Determination of Serum 25-Hydroxyvitamin D_3 by LC/MS/MS and Its Monthly Variation in Sapporo Indoor Workers

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25-Hydroxyvitamin D_3 (25(OH)D_3) as the metabolite of vitamin D, is connected with various of diseases, and important to people with limited sunshine. Thus, the investigation of serum 25-hydroxyvitamin D and its variation in these people is necessary. In this study, a simple, precise, and accurate method for serum 25(OH)D_3 determination by LC/MS/MS was developed. Serum samples were obtained monthly for one year from 11 male and 11 female indoor workers in Sapporo, Japan, and the overall 25(OH)D_3 concentration was 12.9 ± 4.7 ng/mL. The 25(OH)D_3 in females was significantly lower than that in males (14.0 ± 5.0 *vs.* 11.9 ± 4.3 ng/mL). The serum 25(OH)D_3 concentration in males and females were both strongly correlated to UV-B radiation (r^2 = 0.8477 and 0.7384, respectively), with a two-month's lag. Also the monthly change in 25(OH)D_3 in males was more significant than that in females.

Keywords 25-Hydroxy vitamin D_3, LC/MS/MS, UV-B, monthly variation

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Introduction

Vitamin D has been one of the nutrients of public concern that were identified in the US 2010 Dietary Guidelines for Americans.[1] The indication for vitamin D assessment is reported in various disorders, including rickets, osteomalacia, osteoporosis, chronic kidney disease, hepatic failure, malabsorption syndromes, and hyperparathyroidism.[2] The biosynthesis of vitamin D initializes from provitamin D_3 (7-dehydrocholesterol), which exists on the surface of human skin, and converts to previtamin D_3 (precholecalciferol) through a ring-opening reaction mediated by solar ultraviolet (UV)-B radiation. On the other hand, vitamin D is converted to the storable 25-hydroxyvitamin D in the liver, and is further converted to physiologically active 1α,25-dihydroxyvitamin D (25(OH)D_3, calcifediol) in the kidney.[3] Serum 25(OH)D_3 insufficiency is observed for people who live at high latitudes is where sunshine hours are short in the winter. However, related research activity is limited.

Thanks to the development of liquid chromatography combined with the mass spectrometry (LC/MS) technique in recent years, researchers can successfully determine various of analytes, ranging from the endogenous metabolites to the exogenous drugs in different biological samples, with or without derivatization.[4–7] Although there have been commercial kits for 25(OH)D_3 based on the electrochemiluminescence immunoassay (ECLIA), a simple, precise, and accurate quantitative method by LC/MS is required.

The aim of this study is to find out whether age, sex, and monthly variation influence the concentration of 25(OH)D_3 in serum of Sapporo indoor workers. In our present report, the effects of these variable factors on the serum 25(OH)D_3 concentration were studied for every month through one year in the same people working in a medical laboratory in Sapporo, Japan. Here, we report on a gender-wise seasonal variation in serum 25(OH)D_3, and its possible association with UV-B radiation.

Experimental

Reagents and chemicals

Formic acid, zinc sulfate, ultrapure water, LC-MS grade acetonitrile were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The 25(OH)D_3 and 25(OH)D_3-d_3 (both were of 100 µg/mL in ethanol) were purchased from Polysciences, Inc. (Warrington, PA, USA).

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Specimens

Ethical approval for this study was obtained from the Ethical Committee of (1) Faculty of Health Sciences, Hokkaido University (Approval No. 13 – 92) and (2) Hokkaido University hospital (Approval No. 012-0081). Blood was drawn without fasting from 22 Japanese volunteers (11 males, 34.9 ± 10.4 years old, and 11 females, 34.2 ± 6.5 years old, their detailed age and gender information are listed in Table S1 in Supporting Information). All of the volunteers were working in the Division of Laboratory and Transfusion Medicine, Hokkaido University Hospital, Sapporo, Japan. None of the subjects was taking any supplement or being pregnant during the study. The blood samples were collected every month between February 2013 and January 2014. All of the serum was separated by centrifugation within 30 min of collection, and then stored at –80°C until being used.

