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Original Article

Using gas chromatography and mass spectrometry to determine 25-hydroxyvitamin D levels for clinical assessment of vitamin D deficiency

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ARTICLE INFO

Article history:
Received 21 June 2018
Received in revised form
23 December 2018
Accepted 24 December 2018
Available online 8 January 2019

Keywords:
Osteoporosis
Vitamin D metabolites
Clinical specimen
Derivatization
Vegetarian

ABSTRACT

Vitamin D is responsible for multiple metabolic functions in humans. Rickets are the most common disease caused by vitamin D deficiency. It is caused by poor calcium intake resulting in poor serum-ionized calcium. The purpose of this study is to develop a rapid, sensitive, and feasible method to determine the 25-hydroxy-vitamin D3 (25(OH)D3) levels in blood samples for clinical assessment. In this study, gas chromatography coupled mass spectrometry with trimethylsilyl derivatization (TMS-GC-MS) is the most suitable protocol for quantitative analyses of 25(OH)D3. Performance of method was evaluated and compared with liquid chromatography and immunoassay. Method validation has been carried out with plasma specimens. The limit of quantitation of TMS-GC-MS method is 1.5 ppb with good linear correlation. Furthermore, the dietary intake and nutritional status of vegetarian and non-vegetarians in Taiwan were assessed by our validated method. As a result, this vitamin D nutrition survey demonstrates that most Taiwanese people have insufficient vitamin D. Due to dietary habits; the male vegans may have the highest risk of vitamin D deficiency.

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https://doi.org/10.1016/j.jfda.2018.12.010

1. Introduction

Vitamin D is a group of fat-soluble vitamins which are responsible for mineral (calcium, magnesium, phosphate, and zinc) absorption and multiple biochemical functions in humans [1]. In humans, vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol) are the major compounds in this group. Both vitamins can be acquired from dietary intake and supplements. Vitamin D2 can only be procured through diet and supplementation, and parts of vitamin D3 can be synthesized through sun exposure in the skin of mammals [2].

Clinical studies suggest that vitamin D levels can affect cancer, cardiovascular disease, hypertension, depression, and immunity [3]. Vitamin D deficiency leads to chronic conditions and may lead to lethal effects. In children, asthma is a major risk cause by vitamin D deficiency [4,5]. However, the excess of vitamin D through diet could cause hypercalcemia [6,7].

The molecules of vitamin D and its metabolites are shown in Fig. 1. Vitamin D from the diet or skin synthesis is biologically inactive. Vitamin D will be hydroxylated to the 25-hydroxy-vitamin D (25(OH)D) form that circulates in the blood and the active form is1,25-dihydroxy-vitamin D ($1,25(OH)_2D$) which is synthesized by enzymatic conversion in the liver and kidneys [8]. 25(OH)D has a long plasma half-life of 2–3 weeks compared to the shorter half-life of $1,25(OH)_2D$ metabolites in circulating blood [9]. The total 25(OH)D is the sum of 25(OH)D3 and 25(OH)D2 levels, and is considered as the best biomarker to evaluate vitamin D levels in biological fluids [10]. Vitamin D (VitD) could metabolize to 25(OH)D in liver, and convert to active form through 1-alpha hydroxylase (CYP27B1), which was vitamin D activating enzyme. 25(OH)D2 and 25 (OH)D3 were sum of the 25(OH)D. The 25(OH)D3 was major amount (Appendix Table S1) and high half-life than 25(OH)D2 in blood [11]. The biomarker development must be considered the specific characteristics include sensitivity, specificity, and robustness. As discussed above, 25(OH)D3 was suitable to be monitoring the VitD level than 25(OH)D2. Mass spectrometry can distinguish these three metabolites without doubt, but the focus of this study is on the comparison of mass spectrometry methods, so we compare 25(OH)D3 only.

This study developed a derivatization method to enhance the sensitivity of 25(OH)D3 in the analytical instrument and compare the commercial derivatization reagents. The 25(OH)D3 and 25(OH)D2 had similar structure (as show in Fig. 1) and could separate by LC-MS/MS [12]. The amount of 25(OH)D2 and 25(OH)D3 in human body was ppt and ppb level, respectively, and the average content of 25(OH)D2 was lower than 25(OH)D3 about 59 times [13]. This study selected the 25(OH)D3 without 25(OH)D2 was considerate the various aspects including price of standard and derivatization reagent, detection limit, and operational convenient.

Otherwise, many clinical studies have shown that 25(OH)D3 is the major component of 25(OH)D in the blood, accounting for more than 95% of the relative abundance. Differences in the proportion of 25(OH)D constituents are not dominated by gender, race, diet, and geography. Thus, the quantitative analysis of 25(OH)D3 has also been applied to the estimation of vitamin D in serum or plasma samples [14].

The two main types of analytical techniques developed to assay the vitamin D levels in serum or plasma are immunoassay and those based on chromatographic separation followed by non-immunological detection [15]. Establishing an accurate base is important for nutrition surveys and studies with collaborative proficiency testing schemes, such as the Vitamin D External Quality Assessment Scheme (DEQAS) [16,17].

