Jordan Journal of Pharmaceutical Sciences, Volume 6, No. 2, 2013

The Determination of 25-OHVitamin (D2/D3) in Human Serum by Liquid Chromatography Tandem Mass Spectrometry with Comparison to IDS Enzyme Immunoassay

Samar J. Melhem[1], Khaled M. A. Aiedeh[2] , Kamal A. Hadidi[3]✉ FerasAlali[4]

[1] Department of Clinical Pharmacy, Faculty of Pharmacy, University of Jordan, Amman 11942, Jordan.
[2] Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Jordan , Amman 11942, Jordan.
[3] Department of Pathology, Microbiology & Forensic Medicine, Faculty of Medicine, University of Jordan, Amman 11942, Jordan
[4] Faculty Of Pharmacy – The Jordan University of Science And Technology

ABSTRACT

The proper assessment of the status of vitamin D requires the accurate measurement of both 25-OH vitamin D2 and 25-OH vitamin D3, which collectively constitute 25-OH vitamin D, the best indicator of vitamin D status. Currently, numerous assay methods are available for 25-OH vitamin D measurement but their comparability is uncertain. We employed isotope dilution liquid chromatography coupled with tandem mass spectrometry (ID-LC-MS/MS) to quantify 25-OH vitamin D2 and 25-OH vitamin D3 in human serum. Hexadeuterium labeled 25-OH vitamin D3 internal standard was added to calibrators, controls prepared in 6% bovine serum albumin in phosphate buffered saline, and patients' sera. Zinc sulphate was added to release 25-OH vitamin D metabolites for vitamin D binding protein, followed by a precipitation step withthe addition of acetonitrile. Subsequent online phase extraction by trap column followed by chromatographic separation on a C-8 column using a water/acetonitrile gradient was employed. Detection was performed using Atmospheric Pressure Chemical Ionization (AP-CI) in a Multiple Reaction Monitoring (MRM) mode. The method was linear from 4 to 70 ng/mL. The intra and inter-day CV% were ≤ 10 for both 25-OH vitamin D2 and 25-OH vitamin D3. Recoveries ranged between 39.09 % to 64.31 % for 25-OH vitamin D2 and 30.44 % to 58.66 % for 25-OH vitamin D3, while recoveries from hexadeuterium 25-OH vitamin D3 ranged from 44.11 % to 67.5%.We compared the newly validated LC-MS/MS with a commercial Enzyme Immunoassay from Immunodiagnostic Systems (IDS EIA) in terms of inter-method agreement, correlation, and impact of assay variation on the diagnostic categorization of vitamin D status through the measurement of 182 subjects' sera. The mean bias % of the IDS EIA relative to the LC-MS/MS was -34.28 ± 10.15 (mean ± std) with 95% CI [-24.13 to 44.43]. The two methods were ingood agreement with reasonable correlation (r^2=0.82, P value = 0.000). Inter-method diagnostic categorization was variableand depended on the type of assay method and the applied cut offs used. Cross calibration and standardization of vitamin D assay methods is crucial for proper clinical assessment of vitamin D status. This LC-MS/MS method is reliable and robust for the measurement of both 25-OH vitamin D2 and 25-OH vitamin D3 in human serum.

Keywords: 25-hydroxyvitaminD, LC-MS/MS,enzyme immunoassay, method comparison, diagnostic categorization of vitamin D status.

INTRODUCTION

Vitamin D deficiency is now recognized as a worldwide problem with estimations of 1 billion people worldwide vitamin D deficient or insufficient by several studies.[1-4]In addition to the well- known effects of vitamin D on bone metabolism,[5,6]there is now an emerging body of evidence that delineates the role of vitamin D deficiency in other chronic diseases such as certain cancers, autoimmune, cardiovascular diseases,

✉ Khadidi@ju.edu.jo
Received on 31/10/2012 and Accepted for Publication on 6/5/2013.

and diabetes.[1,5,7-11]Consequently, the volume of vitamin D laboratory testing has markedly increased in the last decade as vitamin D deficiency is thought to be more prevalent than earlier assumed.

Vitamin D exists in two forms;vitamin D2 (ergocalciferol) obtained exogenously from the diet and vitamin D3 (cholecalciferol) produced endogenously in the skin in response to sun exposure and both forms are found in many supplementation products. Vitamin D when ingested undergoes metabolism in the liver to form 25-OH vitamin D which is subsequently metabolized to 1, 25 -OH – vitamin D in the kidney to constitute the active metabolite. To date, there is no consensus on the optimal circulating 25-OH-vitamin D concentrations. Nevertheless, most experts define vitamin D deficiency by serum levels below 20 ng /ml.[1,12-14]

For the assessment of vitamin D status, it is widely acknowledged that the measurement of 25-OH vitamin D is the best indicator of vitamin D's nutritional bodily stores.[1]Currently, there arenumerous vitamin D assay methods used for the quantification of 25-OH vitamin D. These include immunoassays and methods based on chromatographic separation (LC-MS/MS and HPLC). However, these methods are not standardized against reference method(s), reference material(s),and reference interval(s) in addition to the inter-laboratory and inter-assay variability which may confound the definition of vitamin D deficiency.[15-18,60]In addition, the hydrophobic characteristics of 25-OH vitamin D metabolite, along withits existence in two distinct hydroxyl metabolites (25-OH vitamin D2 and 25-OH vitamin D3) in nanomolar concentrations and its strong binding properties, imparts its measurement in biological matrices with technical demand.[19-20,59]We developed a newly validated isotope-dilution LC-MS/MS for the simultaneous determination of both 25-OH vitamin D2 and 25-OH vitamin D3 in human serum. The newly validated LC-MS/MS was compared with the popularIDS enzyme immunoassay (IDS EIA) assay method in terms of agreement, correlation, and impact of the assay variation on the diagnostic categorization of vitamin D status.

MATERIALS AND METHODS

Materials and Reagents

The 25-OH vitamin D2 was purchased from Sigma-Aldrich (Germany). The internal standard (IS) 26, 27-hexadeuterium labeled 25-OH vitamin D3 and 25-OH vitamin D3 were purchased from Toronto Research Chemicals (TRC). Double distilled deionized water was prepared by Nanopure (Fisher Scientific and Merck). Acetonitrile and methanol of advanced gradient grade were from Fisher Scientific andMerck. Na2HPO4 and KH2PO4were from Sigma-Aldrich as well asalbumin from bovine serum lyophilized powder(≥ 98 %, agarose gel electrophoresis)and zinc sulphate. KCl was from Fisher.

Stock Solutions, Calibration Standard Solutions,and Control Samples

Stock solutions of approximately 1 mg/ml for both 25-OH vitamin D2 and 25-OHvitamin D3 were prepared in MeOH. Six calibration standards were prepared by diluting both stock calibrator solutions in 6% bovine serum albumin (BSA) in 0.01 M phosphate buffered saline (PBS) to give final concentrations of 4-70 ng/ml for both 25-OH vitamin D2 and 25-OH vitamin D3.A 0.5 mg/ml IS stock solution was prepared in MeOH. A working solution of IS was prepared by diluting the stock solution with (1:1) (MeOH: water) to give 150 ng/ml. Stock and working solutions were stored frozen at -78 ºC. At this temperature these are stable for at least 6 months. The quality control samples (QCs) of low, medium, and high concentrations were prepared from 6% (bovine serum albumin) BSA in 0.01 phosphate buffered saline (PBS). These contain 12, 25, and 55 ng/ml of both 25-OH vitamin D2 and 25-OH vitamin D3.