Extraction and purification of 25-(OH)D_3

A portion of 100 μL serum sample with 25(OH)D3-d_3 as the internal standard (500 ng/mL, 10 μL) was mixed with 100 μL of 0.2 mol/L zinc sulfate and 100 μL of acetonitrile, and then extracted by vortex for 1 min. After centrifugation (10000 rpm, 5 min), the supernatant was transferred to another tube, and 200 μL of water was added. The solution was applied to a MonoSpin® C18 solid phase extraction cartridge (sorbent, 1 mg; surface area, 350 m^2/g; sample volume, 50 – 800 μL), which was previously conditioned with 200 μL of water. The targeted 25(OH)D_3 was eluted by a 100-μL of mixture of acetonitrile and water (80:20, v/v) after the solid phase was washed with 200 μL of a mixture of acetonitrile and water (20:80, v/v). Also, 10 μL of the elution was subjected to the LC-MS/MS system.

LC-MS/MS

The LC-MS/MS system consisted of an Accela pump system and a TSQ Quantum Access triple stage quadrupole mass spectrometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) with an atmospheric pressure chemical ionization (APCI) source in the positive ion mode. A Hypersil GOLD column (1.9 μm, 50 × 2.1 mm, Thermo Fisher Scientific, Inc.) was equipped, and the oven temperature was set at 60°C. Gradient elution was performed using a mobile phase composed of a 0.1% formic acid aqueous solution (solvent A) and acetonitrile (solvent B), with a total flow rate of 600 μL/min. The gradient was programmed as follows: 0 min 70% solvent A and 30% solvent B; 0 – 4 min 100% solvent B. For MS detection, nitrogen was used as the sheath gas and the auxiliary gas (set at 40 and 10 psi, respectively). The vaporizer temperature and the capillary temperature were set at 300 and 200°C, respectively. Argon was used as the collision gas at 1.5 mTorr, and the corona discharge was set at 2 μA. Integration of the peak area and the concentration calculation was done by using the workstation Xcalibur 2.0.7 software.

Method validation

To draw a calibration curve, 25(OH)D_3 standard solutions with a series of diluted concentration were prepared. For a reproducibility study, the serum 25(OH)D_3 concentration was determined six times on the same day (intra-day assay) and on the different days (inter-day assay), respectively. The analytical recovery (%) was obtained with the test sample (n = 6), in which 100 μL of serum was spiked with 0.2 ng of the 25(OH)D_3 standard as a low level, and 1 ng as a high level, respectively. The analytical recoveries throughout the extraction and LC-MS/MS procedures were obtained by calculating with the following formula:

$$\text{Recovery (\%)} = [(\text{amount found} - \text{amount original})/\text{amount spiked}] \times 100\%.$$

Data analysis and statistics

A statistical analysis was conducted using the unpaired two-tailed t-test and two-way ANOVA (using the Tukey *post hoc* test), which was performed by Prism 5.0 (GraphPad Software, Inc., La Jolla, CA). $P < 0.05$ was considered to be statistically significant. The 25(OH)D_3 seasonal variation analysis was based on the Akaike Information Criteria (AIC).[8] Local information of the UV-B radiation and the sunshine duration at Sapporo during the studied months were provided by Sapporo Regional Headquarters, Japan Meteorological Agency (data shown in Table S2 in Supporting Information).

Results and Discussion

Optimization of LC/MS condition and method validation

In our preliminary study, electrospray ionization (ESI) was applied for detecting 25(OH)D_3. However, the ionization efficiency seemed to be unsatisfied under the ESI mode, with the pseudo molecular ion peak being difficult to be observed; thus, the LOD in the ESI mode could not be accepted. Previous studies by Ogawa *et al.*[7] solved this problem by developing a novel Cookson-type reagent for enhancing the sensitivity with ESI, and the reported limit of detection (LOD) of 25(OH)D was approximately 0.1 pg. There are also some reports[9–14] on the use of APCI-MS to detect non-derivative 25(OH)D in serum effectively. Therefore, in the present LC-MS/MS method, APCI was selected for the ionization of 25(OH)D_3. The signal intensities of $[M–H_2O+H]^+$ (m/z 383.4 for 25(OH)D_3, and m/z 386.4 for 25(OH)D_3-d_3, respectively) were much higher than those of $[M+H]^+$, and hence selected as the precursor ions in SRM, as reported by Garg *et al.*[14] The optimized SRM parameter for 25(OH)D_3 was set as m/z 383.4 for the precursor ion, m/z 365.4 for the product ion, and 18 V for the collision energy. Also, for 25(OH)D_3-d_3, it was m/z 395.4 → 377.3 (15 V). Under this condition, the representative chromatograms are shown in Figs. 1A – 1D, indicating good selectivity.