Most studies focus on 25(OH)D3 and 25(OH)D2 to perform quantitative analysis with various analytical tools [18]. To analyze a wide range of compounds, liquid chromatography

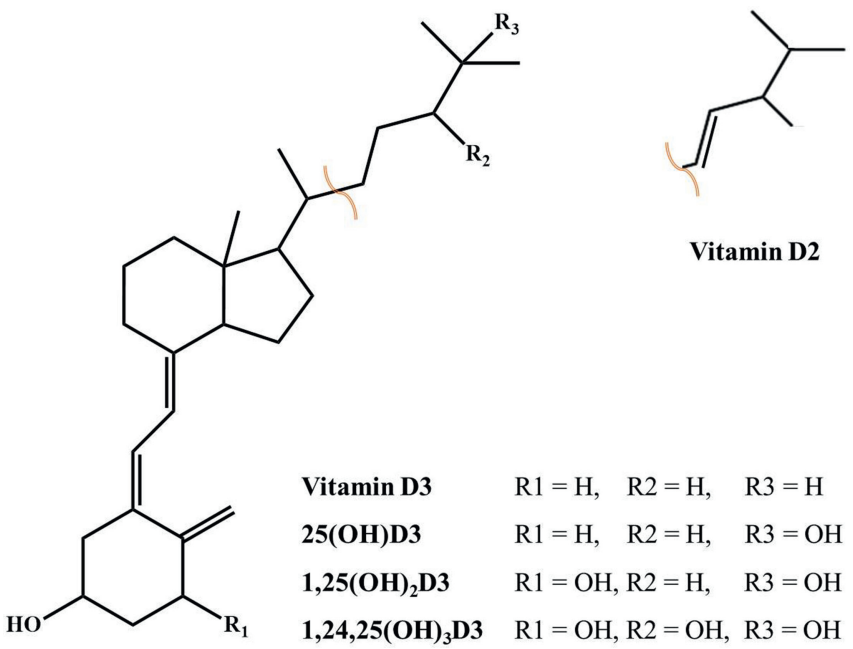

Fig. 1 – The molecular structures of the vitamin D2, D3, and its metabolites.

tandem mass spectrometry (LC-MS/MS) is the most recommended technique for quantitation by the NIH office of Dietary Supplement for high sensitivity and specificity from biological matrices [17,19]. Advances in tandem mass spectrometry have enabled the introduction of routine procedures based on LC-MS/MS for measuring vitamin D metabolites and the use of this methodology is increasing [20]. Gas chromatography-mass spectrometry (GC–MS) has also employed to quantify vitamin D3 metabolites [21].

Derivatization is a specialized technique used in chromatographic and mass spectrometric analysis to improve intensity and selectivity. The Amplifex derivatizing agent is available to test 25(OH)D derivatives with LC-MS [22]. Trimethylsilyl (TMS) derivatization can be utilized for gas chromatography (GC) analysis to increase volatility and to block activate chemical group.

Since vegetarianism has many benefits for health and environmental protection, the vegetarian population in Taiwan has been growing. However, some studies have shown that vegetarian diets have the risk of partial nutrient deficiency. Vegetarian diet effects on growth and development are a concern for many people.

The purpose of this study is to develop a rapid, sensitive, and feasible method to determine the 25(OH)D3 levels in blood sample for clinical assessment. Method validations have been carried out in plasma and serum samples. Furthermore, the validated method was used to evaluate and compare the dietary intake and nutritional status of vegetarians and non-vegetarians in Taiwan.

2. Material and methods

2.1. Chemicals

25-hydroxyvitamin D3 was purchased from Toronto Research Chemicals (Canada). An isotopic standard solution (d_6-25-hydroxyvitamin D3), *N,O*-bis-(trimethylsilyl)-acetamide (BSA), pyridines, formic acid, methanol, acetonitrile, hexanes were purchased from Sigma–Aldrich (USA). Amplifex™ diene reagent was purchased from AB Sciex (USA). K_2EDTA anticoagulant was purchased from Becton Dickinson (USA).

2.2. Specimens collection

The study protocol (05-M03-028) was been approved by the Institutional Review Board of Taipei Tzu-Chi Hospital, Buddhist Tzu-Chi Medical Foundation on June 15, 2016. The constitution and operation of this review board are according to the guidelines of the ICH-GCP.

Two blood samples were provided as control standards from our laboratory members. One sample is labeled as Specimen #1 from a male, and the other is labeled as Specimen #2 from a female.

The 117 blood samples from clinical subjects were collected in vacuum blood collection tubes with anticoagulant in Tzu-Chi Hospital, Taipei, Taiwan. Collections were made from July to September in 2016. There were 44 males and 73 females in this clinical study (Appendix Table S2). It includes the patient's information with gender, age, and diet type. The specimens were centrifuged immediately at 2600 G for 10 min, and then the plasma was collected and stored at −20 °C until analysis.

2.3. Sample preparation

Before chromatographic analysis, a preparation procedure was carried out to extract the 25(OH)D3 from the plasma. 0.2 mL of plasma was mixed with 0.2 mL of acetonitrile, and then the solution was placed at 4 °C for 10 min for protein precipitation. 1.8 mL of hexane was added to the specimens and vortexed for 10 min. The organic layer of the sample was collected after centrifugation at 500*g* for 5 min at 4 °C.