Sample Preparation

Because it is difficult to obtain biological matrices that are free of vitamin D and its metabolites, a 6% bovine serum albumin (BSA) in a 0.01M phosphate buffered saline (PBS) solution was spiked with standards and extracted. Sample preparation consisted of the addition of 50 µL IS (150 ng/mL) solution to 100 µL of patients' sera, controls, or calibrators in 1.5 ml Eppendorf tubes followed by the addition of 20 µL of 1 Mzinc sulfate and vortex mixed briefly. Then, 0.5 ml

acetonitrile for protein precipitation was added and vortex mixed for 1 min before centrifugation at 10,000 rpm for 10 min. The upper layer was transferred to a glass tube, evaporated under steam nitrogen at 50 ºC and the samples were reconstituted with 100 µL of (50:50) (acetonitrile: deionized water) and vortex mixed.The content of the glass tubes was then transferred to LC-vials which were sealed and then centrifuged at 13,000 rpm for 5 min and 75 µL were injected on the LC-MS/MS using the method of analysis. Total run time was 5 minutes, the analytes of interests eluted at 4:10 minutes and 4:15 minutes for 25-OH vitamin D3 and 25-OH vitamin D2, respectively. Calibrators (n=6) and controls (n=3) were run with every batch (up to 50 patients' sera) at the beginning as well as the end of the working list.

Experimental

LC-MS/MS System

An API 3200 Q-trap mass spectrometer (Applied Biosystems® Inc.) interfaced with an APCI source (Applied Biosystems® Inc.) coupled with an Agilent® 1200 series Thermo stated column compartment (Agilent® Technologies, Switzerland) equipped with a binary pump controlled with a six-port valve (Agilent® 1200 series Technologies), an on-line vacuum Degasser (Agilent® 1200 series technologies), and a 75 µL fixed volume injector (Agilent® 1200). An auto-sampler (Agilent® 1200 series Technologies) was used and controlled by Computer Analyst 1.5 Data Management Software.

A two dimensional LC (liquid chromatography) was conducted on C8 column ACE® (100× 2.1 mm, 5 µm) maintained at 40 °C coupled to a trap column for online phase extraction (Chromsystems® order no.62110) through a six-port valve for optimized sample extraction and controlled by a binary pump. A total of 75 µL of each sample was injected onto the trap column for sample clean up and then to the analytical column. The mobile phase operated at a flow rate of 1 mL/min and consisted of 100% deionized water as solvent A and acetonitrile (9:1) (v/v) in water as solvent B under gradient elution. The gradient program is shown in table 1 and the switching valve in table 2.

Table 1 : Gradient elution program.

Step	Total time (min)	Flow rate (ml/min)	Mobile phase A%	Mobile phase B%
0	0.00	1	100.0	0.0
1	2.00	1	100.0	0.0
2	2.10	1	0.0	100.0
3	3.50	1	0.0	100.0
4	3.60	1	100.0	0.0
5	5.00	1	100.0	0.0

Table 2: Column switching valve installed.

Total time (min)	Valve position
0.00	Right
3.00	Left
4.70	Right

Optimization of the LC-MS/MS Parameters

Tuning parameters were achieved by injecting 10 µg/ml of both 25-OH vitamin D2 and 25-OH vitamin D3 in a solution (1:1) (MeOH: deionized water) into the LC-MS/MS operating in the positive APCI mode using the built-in infusion syringe pump. The following tuning parameters were obtained for 25-OH vitamin D2: DP: 42:00, EP: 6.00, CEP: 20:00, CE: 26:00, and CXP: 4:00.

For 25-OH vitamin D3, the following tuning parameters were DP: 32.00, EP: 10:00, CEP: 23:00, CE: 32:00, and CXP: 3.00. For hexadeuterium 25-OH vitamin D3 (IS), the parameters were: DP: 32:00, EP: 10:00, CEP: 20:59, CE: 32:00, and CXP: 3:00. Curtain gas (CUR) was 15.00, collision activation energy was medium, temperature (TEM) was250°C, GAS1 (heater gas) was40:00, GAS 2 (nebulizer gas) was 0.00, the interface heater wason, and the corona's discharge current was4:00 microamperes. The mobile phase composition was a mixture of (deionized water: (9:1) acetonitrile: deionized water). Detection of 25-OH vitamin D2, 25-OH vitamin D3, and the internal standard was achieved using AP-CI (+) Multiple Reaction Monitoring (MRM) Mode, for 25-OH vitamin D2 at m/z 395.40→269.50, for 25-OH vitamin D3 at m/z 383.3→211, and for hexadeuterium25-OHvitamin D3 at m/z 389.4→211.40. The peak areas were measured and the ratios of the two analytes (25-OH vitamin D2and 25-OH vitamin D3) to the internal standard were calculated by computer software.The relationship between the concentration and peak area ratio was found to be linear within the range of 4 to 70 ng/ml for both 25-OH vitamin D2 and 25-OH vitamin D3. The limit of quantification was 4 ng/ml.

Method Validation

The validation of the analytical method was conducted in compliance with the Food and Drug Administration guidelines.[21]Validation tests included tests for linearity/calibration curve, accuracy, intra-and inter-assay reproducibility, extraction recovery, sample dilution, matrix effect, short term stability, dry extract stability, post preparative stability, and long term and stock solution stability in blank human serum (6% BSA in PBS).

Linearity/Calibration Curve

Linearity was evaluated by measuring three replicates of six dilutions of 25-OH vitamin D3 and 25-OH vitamin D2 PBS containing 6% BSA in the range of 4-70 ng/ml. The responses were considered to be linear if the correlation coefficient (r^2) was greater than 0.99, calculated by least-squares linear regression with a weighing factor equal to 1/X using Analyst 1.5 software.

Detection Limits

The lower limit of detection (LOD) and the limit of quantification (LOQ) were determined by diluting a low standard solution that contained 25-OH vitamin D2 and 25-OH vitamin D3 and a low serum control pool for both analytes.LOD and LOQ were defined as the injected amount that produced a signal to noise ratio of 3 and 10, respectively.

Intra- and Inter-assay Imprecision

Intra-assay and inter-assay imprecision expressed as coefficient of variance (CV %) weretested by analysis of three in house freshly prepared control serum samples with low, medium, and high concentrations of 25-OH vitamin D2 and 25-OH vitamin D3. Each level was assayed six times within a run over a total of 3 days.

Extraction Recovery, Matrix Effects Stock Solution(s) Stability

The extraction efficiency was evaluated using serum samples of the calibration curve at concentrations of 12, 35, and 55ng/ml. The reference samples of extraction efficiency were prepared by adding 25-OH vitamin D2/D3 and IS working solutions to blank human serum. The recovery percent was determined by comparing the absolute peak area of extracted serum samples to absolute peak areas of an equivalent aqueous standard. To investigate the matrix effect, the reference solutions of extraction efficiency were compared to reference solutions without matrix. Stock solution stabilities of 25-OHvitamin D2 and 25-OH vitamin D3 and IS were demonstrated following storage at – 78 ˚C.

Dilution Integrity

Dilution integrity was investigated by a threefold dilution factor by spiking 100 µl from point 6 serial solution containing 1600 ng/ml 25-OH vitamin (D2/D3) into 900 µl blank bovine serum and then vortex mixed well to give a final concentration of 140 ng/ml.Then the 100 µl volume was taken from the previous concentration (140 ng/ml) and added to 200 µl human serum.

