which involves release of the compound from the vitamin D binding protein followed by its chromatographic separation and detection by a Mass Spectrometer. As no such studies of the use of liquid chromatography mass spectrometry for the detection of vitamin D2 and D3 are available in Pakistan, so this analysis was carried out.

METHODOLOGY

This cross sectional validation study was conducted in department of Chemical Pathology and Endocrinology Armed Forces Institute of Pathology (AFIP), Rawalpindi, from March 2019 to March 2020 after approval from Institutional Review Board (IRB). The technique of non-probability convenient sampling was incorporated. A total of 120, otherwise healthy indivi-
Vitamin D Analysis in blood

Participants reporting for vitamin D analysis to Armed Forces Institute of Pathology between the ages of 18-80 years were included so that the reference range can be established for future use in clinical labs7. Whereas, pregnant and lactating women, patients with acute and chronic illness (tuberculosis, CKD, CCF, CLD) and history of alcohol intake were excluded from the study. Five ml baseline venous blood sample was taken in clot separator /gel tube at the start of study. After centrifugation serum was separated and stored at temperature-80°C for further analysis of vitamin D by LCMS/MS. Pure calibrators of 25 Hydroxy vitamin D3 (5 μg/ml vial), 25 Hydroxy vitamin D2 (50 μg/ml vial) and 25 Hydroxy vitamin D3-d6 μg/ml vial (internal standard) were obtained by Cerilliant® (Sigma Aldrich) for method development and serial dilutions were prepared accordingly. For 25-hydroxy vitamin D3 calibration curve concentrations of 3.9, 7.8, 20, 50, 100 and 200 ng/ml and for constructing 25-hydroxy vitamin D2 concentrations of 7.8, 15.6, 31.2, 62.5, 125 and 250 ng/ml were prepared by diluting pure standards in methanol9.

Stock solutions were prepared & placed at-20°C in amber vials protected from light. They were found to be stable for a period of 3 months under these conditions. By keeping the solutions in dark, light-induced degradation of the analyte was avoided10. Other reagents used include methanol, N-Hexane, ethyl acetate, formic acid, HPLC grade ultrapure water. All solvents were LC/MS grade. Pipettes with tips, centrifuges, vortex mixer, rotator and dry bath and N2 source for drying were utilized during sample preparation.

Blood sample was collected in serum separator tube. After centrifugation serum was separated. In 500 μl serum 100μl internal standard was added and vortexed briefly, followed by addition of 400μl of methanol and vortexed for 1 min and kept for incubation for 10 minutes at the room temperature. Afterwards, 1000 μl of Ethyl acetate: n- hexane (1:1) mixture was dispensed. The tubes were rotated for 10 minutes followed by 15 minutes centrifugation at 15,000 rpm11. The supernatant was collected in a separate tube and kept for drying under nitrogen for 10 minutes. Finally the sample were reconstituted in 200 μl of methanol: water (75: 25) and injected for analysis. POROSHELL column (120EC-C18) having dimensions 2.1 x75mm, 2.7 micron was utilized which physically separated the metabolites. Injector volume was kept 20μl while the chromatographic column was maintained at a temperature of 50°C. Flow-rate was maintained as 0.5mL/min for achieving separation. Table-I shows the summary of The LC module and MS parameters. An electro spray ionization (ESI) mode with positive polarity was used. While nitrogen gas was utilized as a desolvation and collision gas.

Table-I: LC and MS parameters for 2 Hydroxy D2 and 25 Hydroxy-D3 detection in blood.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injector volume</td>
<td>10 μL</td>
</tr>
<tr>
<td>Temperature (column)</td>
<td>50°C</td>
</tr>
<tr>
<td>Mobile phase (A)</td>
<td>5% methanol in 0.1%</td>
</tr>
<tr>
<td>Mobile phase (B)</td>
<td>Formic acid</td>
</tr>
<tr>
<td>Mobile phase (C)</td>
<td>Methanol</td>
</tr>
<tr>
<td>Flow rate for mobile phase</td>
<td>0.5 ml per minute</td>
</tr>
<tr>
<td>Run (Time)</td>
<td>7 min</td>
</tr>
<tr>
<td>Post-run time</td>
<td>1 min</td>
</tr>
<tr>
<td>Type</td>
<td>MRM mode</td>
</tr>
<tr>
<td>Polarity</td>
<td>Positive</td>
</tr>
<tr>
<td>Temperature (MS module)</td>
<td>25°C</td>
</tr>
<tr>
<td>Flow</td>
<td>10 L / minute</td>
</tr>
<tr>
<td>Temp (Sheath Gas)</td>
<td>325°C</td>
</tr>
<tr>
<td>Flow rate (Sheath Gas)</td>
<td>11 L / minute</td>
</tr>
<tr>
<td>Pressure (nebulizer)</td>
<td>50 –Psi</td>
</tr>
<tr>
<td>Voltage of capillary</td>
<td>5000 –V</td>
</tr>
</tbody>
</table>

The conversion factor for 25-OHD2 and 25-OHD3 is 2.496.12. The measures of validation parameters including limit of detection and quantification, accuracy, precision, analytical specificity, recovery and stability were calculated manually following the FDA guidelines for validation studies of chromatographic assays13,14. Precision was expressed as coefficient of variation while accuracy was expressed as percentage of the relative error. It was determined with the given formula of [(mean measured concentration/nominal concentration)/nominal concentration] × 100. The criterion for acceptance of precision is <15% RSD15. Accuracy with a bias within ± 15% is regarded as acceptable linearity calibration curve was generated by the analyzing equipment.