The 25(OH)D3 standard solutions for calibration were prepared at 0, 12.5, 25, 50, and 100 ng/mL in methanol. 20 μL of a 250 ng/mL d_6-25(OH)D3 solution was spiked in the standard solutions and the specimens before sample preparation as an internal standard (I·S.).

2.4. Derivatizations for LC or GC analysis

Derivatization can alter the structure of the analyte to facilitate its isolation, detection or identification. The 25(OH)D3 derivatives (-AD and -TMS) were utilized for LC-MS/MS and GC–MS/MS analysis, respectively.

The AD derivatization followed the product manual with the following modifications: 0.424 mL of 25(OH)D3 extract was dried by rotary evaporator (RE100-Pro, Scilogex, USA) and re-suspended in 0.1 mL methanol. 0.05 mL of AD reagent was added and reacted at room temperature for 30 min, then 0.5 mL deionized water was added for LC-MS/MS analysis.

The TMS derivatization of 25(OH)D3 was carried out with BSA reagent. The 1.4 mL extract was dried by rotary evaporator and re-suspended with a mixed solution of 0.02 mL BSA and 0.04 mL pyridine. The sample solution was incubated at 60 °C for 40 min, and then analyzed by GC–MS/MS.

2.5. Chromatography and mass spectrometry conditions

The LC-MS system components included a Accela pump with TSQ triple quadrupole MS (Thermo Fisher, USA). 10 μL of sample was injected for LC analysis. The 25(OH)D3 and 25(OH)D3-AD were separated with an Atlantis T3 column (3.0 μm, 2.1 mm id × 100 mm, Waters), and the mobile phase was prepared with deionized water and acetonitrile (with 0.1% formic acid) as solvent A and B, respectively. The gradient elution was as follows: 80% solvent A was held for 1 min, and then linearly increased to 80% solvent B for 4 min and held for 10 min. The HPLC flow rate was 0.3 mL/min. The instrument parameters used for LC-MS/MS to detect the 25(OH)D3-AD were as follows: ionization mode, positive ESI mode; scan time, 0.3 s; capillary temperature, 270 °C; capillary voltage, 3.5 KV; sheath gas flow rate, 40; Q1 and Q3 peak width, 0.7; the collision gas (He) pressure was set at 1.5 mTorr; MS transition mode and collision energy [15]: 25(OH)D3, 401.3 → 365.3 (CE:13 eV), 401.3 → 383.3 (CE:10 eV); d_6-25(OH)D3, 389.3 → 371.1 and 221.2 (CE: 10 eV); 25(OH)D3-AD, 733.8 → 217.0 (CE: 30 eV), 733.8 → 674.7 (CE: 25 eV); d_6-25(OH)D3-AD, 736.9 → 217.1 (CE: 30 eV), 736.9 → 680.7 (CE: 25 eV).

GC–MS/MS analysis was performed on a trace ultra GC with a TSQ triple quadrupole MS (Thermo Fisher, USA). The 25(OH)D3-TMS was separated with a HP-5MS column (25 m, 0.2 mm id, 0.33 μm film thickness; J&W Scientific). The carrier gas used was helium gas and the flow rate was 0.7 mL/min. The GC oven temperature was initially at 150 °C and held at this temperature for 1 min, increased to 200 °C at 10 °C/min and then increased to 290 °C at 20 °C/min and held at this temperature for 7 min. The inlet temperature was 280 °C 2 μL of sample was injected in PTV splitless mode. The instrument parameters used to detect the 25(OH)D3-TMS for GC–MS/MS were as follows: ionization mode, positive mode; scan time, 0.3 s; emission current, 50 μA; source vaporizer temperature, 290 °C; Q1 peak width, 0.7; MS transition mode and collision energy: 25(OH)D3-TMS, 544.4 → 439.4 (CE: 10 eV), 544.4 → 413.4 (CE: 10 eV) and d_6-25(OH)D3-TMS, 445.3 → 143.0 (CE: 20 eV), 445.3 → 140.9 (CE: 10 eV). All the analytical data acquisition in this experiment was performed by Xcalibur 2.2 software.

2.6. Immunoassay

Examination of the control standards, Specimen #1 and #2, was outsourced to the Union Clinical Laboratory (Taipei, Taiwan) to perform a Liaison Total 25OHD assay (DiaSorin Ltd., UK). The method is a chemiluminescent immunoassay (CLIA) on an automated platform for quantitative determination of 25(OH)D. During the incubation, 25(OH)D is dissociated from its binding protein and competes with the isoluminol labeled vitamin D for binding sites on the specific antibody. After the incubation, the unbound material is removed with a wash cycle. Subsequently, the starter reagents are added and a flash chemiluminescent reaction is initiated. The light intensity is measured in relative light units (RLU) and is inversely proportional to the concentration of 25(OH)D present.

2.7. The evaluation criteria of vitamin D content

In this study, the evaluation criteria of vitamin D was followed the nutrition and health survey in Taiwan and the Endocrine Society in USA (Appendix Table S3).