Application to Clinical Study

This method was applied to the analysis of serum samples obtained from 182 male and female healthy subjects between 18 and 79 years. The subjects signed an

informed consent and the study protocol was approved by the Ethics Committee of Jordan University Hospital (JUH). Venous blood samples (5 mL) were collected from each subject who came to JUH outpatient laboratories between the 28th of February andthe 15th of May 2011 to test vitamin D deficiency. The participants were not required to fast for blood collection, which was performed by routine venipuncture. Samples were collected in plain gel tubes to separate serum from red cells, allowed to clot for 30–45 min at room temperature, and centrifuged at 4000 rpm for 5 minutes.Thenthe serum layer was transferred to glass tubes, protected from light, and stored at -78ºC until analysis. The same samples were analyzed by an IDS EIA kit (a diagnostic vitamin D assay method approved by the FDA in 2002 for vitamin D deficiency) for inter-method comparative purposes in terms of agreement, correlation, and impact of assay variation on the diagnostic categorization of vitamin D status.

Statistical Analysis

Data was statistically analyzed using Microsoft EXCEL (2007) software.The chi square test was used to test the association between categorical variables, and thetwo tailed P value < 0.05 was considered statistically significant.

RESULTS

The 25-OH vitamin D2 and 25-OH vitamin D3 were resolved chromatographically (figure 1/a and 1/b; respectively). The retention time for 25-OH vitamin D2 was 4.15 minutesand for 25-OH vitamin D3 was 4.10 minutes with a reproducible, good peak shape. Because LC-MS/MS is an inherently specific method, the specificity of the mass selection and fragmentation (m/z transitions) gave the necessary compound specificity and selectivity for the molecules of interest.

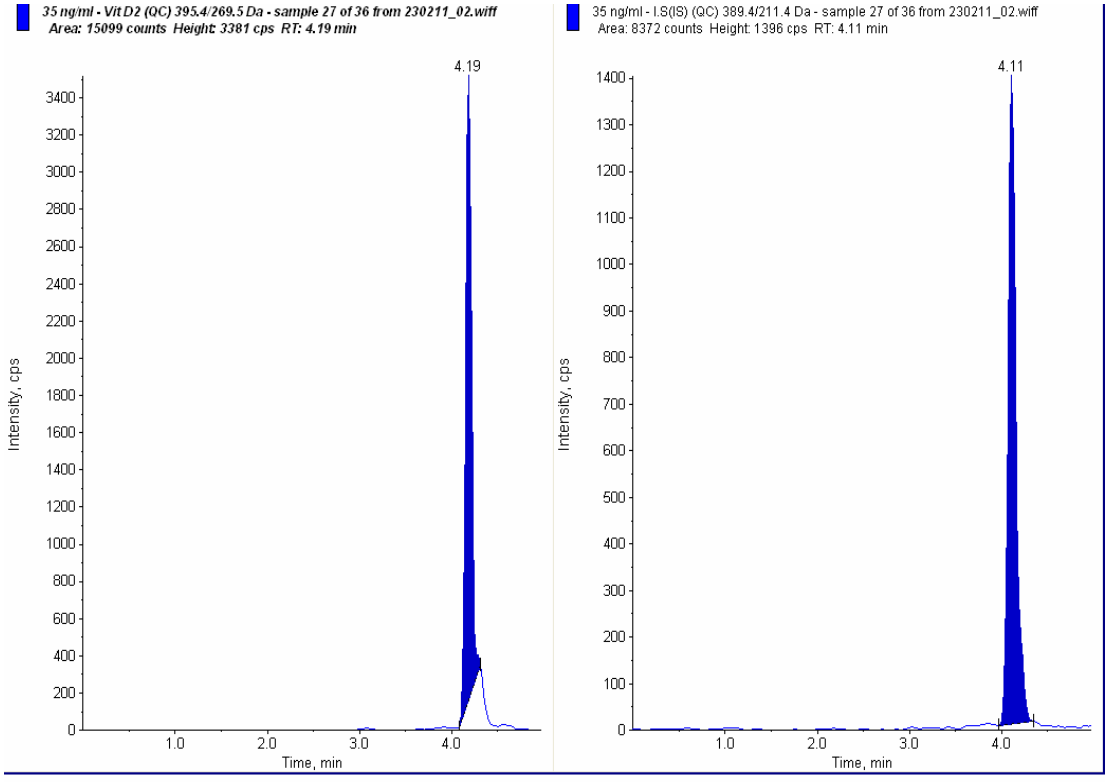

Fig. (1/a). Chromatogram of a serum control sample containing 35 ng/ml 25-OH vitamin D2.Registrations of peaks are shown with the *m/z* transitions:395.4/269.5 for 25-OH vitamin D2 (left) and 389.4/211.4 for the IS (right).