Under this condition, the calibration curve of 25(OH)D_3 showed satisfied linearity within the range of 0.04 – 2.0 ng ($y = 0.0194x + 0.0145$, $r^2 = 0.9999$, shown in Fig. S1 in Supporting Information). The LOD was 16 pg (0.8 ng/mL) with an S/N ratio of 4.6, while the limit of quantitation (LOQ) was 40 pg (2 ng/mL) with an S/N ratio of 10. The analytical recoveries of 25(OH)D_3 were 89.9 ± 15.6% (n = 6) for low level, and 91.2 ± 2.0% (n = 6) for high level, suggesting the acceptable accuracy and precision.

Several liquid–liquid extraction and solid phase extraction methods for 25(OH)D reviewed by Musteata *et al.*[15] were examined in this study. However, only low recoveries (15 – 50%) were obtained by the reported methods (data not shown), presumably due to the strong binding of vitamin D to vitamin D binding protein (DBP).[16] In our study, deproteinization with both zinc sulfate and acetonitrile successfully dissociated 25(OH)D from DBP, and good recovery was obtained using the C18 solid phase.

Serum 25(OH)D_3 concentration

The serum 25(OH)D_3 values among 22 volunteers in every month of one year were determined with the validated LC-MS/MS method in this study, and the overall serum 25(OH)D_3 concentration was 12.9 ± 4.7 ng/mL. This result was in accordance with Trang *et al.*[17] (16.6 ± 7.8 ng/mL), but lower

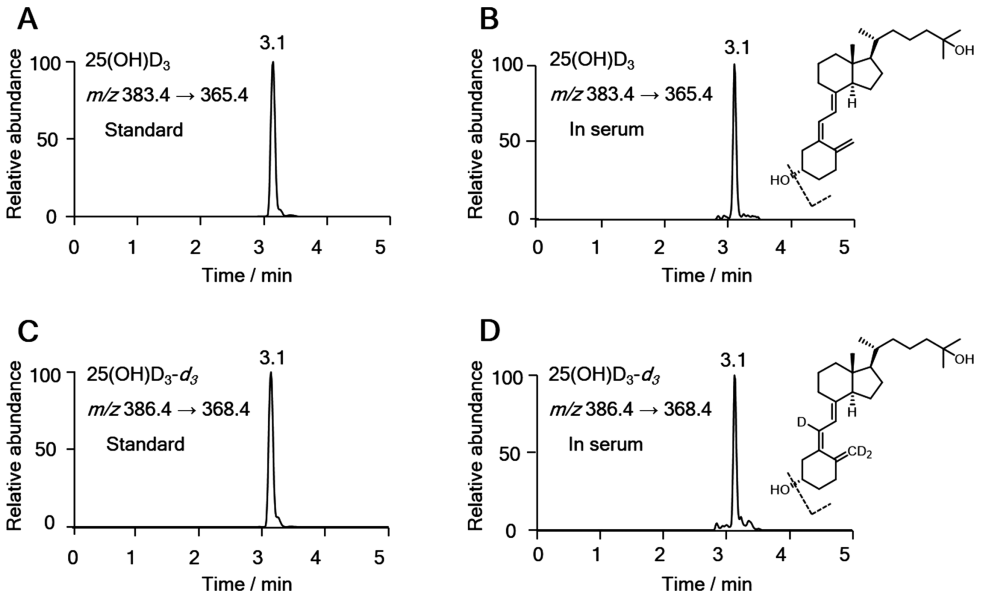

Fig. 1 SRM chromatograms of 25(OH)D_3 (A, B) and the internal standard 25(OH)D_3-d_3 (C, D) in the standard solution and in serum, respectively, together with their structures. The calibration curve under the condition is shown in (E).