3. Results and discussion

3.1. LC-MS/MS analysis of 25(OH)D3 and 25(OH)D3-AD

As shown in Fig. 3, the 25(OH)D3 could be detected directly by LC-MS/MS in multiple reaction monitoring mode (MRM). The parent and product ion of 25(OH)D3 are identified at $m/z = 401.3$ and 383.3, respectively. The MRM mode enhanced the signal to noise (S/N) ratio and detection limit for the analyzed compound. A calibration curve with $R^2 = 0.9418$ was obtained. 50 ng/mL of 25(OH)D3 could be monitored as the limit of quantitation (LOQ) at the lower end. However, the precision of a direct LC-MS/MS method (without derivatization) is insufficient for clinical assessments.

The 25(OH)D3-AD was derived from reacting 25(OH)D3 with Amplifex™ Diene at room temperature for 30 min. A mass loss of 59 Da from 25(OH)D3-AD corresponds to a loss of C_3H_7O as shown in Fig. 4. For quantitation, at the lower limit, 6.25 ng/mL of 25(OH)D3-AD could be detected, achieving a good linear relationship and better precision than other methods. The calibration curve had a 0.9999 R-squared value.

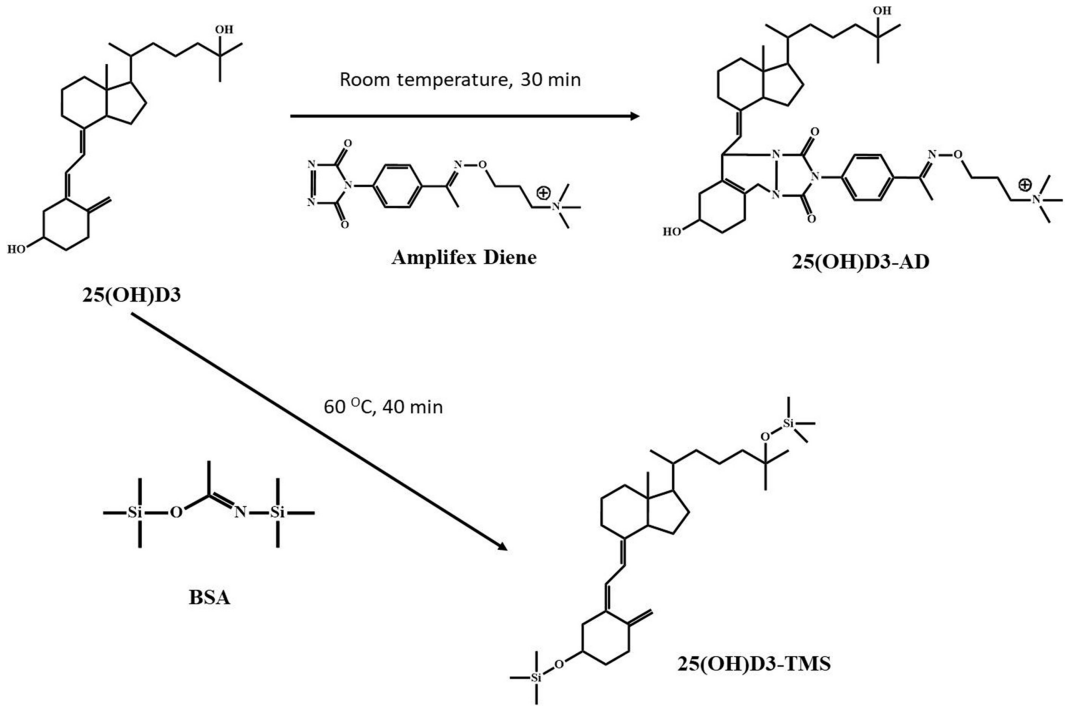

Fig. 2 – 25(OH)D3 derivitizations by Amplifex™ Diene Reagent and TMS, respectively.

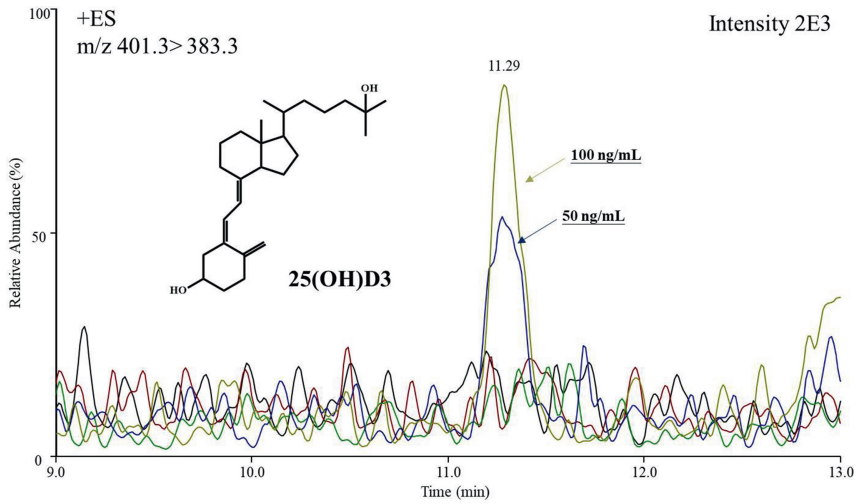

Fig. 3 – Calibrated concentrations of 25(OH)D3 were detected in 6.25, 12.50, 25, 50 and 100 ng/mL and quantified by LC-MS/MS with the MRM transition *m/z*: 401.3 → 383.3.