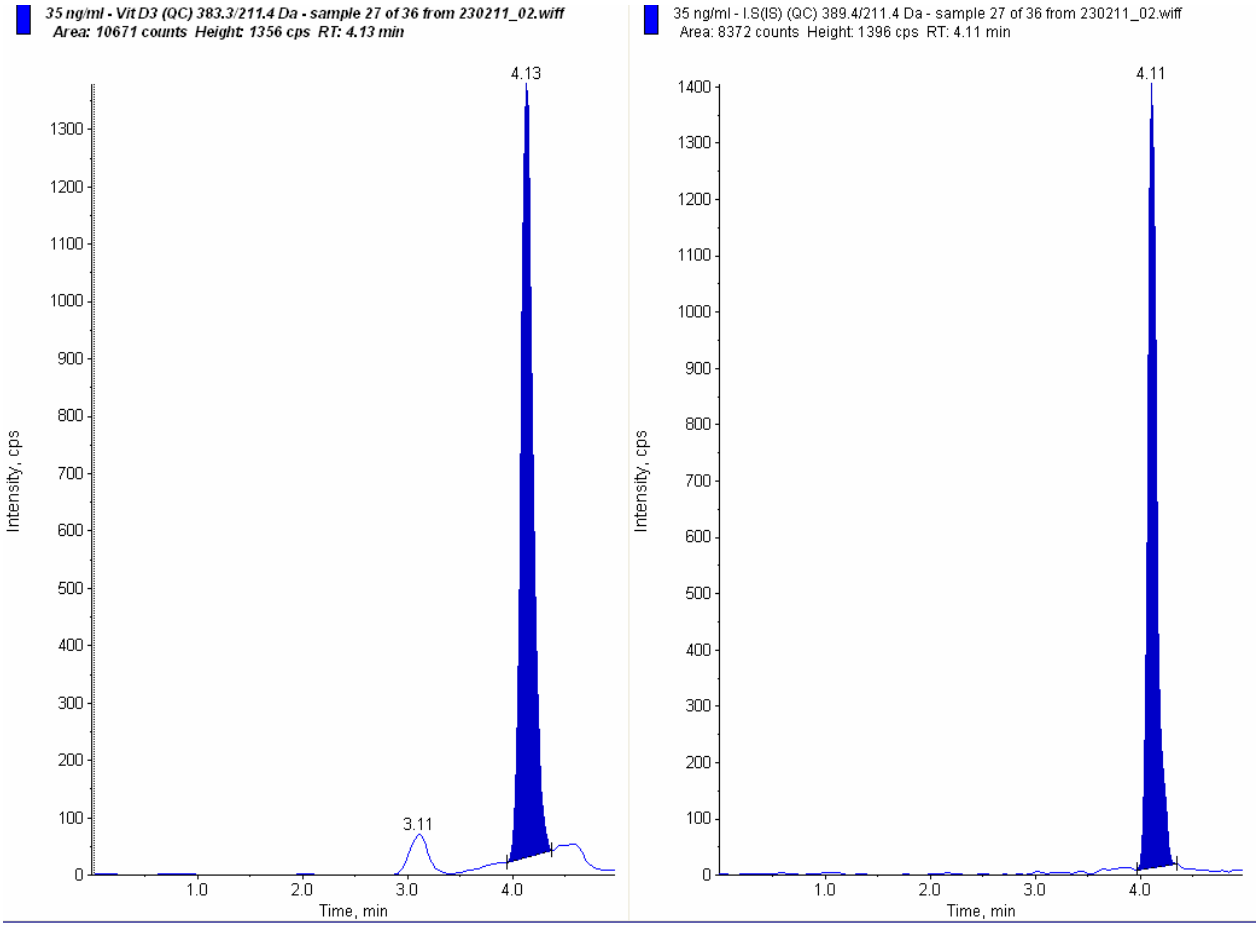

Fig. (1/b). Chromatogram of a serum control sample containing 35 ng/ml 25-OH vitamin D3.Registrations of peaks are shown with the *m/z* transitions: 383.3/211.4 for 25-OH vitamin D2 (left) and 389.4/211.4 for the IS (right).

Validation Parameters

The calibration curve for 25-OH vitamin D2 and 25-OH vitamin D3 was established in the range of 4-70 ng/ml and exhibited good linearity with the correlation coefficient of 0.9947. The intra- and inter-assay reproducibility of the assay were investigated in terms of accuracy and precision from three controls' concentrations of 12, 35, and 55 ng/ml. Intra-day accuracy for 25-OH vitamin D2 and 25-OH vitamin D3 was 85.49% to 97.67% and 89.86% to 112.03%, respectively. Inter-day accuracy for 25-OH vitamin D2 and 25-OH vitamin D3 was 85.49% to 114% and 85.42% to 112.03 %, respectively. The intra-day CV% for 25-OH vitamin D2 and 25-OH vitamin D3 was 4.35% to 9.98% and 3.93% to 7.68 %, respectively. The inter-day CV% for 25-OH vitamin D2 and 25-OH vitamin D3 was 4.07% to 9.98% and 3.36% to 7.68%, respectively. We also studied the stock solution stability in MeOH and long term stability for 6 months at -78˚C. The effects of the freeze thaw cycles were studied and our results confirm that the analytes of interest are stable over three cycles at -78 °C. Stability at room temperature and autosampler stability at 6 ˚C were confirmed to be valid for 4 hours and 24 hours, respectively. Analyte stability in serum extracts was also tested. Extracts held for 2 hours at room temperature and at -20 °C for two days showed no significant difference as compared with fresh extracts. The storage of serum samples for 3 months at -78˚C did

notaffect the assay results of 25-OH vitamin D2 and 25-OH vitamin D3. The mean values of matrix effect for 25-OH vitamin D2 and 25-OH vitamin D3 in serum samples at the concentrations of 12, 35, and 55 ng/ml were 107.46% to 119.23% and 109.47% to 125.58%, respectively. For 25-OH vitamin D3,the mean values of the matrix effects were that the IS was 122.12%. The mean recovery efficiencies weredetermined by spiking human serum with standard solutions (acetonitrile: deionized water) containing both 25 –OH vitamin D2 and 25-OH vitamin D3 at 3 concentrations: QC low, QC medium, and QC high (12 ng/ml, 35 ng/ml, and 55 ng/ml, respectively). Recovery for QClow was 39.09% for 25-OH vitamin D2 and 30.44% for 25-OH vitamin D3, for QC medium recovery was 63.77% for 25-OH vitamin D2 and 58.66% for 25-OH vitamin D3, for QC high the recovery was 64.31% for 25-OH vitamin D2 and 53.35% for 25-OH vitamin D3.

Method Comparison

The serum total of 25-OH vitamin D was measured in the 182 specimens using the IDS EIA method. Our validated LC-MS/MS method is capable of resolving 25-OH vitamin D2 and 25-OH vitamin D3 when applied to the same specimens, and the two forms were added together to calculate the total 25-OH vitamin D. The mean ± (std) LC-MS/MS for this set of 182 serum specimens was 13.5± 7.8 and the range was 4 to 68.94 ng/ml, the values 4 ng/ml werebelow the limit of quantification (BLQ). The IDS EIA mean ± (std) for this set was 10.58 ± 7.8 ng/ml. Of the 182 serum specimens, the maximum value measured by LC-MS/MS was 68.94 ng/ml whereas the same value was measured by IDS EIA kit as 50.16 ng/ml. The lowest value measured by LC-MS/MS was 4 ng/ml and 2 ng/ml for IDS EIA which is considered as the LLOQ. Descriptive statistics are represented in table 3 of the 182 specimens in which 25-OH vitamin D results were compared, and none had detectable 25-OH vitamin D2 at baseline levels.

Table 3: Descriptive Statistics of 182 sample(s) analyzed at basal levels.

Method used to Measure Total 25-OH vitaminD (ng/ml)	No. of samples	Working range	Min. value of 25-OH vitamin D measured	Max. value of 25-OH vitamin D measured	Mean Value of 25-OH vitamin D		
			Min. value	Max. value	Mean	SE	STD
LC-MS/MS	182	(4 to 70)	4 (LLOQ)*	68.94	13.46	0.87	11.72
IDS EIA	182	(2 to 144)	2 (LLOQ)*	50.16	10.59	0.58	7.83
Total number of samples	364						

* LLOQ: lowest limit of quantification for the 182 samples analyzed by IDS EIA and LC-MS/MS,

Inter-Method Agreement

Inter-Method agreement was assessed using the below illustrated scheme (fig. 3). The Y axis represents the differences in 25-OH vitamin D measurements between the two methods (total 25-OH vitamin D measured value by IDS EIA - total 25-OH vitamin D measured value by LC-MS/MS) while the X axis represents the averages of 25-OH vitamin D measured by the two methods. Figure 3 shows the agreement between the newly validated LC-MS/MS and the IDS EIA method(s). The figure demonstrates that there is a good agreement between the two methods (IDS EIA versus LC-MS/MS) as most average 25-OH vitamin D values are clustered around the (mean±1 std).Thisimplies that the probability that the average value of 25-OH vitamin D measured by both methods resides between 2.45 - 22.05 ng/ml is 68%. The average 25-OH vitamin D measurement by the two methods is 12.25± 9.8 ng/ml with 95% CI [11.25-13.25].