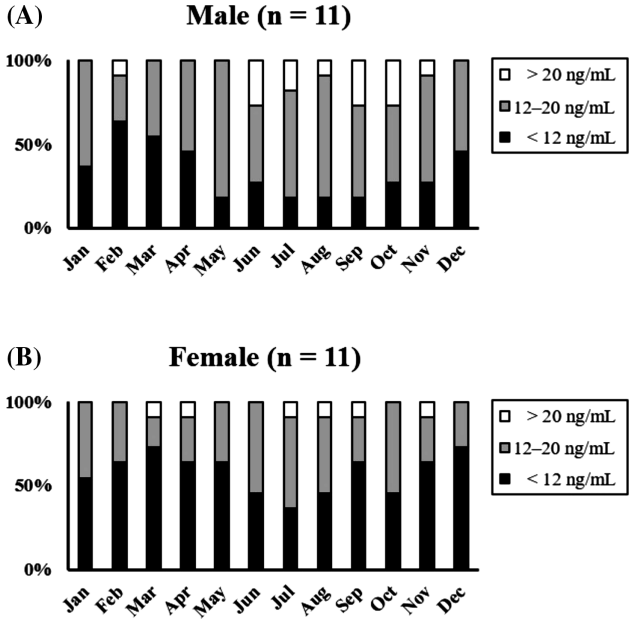

Fig. 2 25(OH)D_3 deficiency rates of males (A) and females (B) in every month. White, gray, and black bars show 25(OH)D_3 of more than 20 ng/mL, between 12 and 20 ng/mL, and less than 12 ng/mL, respectively.

than those in Cigolini's report[18] (24.1 ± 9.1 ng/mL) and Targher's reports[18,19] (31.0 ± 6.2 and 26.1 ± 9.6 ng/mL, respectively). Although most of the studies demonstrated that the decreasing of serum 25(OH)D_3 was related to diseases such as non-alcoholic fatty liver disease (NAFLD) and type 2 diabetes,[18-20] in this study, even all of the serum samples came from healthy volunteers, there was a significant difference observed between males and females (14.0 ± 5.0 *vs.* 11.9 ± 4.3 ng/mL, $P < 0.05$). While there was no significant difference in the 25(OH)D_3 concentration among subjects of different ages ($P = 0.097$) (The detailed data were shown in Table S3 in Supporting Information).

A normal level of vitamin D is usually defined as a 25(OH)D concentration higher than 30 ng/mL (75 nmol/L).[21] Vitamin D insufficiency and deficiency are usually defined as a 25(OH)D concentration of 20 to 30 ng/mL (50 to 75 nmol/L) and that less than 20 ng/mL (50 nmol/L), respectively.[2] A 25(OH)D concentration of less than or equal to 12 ng/mL (30 nmol/L) can strongly indicate vitamin D deficiency.[22] In this study, the average of 87% in male subjects for all months showed a high incidence of vitamin D deficiency, diagnosed by 25(OH)D_3 concentration lower than 20 ng/ml, and 33% of them were less than 12 ng/mL (shown in Fig. 2A). The situation got even worse in the investigated female subjects, of whom 93% exhibited an extremely high incidence of vitamin D deficiency with 25(OH)D_3 less than 20 ng/mL, and 58% even less than 12 ng/mL (shown in Fig. 2B).

Monthly variation of serum 25(OH)D_3

The serum 25(OH)D_3 concentration of both males and females changed to be higher toward the end of summer, and become lower toward the end of winter (shown in Fig. 3A). The highest monthly serum 25(OH)D_3 concentration reached 17.6 ± 6.8 ng/mL in September for males, and 13.3 ± 5.8 ng/mL in August for females, showing a statistically significant difference ($P < 0.05$). However, the lowest monthly concentration was 11.0 ± 3.4 ng/mL in March for males, and 10.2 ± 4.2 ng/mL in February for females, showing no statistically significant difference ($P = 0.31$). Interestingly, it seemed that the maximum/minimum level of serum 25(OH)D_3 appeared 2 months after the strongest/weakest sun shined. Because 25(OH)D_3 biosynthesis is known to be closely related to sunshine, the local UV-B radiation and sunshine duration data (shown in Fig. 3B) during the studied months were considered into further correlation analysis.