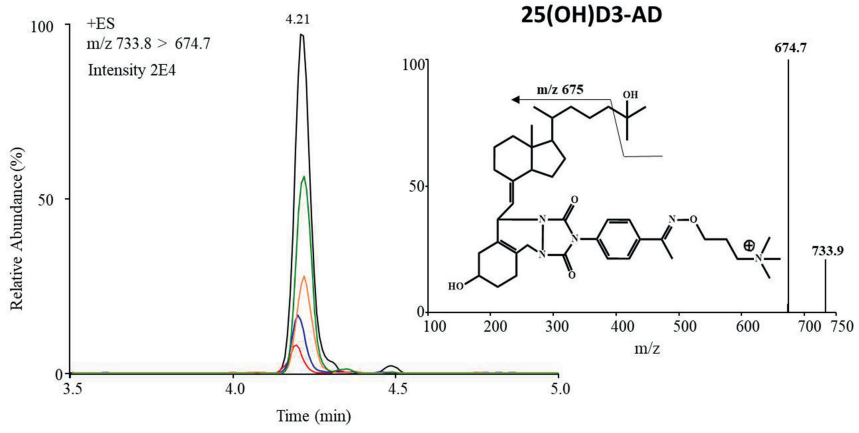

Fig. 4 – Calibrated concentrations of 25(OH)D3-AD were detected in 6.25, 12.50, 25, 50 and 100 ng/mL and quantified by LC-MS/MS with the MRM transition *m/z*: 733.8 → 674.7.

3.2. *GC–MS/MS analysis of 25(OH)D3-TMS*

BSA is used to react the hydroxyl group of 25(OH)D3 with the trimethylsilylation as shown in Fig. 2. This process causes 25(OH)D3 to convert to 25(OH)D3-TMS, enhancing the ionization ability of the compound significantly. The parent and product ions of 25(OH)D3-TMS were identified as $m/z = 544.4$ and 439.6, respectively. The predominant fragmentation patterns of 25(OH)D3-TMS could be characterized by masses of $m/z = 413.3$, 439.6, and 454.4 as shown in Fig. 5. Concentrations as low as 6.25 ng of 25(OH)D3-TMS could be detected, achieving a good linear relationship and good precision in our GC–MS/MS method. The calibration curve had an R-squared value of 0.9984. The CLIA for 25(OH)D3 analyze is a standard method in clinical assessment. This study developed a 25(OH)D3 diagnosis method with TMS-GC-MS and compared with CLIA and AD-LC-MS. The recovery in TMS-GC-MS was selected 3 concentrations include 25, 50, and 100 ng/mL 25(OH)D3 and the result was between 95 and 112% (Appendix Table S4). The analysis time was 9 min and LOD 1.5 ng/mL. On the other hand, this study was used to 2 specimens to compare three analytical methods. These data were difference less than 6%. The result was indicated TMS-GC-MS method has similar ability with CLIA for 25(OH)D diagnosis (Appendix Table S5).

3.3. *Validation with clinical specimens*

Vitamin D deficiency is defined as levels below 30 ng/mL of 25(OH)D in serum. Clinical practice related to vitamin D has been hampered because of difficulty measuring its active forms in serum. Thus, a clinical method must meet sufficient accuracy, specificity, and sensitivity standards. Although, the LC-MS method can directly measure the various vitamin D metabolites, its sensitivity is insufficient to be used in a clinical setting.

In terms of quantitation, our methodology demonstrates that GC–MS/MS with TMS derivatization (TMS-GC-MS) can achieve a similar sensitivity as LC-MS/MS with AD derivatization (AD-LC-MS). Two blood samples were used to evaluate the practical performances of TMS-GC-MS and AD-LC-MS, and