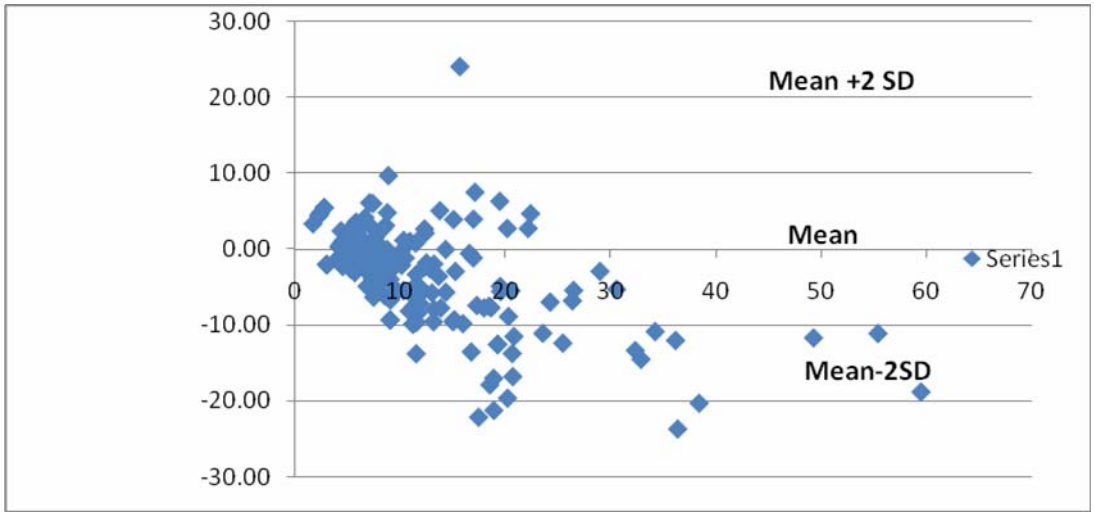

Fig. 2. Agreement between (IDS EIA vs. LC-MS/MS) Plot of Difference (Y axis) against average (X axis) for 25 (OH) D concentrations(s) Correlation between was calculating (Person correlation r^2) using two tailed test. The r^2 of the

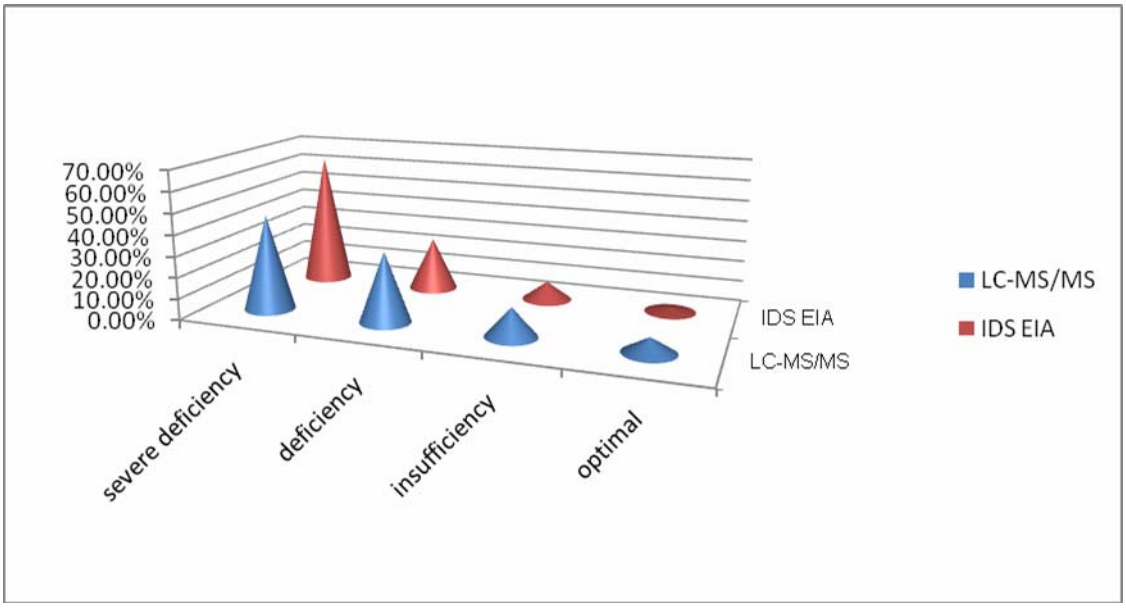

Fig. 3. Vitamin D status assessment of 182 serum specimens by LC-MS/MS vs. IDS EIA methods.

Inter-Method Correlation

Correlation between the two methods was calculated(Pearson correlation r^2) using the two tailed test. The r^2 of the two methods was found to be 0.82 with a P value =0.000, which implies that the two methods are of significant reasonable correlation at basal levels. The results for the correlation between IDS EIA and LC-MS/MS methods are shown in table 4.

Table 4: Correlation between LC-MS/MS and IDS EIA methods

Correlations			
Method (s)		**LC-MS/MS**	**IDS EIA**
LC-MS/MS	Pearson Correlation	1	. 823**
	Sig. (2-tailed)		.000
	Number	182	182
IDS EIA	Pearson Correlation	.823**	1
	Sig. (2-tailed)	.000	
	Number	182	182

** Correlation is significant at the 0.01 level (2-tailed).

** Correlation is 0.82 with P-value 0.000.

Comparison of Vitamin D Status Assessment Using the Two Methodologies

Clinical vitamin D status has been defined by serum 25-OH vitamin D measurements as follows: An optimal vitamin D status of25-OH vitamin D levels is more than 30 ng/ml.[1]A level of less than 30 ng/ml corresponds to low vitamin D status. See table 5.

Table 5 : Vitamin D status definition

Vitamin D status	Total 25-OH vitamin D ng /ml
Optimal	≥ 30
Vitamin D insufficiency	20-29.99
Vitamin D deficiency	10-19.99
Severe vitamin D deficiency	< 10 ng/ml

Diagnostic Categorization of Vitamin D Status by the Two Methods

When applying the cutoff of 20 ng/ml for defining deficient versus normal results, as proposed by Holick,[1]the LC-MS/MS and IDS EIA classifies 79.1% and 89.0%, respectively as vitamin D deficient (< 20 ng/ml), (P value = 0.02). There were considerable differences between the methods in the proportion of participants classified as "vitamin D severely deficient ", using a 10 ng/ml cut off , 63.7% of our subjects were classified as "severe vitamin D deficiency" using the IDS EIA method compared to 45.6% using the validated LC-MS/MS. Thismay indicate that the IDS EIA clearly overestimated the number of individuals classified with "severe vitamin D deficiency" (P value = 0.0005), whereas there was no significant difference between the two methods regarding the proportion of subjects classified as deficient (25-OH vitamin D > 10 and < 20 ng/ml) as it was 35.5% by LC-MS/MS and 25.3% by IDS EIA (P value = 0.08) as well as subjects classified as "relatively insufficient" as the proportion was 33.5% by LC-MS/MS and 25.3% by IDS EIA with P value = 0.13. On the other hand, only 2.2%, i.e., 4 subjects wereclassified having optimal vitamin D status by IDS EIA method in comparison with 7.1%, i.e., 13 subjects by LC-MS/MS (P value = 0.03). However, when comparing the whole sample measured in a comparative manner by the two methods, we recognized a significant statistical significance (P value = 0.003). In addition, significant difference in diagnostic categorization is observed between the two methods, when applying the 20 ng/ml cut off.The IDS EIA classifies 89.1% of the subjects as deficient (25-OH vitamin D between 0-20 ng/ml), whereas the LC-MS/MS

classifies 79.1% as vitamin D deficient (P = 0.02). Results are represented below in tables 6, 7, 8, 9 and 10

Table 6: Vitamin D status assessment by IDS EIA of 182 subjects' specimens

Vitamin D status*	Number	Percent %	Cumulative %
Severe deficiency	116	63.7	63.7
Deficiency	46	25.3	89.0
Insufficiency	16	8.8	97.8
Optimal	4	2.2	100
Total	182	100	