RESULTS

Linearity obtained for 25 OH D2 & D3 assay was linear over the analytical measurement range (AMR) of 3.9-200 ng/ml while the correlation coefficient was expressed as 0.99 (fig-1(a&b)). The parameters of limit of detection and limit of Quantitation were assessed in a series of experiments by diluting low QC (serum pool) and standard solution for 25 hydroxy D3 and D2. Limit of detection (LOD) was found to be at the level of 2.49 ng/ml and limit of quantitation (LOQ) was analyzed to be 3.9 ng/ml for both of the metabolites.
Accuracy & precision for 25OH vitamin-D2 & 25OH vitamin-D3 LCMS assay were assessed in triplicate in control samples, at three specific concentrations i.e. 10, 50 & 100 ng/ml. All results obtained were satisfactory meeting the above criterion.

For example integrity and stability the samples were analyzed at 2 different temperatures i.e 30°C & 37°C. Freeze and thaw cycles were also assessed. No variability in the results were observed. Stability was determined after the exposure of the spiked samples, 10, 50 and 100 ng/ml at room temperature for 4 & 24 hours. It showed that the given method is stable in freeze and thaw cycle and at room temperature if samples are kept for 4 and 24 hours.

Potentially interfering substances were assessed by analyzing hemolytic and icteric samples i.e spiked standards of vitamin D3 at 10 ng/ml concentration. The RSD% for triplicate hemolytic and icteric samples is 4.35% and 2.25% respectively.

### DISCUSSION

This study illustrates the use of LC/MS for detection of 25 hydroxy vitamin D2 and D3 in serum. The detection of vitamin D metabolites through LC/MS is a challenging task in analytical chemistry. This is attributed to the lipophilic nature and low ionization properties of vitamin D and its metabolites. The data regarding its estimation by LC/MS is sparse in our region. Currently majoriy of the vitamin D analysis in our country is carried through antibody based immunoassays. Immunoassays no doubt are robust, time saving and involves less analytical expertise but on the other hand have certain limitations. They do not differentiate between vitamin D3, D2 and other vitamin D metabolites. It suffers from interference issues due to cross reactivity with other related vitamin D metabolites and sterols.

Watson et al, reported the use of LCMS/MS for the accurate estimation of vitamin D for the first time. Since then a lot of advancements have been made to improve the throughput and sensitivity by using automated sample preparation techniques. Clarke et al, used 750 mL hexane: ethyl-acetate v/v in 4:1 along with 50ng mL-1 of each deuterium labeled internal standard to attain 60-90% recovery. In our study we

### Table-II: Accuracy and precision of 25 hydroxy D 3 and D2 level in triplicate at three concentrations.

<table>
<thead>
<tr>
<th>Level of Concentration (ng/ml)</th>
<th>Accuracy and Precision of Vitamin D3</th>
<th>Accuracy and Precision of Vitamin D2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed mean ng/ml ± SD</td>
<td>RSD (Precision)</td>
</tr>
<tr>
<td>Inter Assay</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 ng/ml</td>
<td>9.73 ± 0.06</td>
<td>0.59%</td>
</tr>
<tr>
<td>50 ng/ml</td>
<td>48.23 ± 0.29</td>
<td>0.59%</td>
</tr>
<tr>
<td>100 ng/ml</td>
<td>98.17 ± 0.76</td>
<td>0.78%</td>
</tr>
<tr>
<td>Intra Assay</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 ng/ml</td>
<td>10 ± 0.10</td>
<td>1.0%</td>
</tr>
<tr>
<td>50 ng/ml</td>
<td>49.57 ± 0.38</td>
<td>0.76%</td>
</tr>
<tr>
<td>100 ng/ml</td>
<td>98.53 ± 1.36</td>
<td>1.38%</td>
</tr>
</tbody>
</table>

Satisfactory results for both D2 and D3 were obtained. For D3 observed recovery was 98% and 97.5% respectively while for D2 the recovery was calculated to be 97% and 98%. Percentage RSD was 0.8% and 1.3% respectively.

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used n-hexane: ethyl acetate in 1: 1 (v/v) and used 25-hydroxyvitamin D3-d6 as an internal standard. The observed recovery was 97% for 25 hydroxy D2 and 98% for 25 hydroxy vitamin D3.

Rola et al reported a fast and reliable method using combination of an organic solvent and acetonitrile to serum in ratio of 8:1. Highest recovery was achieved using larger proportion of organic solvent during extraction. Total run time was 5.5 minutes in their study while in our study the analysis time is 8 minutes which is quite satisfactory and consistent with their findings.

Despite of being specific our method has a limitation in pediatric population due to interference of circulating 3 epi 25 hydroxy D3. This metabolite typically occurs in babies <1 year of age. Shah et al, reported a novel assay which overcame epimer (C3 epimer specially) by use of specially arranged tandem columns. They coupled a high resolution (ZORBAX-C18) column with a specially designed ULTRON-chiral with guard type of column having an inlet filter. These 2 special columns eliminates co eluting isomers thus improving specificity. The drawback of this method was the use of expensive cartridges for solid phase-extraction.

CONCLUSION

The described method utilizing LCMS technology allows detection and quantitation of 25 Hydroxy vitamin D2 and D3, separately in human blood. This LC-MS/MS method is highly sensitive, specific and has less cross reactivity with other vitamin D metabolites and can give highly cost effective standardized results of Vitamin D at a tertiary care setting with mega workload as compared to costly Immunoassay method.

CONFLICT OF INTEREST

This study has no conflict of interest to be declared by any author.

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Vitamin D Analysis in Blood

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