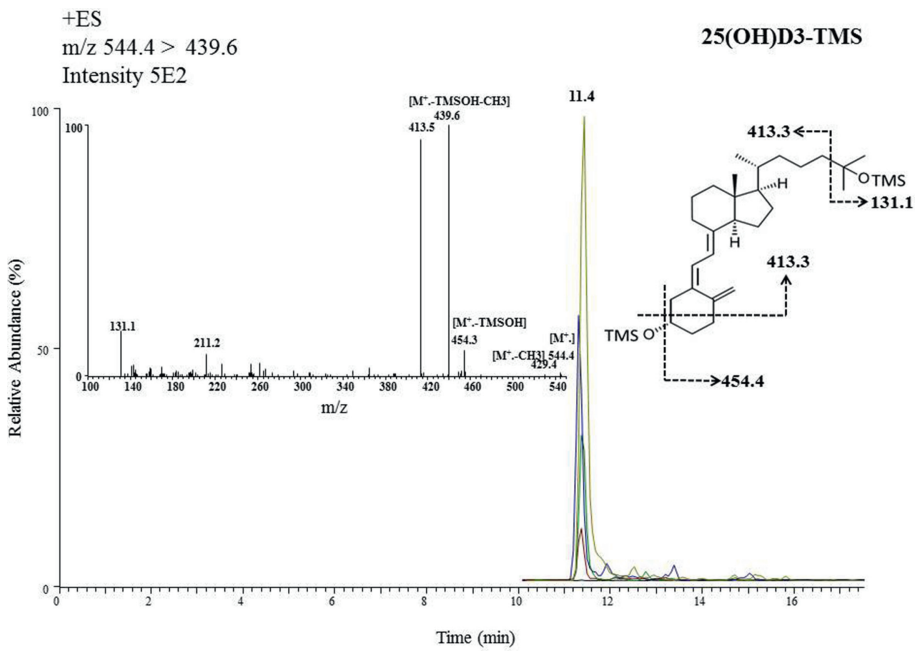

Fig. 5 – Calibrated concentrations of 25(OH)D3-TMS were detected in 6.25, 12.50, 25, 50 and 100 ng/mL and quantified by GC–MS/MS with the MRM transition *m/z*:544.4 → 439.6. The predominant GC–MS/MS fragmentation patterns of 25(OH)D3-TMS are shown.

compared them to the traditional immunoassay. The measured amounts of 25(OH)D3 for Specimen #1 and #2 are shown in Fig. 6 from a triplicate experiment. The data resulting from the CLIA method measures the total amount of 25(OH)D (=25(OH)D2+25(OH)D3). Thus, our two chromatographic methods have a slight underestimation bias when compared to the immunoassay.

These results indicate that the male specimen (#1) has a higher level of 25(OH)D3 than the female specimen (#2). Of note, the AD-LC-MS method presents a large difference in the

Method	CLIA	TMS-GC-MS	AD-LC-MS
Specimen #1	19.8	19.2	19.5
Specimen #2	15.7	15.2	14.9

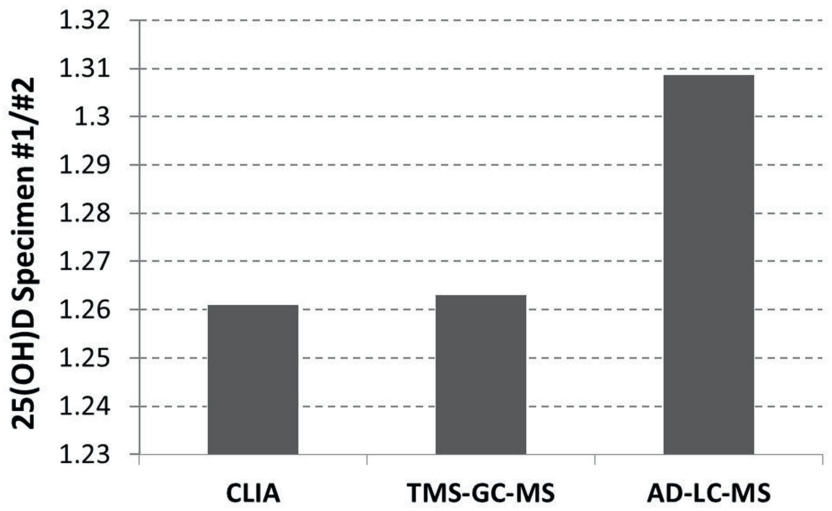

Fig. 6 – Cross-evaluation of methods, the ratios of 25(OH)D3 levels in two independent specimens were compared using three clinical assays: CLIA, TMS-GC-MS, and AD-LC-MS.

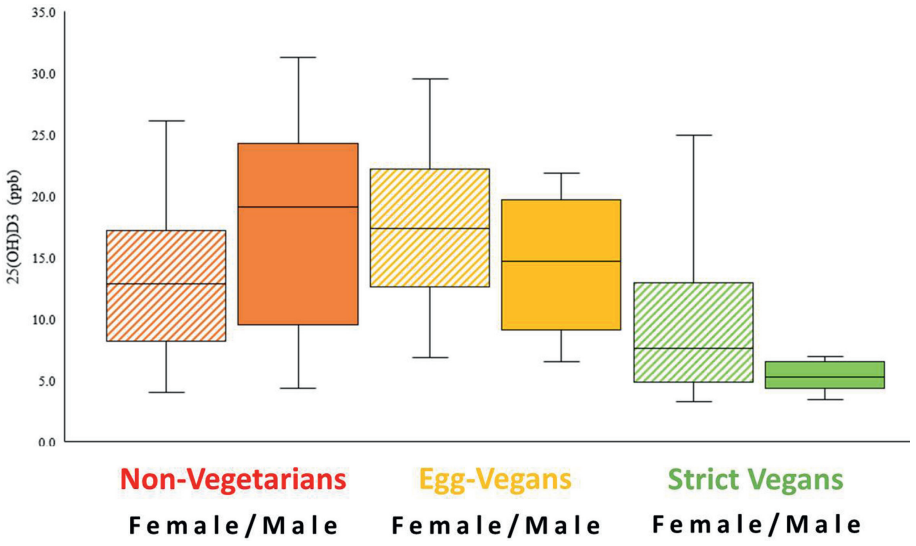

Fig. 7 – The variations of 25(OH)D3 level in blood were measured by GC–MS/MS with TMS derivatization for 115 specimens. The data was analyzed and grouped by dietary types and genders.

structural relationship within samples among these clinical assays. Authors consider that the system deviation is induced by the incomplete derivatization by the diene adduct (AD). The suitability of these analytical methods was evaluated in terms of cost, performance and operational feasibility. Finally, the TMS-GC-MS method had been employed for a clinical nutrition survey of vitamin D.