** Severe deficiency = < 10, Deficiency = (10 - 19.99), Insufficiency = (20- 29.99), Optimal ≥30*

Table 7: Vitamin D status assessment by *LC-MS/MS* of 182 subjects' specimens

Vitamin D status*	Number	Percent %	Cumulative %
Severe deficiency	83	45.6	45.6
Deficiency	61	33.5	79.1
Insufficiency	25	13.7	92.9
Optimal	13	7.1	100
Total	182	100	

***** *Severe deficiency = < 10, Deficiency = (10 - 19.99), Insufficiency = (20- 29.99), Optimal ≥30*

Table 8: Vitamin D status assessment of 182 serum specimens by LC-MS/MS vs. IDS EIA method(s*)*

Method	Severe deficiency	Deficiency	Relative insufficiency	Optimal
LC-MS/MS	45.6%	33.5%	13.7 %	7.1%
IDS EIA	63.7%	25.3%	8.8%	2.2%
Chi test	P=0.0005*(S)*	P=0.08 *(NS)*	P=0.13 *(NS)*	P=0.03 *(S)*

**Severe deficiency ≤ 10, Deficiency = (10-19.99), Insufficiency= (20- 29.99), Optimal ≥ 30*

S: significant; NS: not significant

Table 9: The definition of vitamin D deficiency by using 20 ng/ml cut off.

Method	deficiency	Relative insufficiency	Optimal
LC-MS/MS	79.1%	13.7 %	7.1%
IDS EIA	89.0%	8.8%	2.2%
Chi test	*P=0.02 (S)*	*P=0.13 (NS)*	*P=0.03 (S)*

*****Deficiency (0-19.99), Insufficiency = (20- 29.99), Optimal ≥30*

Table 10: Vitamin D status assessment of 182 serum specimens By LC-MS/MS *vs.* IDS EIA

Vitamin D Status	Assessment Method	
	LC-MS/MS Number (%)	IDS EIA Number (%)
Severe Deficiency	83(45.6)	116(63.7)
Deficiency	61(33.5)	46(25.3)
Relative Insufficiency	25(13.7)	16(8.8)
Optimal	13(7.1)	4(2.2)
Total (100%)	182(100)	182(100)

** Severe deficiency ≤ 10, Deficiency = (10 - 19.99), Insufficiency = (20- 29.99), Optimal ≥ 30*

For table (10) as a whole differences are significant, Chi test P value=0.00

For the assessment of concordant vitamin D status categorization by both the IDS EIA and LC-MS/MS methods, we compared the duplicated 25-OH vitamin D measurements for each subject by each method (IDS EIA vs.LC-MS/MS) in a head to head manner. A total 105 of the 182 specimens (57.68%) measurements by the two methods were concordant in the diagnostic categorization of vitamin D status. For example, the two methods classify (76/182), i.e., 41.76% of subject to have vitamin D severe deficiency, whereas (20/182) constituting 10.89% were categorized as vitamin D deficiency. Only 4 of 182 (2.19%) and (5 of 182) (2.75%) were classified as optimal and relatively insufficient. Results are summarized in table 11.

Table 11: Concordant categorization of vitamin D status by LC-MS/MS & IDS EIA

Subjects with concordant categorization of vitamin D Status by both methods (N=182,100%)		
Category*	Number	%
Severe deficiency	76	41.76
Deficient	20	10.98
Relatively insufficient	5	2.75
Optimal	4	2.19
Total	105	57.68

***** Severe deficiency ≤ 10, Deficiency = (10 - 19.99), Insufficiency = (20- 29.99), Optimal ≥ 30

However, when we apply the 20 ng/ml cut off to define vitamin D deficiency, 81.9% of subjects were categorized concordantly by the two methods as 140/182 of the subjects were classified as vitamin D deficient using this cut off. This can be shown in the table 12.

Table 12 : Concordant categorization of vitamin D status by LC-MS/MS & IDS EIA by using 20 ng/ml cut off.

Subjects with concordant categorization of vitamin D Status by both methods (N=182,100%)		
Category*	**Number**	**%**
Severe deficiency	76	41.76
Deficient	20	10.98
Relatively insufficient	5	2.75
Optimal	4	2.19
Total	105	57.68

***Deficiency = (0 - 19.99), Insufficiency = (20- 29.99), Optimal ≥30*

The LC-MS/MS gave higher values in 122/182, comprising 67% of total measurements, than the corresponding values measured by IDS EIA, whereas the IDS EIA gave higher values in 57/182 measurements, constituting 31.30% of the total specimens. Results are summarized in table 13.

Table 13: Variation of measurements by the two methods.

Variations of measurements of LC-MS/MS vs.IDS EIA	Number	%
1. measurements that are *higher* by LC-MS/MS than EIA =	122	67.00%
2. measurements that are *higher* by EIA than LC-MS/MS =	57	31.30%

The mean bias was calculated by the following formula:

Bias = value of 25-OH D measured by IDS EIA- value of 25-OH D measured by LC-MS/MS

Value of 25-OH vitamin D measured by LC-MS/MS

% mean bias = the mean of all bias' values *100%

The mean % bias of the 182 specimens was - 34.28 ± (69.39), the 95% confidence interval of the mean of bias% - 34.28±10.15, i.e., 95% CI [- 24.13 to - 44.43].

DISCUSSION

In this study, we describe a newly validated ID-LC-MS/MS for the simultaneous determination of 25-OH vitamin D2 and 25-OH vitamin D3 in human serum. In addition, we assessed the vitamin D status in 182 subjects using two contemporary vitamin D assay methods, i.e., IDS EIA and the newly validated LC-MS/MS in a comparative manner, to observe the impact of the analytical method used on the clinical categorization of vitamin D status, i.e., severe deficiency, deficiency , insufficiency and optimal.

Method Development

Several LC-MS/MS methods have recently been described for the determination of 25-OH vitamin D[19,23-25,49-55]with an inter-laboratory imprecision similar to most immunoassays as demonstrated by data derived from laboratories participating in DEQAS.[56]

In the present study, we describe a method involving minimal sample preparation for the determination of serum 25-OH vitamin D2 and 25-OH vitamin D3 using LC-MS/MS. The analytes were extracted from the precipitated serum supernatant using an online solid phase extraction procedure started from the work of Vogeser et al., with some modifications.[19]Washing was followed by elution of the compounds with acetonitrile/deionized water (90:10, v/v) and subsequent introduction into the mass spectrometer by using a 6-port high pressure switching valve. Under these conditions, the retention time for 25-OH vitamin D2and 25-OH vitamin D3, and internal standard was between 4 and 4.15 minutes while the total run time was 5 minutes. The two 25-OH vitamins D3 co-eluted and were detected as

different m/z transitions. As the method required minimal sample preparation work and a short run time. Up to 70 subjects were measured per day. The extraction efficiency was fair enough to attain a Limit of Quantification (LLOQ) of 4 ng/ml with valid accuracy and precision. The LLOQ is defined as 10:1 signal to noise and the limit of detection as 3:1 signal to noise.[22]We investigated the choice of the calibrator matrix. Ethanol and methanol are often used to prepare calibrators in LC-MS/MS methods for measurement of 25-OH vitamin D2 and 25-OH vitamin D3.[19,23-25]We decided to calibrate our newly developed LC-MS/MS assay on dilutions of pure standards of 25-OH vitamin D2 and 25-OH vitamin D3 in phosphate buffered saline containing 6% albumin. Over the concentration range from4 to 70 ng/ml, positive ion AP-CI produced a linear response, and the correlations between response and concentrations of 25-OH vitamin D2 and 25-OH vitamin D3 were excellent. Over 5 days (table 14), the following characteristics for a 25-OH vitamin D3 calibration curve were recorded (peak area ratio/concentration). The slope was 0.0382 ± 0.0042 (mean ± std), the interceptwas 0.0412 ± 0.0266, and the correlation coefficient was0.9972 ± 0.0022. Similarly, 25-OH vitamin D2 calibration curve characteristics were slope at0.0426±0.0071, intercept at- 0.001 ± 0.0161, and correlation coefficient at0.9982 ± 0.0011.