3.4. *A clinical assessment of diet and gender effects on vitamin D levels*

The 25(OH)D3 levels in the blood of 44 males and 73 females were determined through the TMS-GC-MS method. The volunteers were categorized by dietary behavior into the non-vegetarians, egg-vegans, and strict vegans groups. The 25(OH)D3 levels in the blood of strict vegans were the lowest in this clinical study are shown in Fig. 7. The box chart display variation in samples of a statistical population without making any assumptions of the underlying statistical distribution [23]. The box chart displayed the distribution of 25(OH)D3 with different subjects in diet type and they point out that majority of vegans have low 25(OH)D3. The vegans had vitamin D deficiency more than non-vegetarians were a well-known study [24,25]. The male volunteers with strict vegetarianism had the most significant risk of vitamin D insufficiency. The similar levels of 25(OH)D3 for the non-vegetarians and egg-vegans shows that the egg diets could supplement the amounts of vitamin D. Other than the male strict vegetarians, gender did not show a significant effect on the 25(OH)D3 levels in blood between the non-vegetarians and egg-vegans.

In all age groups, vitamin D deficiency is a public health problem in the worldwide [26]. This study was used TMS-GC-MS to investigate 117 clinical subjects with different diet. Regarding to nutrition and health survey in Taiwan and the Endocrine Society in USA, the 25(OH)D below 20 ppb was vitamin D deficiency. The 25(OH)D3 in clinical subjects with strict vegans display the lowest amount, which were below 20 ppb. Our result was found this phenomenon and provides a cheap methodology for clinical diagnosis. Severe vitamin D deficiency (25(OH)D < 20 ppb) causes rickets in children and osteomalacia in adults. Under this definition, vitamin D deficiency is a common global phenomenon [17]. Vitamin D insufficiency also has been implicated in an extremely wide range of clinical disorders. As a result, this vitamin D nutrition survey demonstrates that most people have insufficient vitamin D intake in Taiwan. The vegans may have a higher risk of vitamin D deficiency due to dietary habits.

4. Conclusion

Understanding the relationship between nutrition and health requires detailed surveys of dietary habits and the health status of the population at large [27]. Vitamin D analysis in human biological fluids is the most conventional procedure.

In this study, the TMS-GC-MS method was developed and validated for a clinical testing of vitamin D. We also compare different instrument and items include LC-MS/MS without derivatization and GC–MS. The AD-LC-MS had lower LOQ than TMS-GC-MS/MS; it was also worth noting that amplifex diene reagent price was over 10 times than BSA (Appendix Table S6). Our method provides a rapid, sensitive and cheap procedure to estimate the 25(OH)D level in blood sample. The LOQ of method is 1.5 ppb and the complete analysis costs is only 0.7 $ per sample.

Following the vitamin D evaluation criteria of nutrition and health survey in Taiwan, most vegans in this study have a vitamin D deficiency which 25(OH)D3 was below 20 ppb. Specific studies need to be conducted to test whether certain policy interventions will have the desired effect. Our results can be used to develop nutritional suggestions and monitor risk to improve health.

Acknowledgments

The authors thank the financial supporting in this study from the Buddhist Tzu-Chi General Hospital (TCMMP105-13-02), Tzu Chi University and the Ministry of Science and Technology (MOST 105-2113-M-320-002), Taiwan.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jfda.2018.12.010.

REFERENCES

[1] Holick MF. High prevalence of vitamin D inadequacy and implications for health. Mayo Clin Proc 2006;81:353–73.
[2] Muller MJ, Volmer DA. Mass spectrometric profiling of vitamin D metabolites beyond 25-hydroxyvitamin D. Clin Chem 2015;61:1033–48.
[3] Hock AD. Review: Vitamin D3 deficiency results in dysfunctions of immunity with severe fatigue and depression in a variety of diseases. In Vivo 2014;28:133–45.
[4] Gupta A, Bush A, Hawrylowicz C, Saglani S. Vitamin D and asthma in children. Paediatr Respir Rev 2012;13:236–43.
[5] Huang H, Porpodis K, Zarogoulidis P, Domvri K, Giouleka P, Papaiwannou A, et al. Vitamin D in asthma and future perspectives. Drug Des Devel Ther 2013;7:1003–13.
[6] Korytnaya E, Rao NG, Mayrin JV. An unusual case of hypercalcemia associated with graves' disease and vitamin d deficiency. Clin Med Insights Endocrinol Diabetes 2011;4:25–8.
[7] St-Arnaud R, Arabian A, Yu VW, Akhouayri O, Knutson JC, Strugnell SA. 1alpha,24(S)(OH)2D2 normalizes bone morphology and serum parathyroid hormone without hypercalcemia in 25-hydroxyvitamin D-1-hydroxylase (CYP27B1)-deficient mice, an animal model of vitamin D deficiency with secondary hyperparathyroidism. J Endocrinol Invest 2008;31:711–7.
[8] Pepe J, Romagnoli E, Nofroni I, Pacitti MT, De Geronimo S, Letizia C, et al. Vitamin D status as the major factor determining the circulating levels of parathyroid hormone: a study in normal subjects. Osteoporos Int 2005;16:805–12.
[9] Jones KS, Assar S, Harnpanich D, Bouillon R, Lambrechts D, Prentice A, et al. 25(OH)D2 half-life is shorter than 25(OH)D3 half-life and is influenced by DBP concentration and genotype. J Clin Endocrinol Metab 2014;99:3373–81.
[10] Volmer DA, Mendes LR, Stokes CS. Analysis of vitamin D metabolic markers by mass spectrometry: current techniques, limitations of the "gold standard" method, and anticipated future directions. Mass Spectrom Rev 2015;34:2–23.
[11] Chen H, McCoy LF, Schleicher RL, Pfeiffer CM. Measurement of 25-hydroxyvitamin D3 (25OHD3) and 25-hydroxyvitamin D2 (25OHD2) in human serum using liquid chromatography-tandem mass spectrometry and its comparison to a radioimmunoassay method. Clin Chim Acta 2008;391:6–12.
[12] Thienpont LM, Stepman HC, Vesper HW. Standardization of measurements of 25-hydroxyvitamin D3 and D2. Scand J Clin Lab Invest Suppl 2012;243:41–9.
[13] Schleicher RL, Encisco SE, Chaudhary-Webb M, Paliakov E, McCoy LF, Pfeiffer CM. Isotope dilution ultra performance liquid chromatography-tandem mass spectrometry method for simultaneous measurement of 25-hydroxyvitamin D2, 25-hydroxyvitamin D3 and 3-epi-25-hydroxyvitamin D3 in human serum. Clin Chim Acta 2011;412:1594–9.
[14] Holick MF. Vitamin D status: measurement, interpretation, and clinical application. Ann Epidemiol 2009;19:73–8.
[15] Snellman G, Melhus H, Gedeborg R, Byberg L, Berglund L, Wernroth L, et al. Determining vitamin D status: a comparison between commercially available assays. PLoS One 2010;5:e11555.
[16] Mao X, Zheng H, Liu Z, Wu Y, Na R, Wang C, et al. Analysis of 25(OH)D serum concentrations of hospitalized elderly patients in the Shanghai area. PLoS One 2014;9:e90729.
[17] Sempos CT, Vesper HW, Phinney KW, Thienpont LM, Coates PM, Vitamin DSP. Vitamin D status as an international issue: national surveys and the problem of standardization. Scand J Clin Lab Invest Suppl 2012;243:32–40.
[18] Mineva EM, Schleicher RL, Chaudhary-Webb M, Maw KL, Botelho JC, Vesper HW, et al. A candidate reference measurement procedure for quantifying serum concentrations of 25-hydroxyvitamin D(3) and 25-hydroxyvitamin D(2) using isotope-dilution liquid chromatography-tandem mass spectrometry. Anal Bioanal Chem 2015;407:5615–24.
[19] Grebe SK, Singh RJ. LC-MS/MS in the clinical laboratory - Where to from here? Clin Biochem Rev 2011;32:5–31.
[20] Yeung B, Vouros P, Reddy GS. Characterization of vitamin D3 metabolites using continuous-flow fast atom bombardment tandem mass spectrometry and high-performance liquid chromatography. J Chromatogr 1993;645:115–23.
[21] Kouremenos KA, Harynuk JJ, Winniford WL, Morrison PD, Marriott PJ. One-pot microwave derivatization of target compounds relevant to metabolomics with comprehensive two-dimensional gas chromatography. J Chromatogr B Analyt Technol Biomed Life Sci 2010;878:1761–70.
[22] Hedman CJ, Wiebe DA, Dey S, Plath J, Kemnitz JW, Ziegler TE. Development of a sensitive LC/MS/MS method for vitamin D metabolites: 1,25 Dihydroxyvitamin D2&3 measurement using a novel derivatization agent. J Chromatogr B Analyt Technol Biomed Life Sci 2014;953-954:62–7.
[23] McGill R, McGill R, Tukey JW, Larsen WA. Am Stat 1978;32:12.
[24] Baig JA, Sheikh SA, Islam I, Kumar M. Vitamin D status among vegetarians and non-vegetarians. J Ayub Med Coll Abbottabad 2013;25:152–5.
[25] Outila TA, Karkkainen MU, Seppanen RH, Lamberg-Allardt CJ. Dietary intake of vitamin D in premenopausal, healthy vegans was insufficient to maintain concentrations of serum 25-hydroxyvitamin D and intact parathyroid hormone within normal ranges during the winter in Finland. J Am Diet Assoc 2000;100:434–41.
[26] Palacios C, Gonzalez L. Is vitamin D deficiency a major global public health problem? J Steroid Biochem Mol Biol 2014;144 Pt A:138–45.
[27] Moy FM. Vitamin D status and its associated factors of free living Malay adults in a tropical country, Malaysia. J Photochem Photobiol B 2011;104:444–8.