Table 14: (Slope, intercept, correlation characteristics of the calibration curve during Routine analysis for (25 OH-vitamin D2 and 25-OH vitamin D3).

Days of analysis	25 (OH) Vitamin D3			25 (OH) vitamin D2		
	slope	intercept	Correlation R	slope	intercept	Correlation R
Day 1	0.036	0.033	0.996	0.052	-0.010	0.997
Day 2	0.039	0.057	0.994	0.044	0.011	0.998
Day 3	0.045	0.071	0.999	0.048	0.007	1.000
Day 4	0.034	0.001	0.999	0.036	0.012	0.998
Day 5	0.037	0.044	0.998	0.033	-0.025	0.998
Mean	**0.0382**	**0.0412**	**0.9972**	**0.0426**	**- 0.001**	**0.9982**
±std	**0.0042**	**0.0266**	**0.0022**	**0.0071**	**0.0161**	**0.0011**

Vitamin D Status As Assessed by LC-MS/MS Compared to IDS EIA

We observed high variability between the LC-MS/MS and IDS EIA assay results, emphasizing that a gold standard for the 25-hydroxyvitamin D assay wasneeded to establish consensus on the required level for sufficient vitamin D status. Previous studies support our findings, reporting variability between different 25-OH vitamin D assays, as well as inter-laboratory differences using the same assay.[16,18,26-29,47-48]Binkley et al. reported 18% and 90% insufficiency proportions in two similar populations (n= 20 and 42, respectively) using two RIA assays.[29]Rollins found that in one lab, 60% of the results from an immunoassay method indicated insufficiency, compared to only 30% by LC-MS/MS. Another laboratory had similar discrepancies for sample classification where80% of samples had levels below 32 ng/ml by immunoassay but only 46% of samples by LC-MS/MS.[29]According to Glendenning et al. IDS and DiaSorin-RIA as well as a Nichols Advantage automated protein binding assay detected less than 50% of the changes in 25-hydroxyvitamin D2 detected by HPLC. Lips et al., analyzed samples from a selected population of vitamin D supplement users (n =104) with three different methods. The mean 25-OH vitamin D level was 80% higher when using a competitive protein binding assay as compared with HPLC while intermediate values were found with an RIA assay. The accuracy of the methods was not possible to evaluate. Janssen et al., found that chromatographic methods, and to a lesser

extent the protein binding assay, showed cross-reactivity with 3-epi-25(OH)D3. Agreement of 25-OH vitamin D assays to ID-LC-MS/MS in sorting patients into distinct 25-OHvitamin D categories varied between 53% and 88%.[57]

It is well appreciated that LC-MS/MS can resolve 25-hydroxyvitamin D2 and D3 metabolites whereas the IDS EIA method measures total 25-hydroxyvitamin D levels and other hydroxylated metabolites. In some countries, including the USA, vitamin D2 has been the only form of vitamin D available for prescription, even though both vitamin D2 and vitamin D3 are used as non-prescribed supplements, while in Europe vitamin D3 dominates.[6]LC-MS/MS could therefore have an advantage when evaluating the effect of supplementation with a vitamin D2 form. Nevertheless, none of the samples analyzed at basal levels, for comparative purposes, in our study contained 25-OH vitamin D2concentrations. Thus, in countries where vitamin D2 is not routinely used it may not be of major clinical importance that some assays underestimate D2 or cannot resolve the two hydroxyl metabolites.

Some controversy exists regarding the best method for measuring 25-OH vitamin D levels. Recently, LC-MS/MS is considered the gold standard for the measurement of 25-OH vitamin D levels. Hee-Won Moon et al. showed that 25-OH vitamin D values by LC-MS/MS are close to NIST assigned target values (mean difference + 1.9%). This finding supports the lack of agreement among methods with only the LC-MS/MS assay providing results close to NIST values.[47]

The advantages LC-MS/MS as an analytical method over IDS EIA include the incorporation of a deuterated internal standard that corrects for procedural losses and matrix effects, inherent high specificity, reproducibility, and the incorporation of a standard curve in every assay.However, the need of expertise to operate the system and insufficient throughput limit its employment in routine laboratory diagnostics. There have been numerous reports of discrepancies between the results of LC-MS/MS and immunoassays, with LC-MS/MS reporting serum values up to 40% higher than those reported using an immunoassay.[30]In addition, chromatography and MS/MS conditions cannot distinguish isobaric metabolites such as 3-epi-25 (OH) D3 and interferences can also occur if nonspecific transitions are used.Laboratories should always subscribe to an external quality assurance for 25(OH)D assays.[58]We noted that the IDS EIA gave lower measurements at lower concentrations; this may be concordant with the DEQAS returns of the assay in 2009. These returns constitute 19% and indicate moderately low recovery for this assay (56% for 25-OH vitamin D2 and 79% 25-OH vitaminD3) from exogenously prepared pools that contain low concentration of the two hydroxyl metabolites. (Level 2 SRM).[16]

The newly validated LC-MS/MS used in this study can be considered as a valid measurement method from analytical aspects. Further improvements could be made to ensure analytical reliability and certainty of the newly validated LC-MS/MS. Our results may be considered analytically accurate yet they may still lack clinical accuracy. In this study, we compared the newly validated LC-MS/MS with the IDS EIA (a kit approved for vitamin D status assessment by the FDA since 2002) used in the Jordan University Hospital.

However, For LC-MS/MS labs to have clinical relevance, our results must agree with the DiaSorin RIA which is used in almost every major vitamin D study and was employed to generate currently accepted ranges of serum 25-OH vitamin D3 for optimal health outcomes.[7,13,32-44]There is no well-defined and international accepted definition of optimal serum 25-hydroxyvitamin D value for bone and nutritional health,[1,44,45]but a common definition of vitamin D deficiency is a level of 20 ng/ml although many authors suggest that clinicians should aim at higher levels.[44-46]Overall, our results indicate that there is a good agreement and reasonable correlation (r^2=0.82) between the two methods in vitamin D status classification.

The study by Roth et al., reported a significant bias for the EIA relative to LC-MS/MS (-1%).[15]Similarly, we found a negative bias of the IDS EIA method relative to LC-MS/MS (-34.28), 95% CI [- 44.43 to - 24.13]. When

applying a 20 ng/ml cut off, there is a significant difference in the proportion of subjects classified as vitamin D deficient (P value=0.02). In the 182 specimens compared,89.0% wereclassified as deficient by IDS EIA compared to 79.1% by LC-MS/MS. This implies that 10% of the subjects classified as deficient by the IDS EIA method will be designated as "another category" which may confound clinical decision(s). In addition, the two methods differ significantly in the proportion of subjects designated as "severely deficient" (P value=0.0005) and "optimal" (P value=0.03) vitamin D status. The LC-MS/MS classifies 45.6% of subjects to be severely deficient compared to 63.7% by the IDS EIA method. Concordant vitamin D status categorization differs by the cut off applied.For example, when we apply 20 ng/ml cut off, concordant categorization is achieved in 81.9% of subjects. Whereas upon classifying subjects with severe deficiency (25-OH vitamin D3 < 10 ng/ml), the concordant categorization is achieved in only 41.76% (76/182). Based on this variability, subjects assessed by different assay techniques will continue to be variably identified as having low vitamin D status depending on the laboratory which made the 25-OH vitamin D3 measurement. Our data indicate that widespread clinical application of a single target value, e.g., 30 ng/ml is not appropriate at this time due to marked inter-laboratory variability. Hence, practicing clinicians do not have a reliable tool to detect hypovitaminosis D in their patients.

The recent release of 972 SRM provided by NIST is expected to improve vitamin D testing between assay comparability and reduce inter laboratory variation and contribute in 25-OH vitamin D3 methods standardization which will enable the medical community to define a threshold for optimal vitamin D status using accurate, reproducible assays. These assays must subsequently be internationally standardized and made available to practicing clinicians.

CONCLUSION

In this study we compared a contemporary vitamin D assay, running in routine laboratory setting with IDS EIA, with a newly validated LC-MS/MS. The LC-MS/MS described here provides a rapid, cost-effective, accurate method for the simultaneous determination of 25-OH vitamin D2 and 25-OH vitamin D3 in human serum. In addition, the diagnostic categorization of vitamin D status may vary considerably and depends on the type of assay used. Thus, we conclude that a single threshold value to define an optimal 25-hydroxyvitamin D level is presently impossible to determine because of the differences in assay results. In this regard, the recent release of SRM 962 is expected to improve vitamin D testing, with betterassay comparability and reduced inter-laboratory variability, which will contribute to 25-OH vitamin D3 method standardization. The goal is to have accurate 25-OH vitamin D3 values and to have a precision for 25-OH vitamin D3 testing with a CV%< 1% for optimal patient care.

ACKNOWLEDGMENTS

We thank Dr.Saed Jaradat (Director of Princess Haya Center for Biotechnology/Jordan University of Science and Technology,Irbid,Jordan), Mr.Mamoon Al-Abed (Senior Analyst at Princess Haya Center for Biotechnology) and Mr.Munther Milhem (Technical Manager at Jordan Center for Pharmaceutical Research,Amman,Jordan) for their logistics and technical support and their contribution to the fulfillment of this study.

Abbreviations

AP-CI: Atmospheric Pressure Chemical Ionization
BSA: Bovine Serum Albumin
CE: Collision Energy
CEP: Cell Entrance Potential
CUR: Curtain Gas, rpm: round per minute
CV: Coefficient of variation
CXP: Cell Exit Potential
DEQAS: Vitamin D External Quality assessment scheme
DP: Declustering Potential
EP: Entrance Potential
HPLC: High Performance Liquid Chromatography
IDS EIA: immunodiagnostic systems Enzyme

Linked Immunoassay
IS: Internal Standard
LC: Liquid Chromatography
MeOH: Methanol
MRM: Multiple Reaction Monitoring
MS/MS: tandem mass spectrometry
NIST: National Institute of Standards and Technology
PBS: Phosphate Buffered Saline
std: standard deviation

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تحديد فيتامين د2 ود3 (هيدروكسي 25) في مصل الدم البشري باستخدام تقنية الكراماتغرافيا السائلة مقرونة بقياس الطيف الكتلي (ID-LEMSTMS)

سمر ملحم[1]، خالد اعيده[2]، كمال الحديدي[3]، فراس العلعالي[4]

[1] كلية الصيدلة، الجامعة الأردنية

[2] كلية الصيدلة، الجامعة الأردنية

[3] كلية الطب، الجامعة الأردنية

[4] كلية الصيدلة، جامعة العلوم والتكنولوجيا

ملخـص

تم استخدام تقنية الكرماتغرافيا السائلة مقرونةً بقياس الطيف الكتلي (ID-LC-MS/MS) لقياس نسبة 25 هيدروكسي فيتامين د2 و 25 هيدروكسي فيتامين د3 في مصل الدم البشري .أُضيف سداسي الديتريوم 25 هيدروكسي فيتامين د3 كمادة معيارية داخلية لأنظمة التدريج (كاليبريتور) و التحكم (كونترول) - المحضرة في زلال مصل بقري نسبته 6% وملح الفوسفات كذالك أضيف إلى عينات أمصال المرضى. تمت عملية الفصل الكرمتغرافيبتنقية أولية عن طريق عمود فخ (trap column) يليه الفصل الكروماتوغرافي على عمود C-8 باستخدام تدرج مائي/ الأسيتونيتريل. وقد أجري الكشف عن النواتج الكيميائية بواسطة نظام التأين االكيميائي ذي الضغط الجوي (AP-CI) في رصد ردود الفعل متعددة (MRM) الواسطة. وكانت طريقة الرصد ذات نمط خطي يتراوح ما بين 4 إلى 70 نانوغرام / مليلتر. بلغت النسبة المئوية لمعامل التباين (CV%) خلال اليوم الواحد و خلال عدة أيام ≥ 10% لكل من 25-هيدروكسي فيتامين د2 و 25-هيدروكسي فيتامين د3.تراوح الناتج المسترد ما بين 39.09% إلى 64.31% ل 25-هيدروكسي فيتامين د2 و 30.44% إلى 58.66% ل25 -هيدروكسي فيتامين د3 بينما تراوح الناتج المسترد لسداسي الديتيريوم 25-هدروكسي فيتامين د3 بين 44.11% إلى 67.5% . تمت مقارنة طريقة الكرمتغرافيا السائلة المقرونة بمقياس الطيف الكتلي المستحدثة بتقنية الأنزيم المناعي من شركة Immunodiagnostic systems(IDS)من حيث التوافق والارتباط وتأثير اختلاف طريقة الفحص على التصنيف التشخيصي لحالة فيتامين د بواسطة قياس نسبته في عينات 182 من دم المرضى بطريقة مقارنة؛ كانت نسبة التحيز (Bias %)لتقنية الإنزيم المناعي مقارنةً بطريقة الكرماتغرافيا السائلة10.15 ± 34.28%- بفترة ثقة احتمالية 95 % [-24.13 إلى -44.43] وجد أن هنالك توافقا جيدا مع ارتباط معقول (r^2 = 0.82) , (P value= 0.000) كان التصنيف التشخيصي بين الطريقتين متغيراً، ويرتكز بشكلٍ أساسي على نوع الطريقة المستخدمة و القيم الفاصلة المتبعة لتعريف النقص في فيتامين د لذالك فإن توحيد طرق فحص فيتامين د بطرق معيارية شاملة ضروري جداً من أجل التقييم السريري السليم. تُعتبر طريقة الكرمتغرافيا السائلة المستحدثة متينة من الناحية التحليلية وموثوقا بها مخبرياً لقياس كل من 25-هيدروكسي فيتامين د2 و 25-هيدروكسي فيتامين د3 في مصل الدم البشري.

الكلمـات الدالـة: فيتامين د2 ود3 (هيدروكسي 25)، الطيف الكتلي، مقارنة طريقة، التصنيف التشخيصي لحالة فيتامين د.

تاريخ استلام البحث 2012/10/31 وتاريخ قبوله للنشر 2013/5/6.