Then, the regression model was constructed for predicting of vitamin D based upon the intensity of sunlight. Akaike

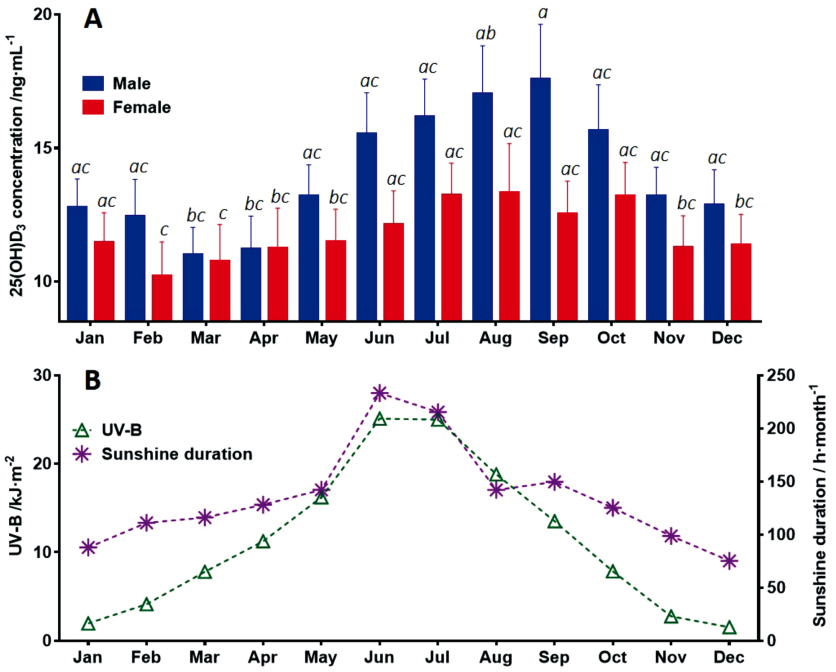

Fig. 3 Monthly variation of serum 25(OH)D_3 concentration in males (blue) and females (red) (A, $n = 10$ or 11, means ± SEM), as well as intensity of sunlight (△ for UV-B radiation, and ∗ for sunshine duration) (B). Columns without a common letter represent significant difference at the 0.05 probability level.

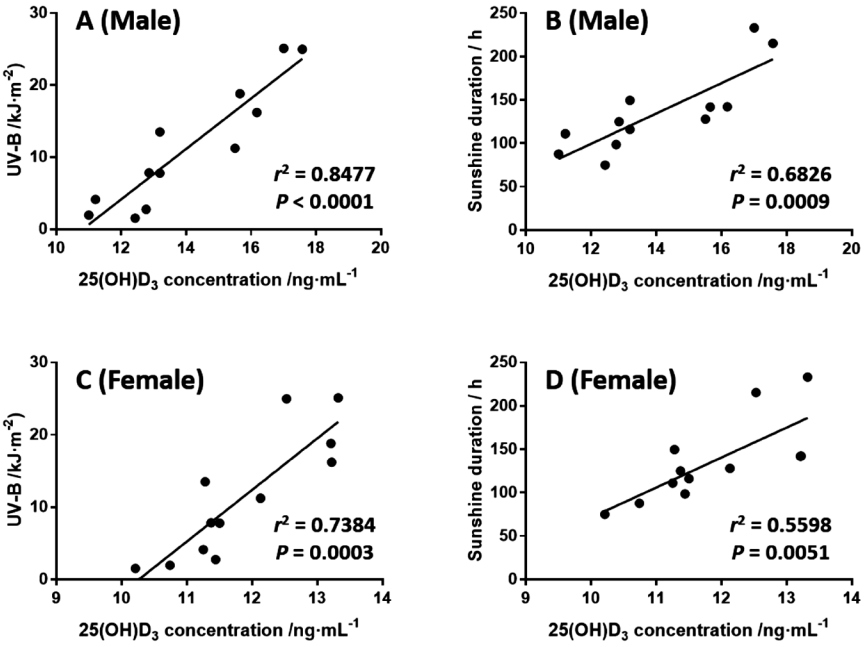

Fig. 4 Correlation between the intensity of sunlight (2 months earlier) and the serum 25(OH)D_3 concentration in males (A, B) and females (C, D).

information criteria showed that it was enough to employ 2 months earlier of UV-B radiation or sunshine duration value to predict 25(OH)D_3 in both males and females. The observed lag time between the sunshine and the serum 25(OH)D_3 concentration could be attributed to the time required for biosynthesis from provitamin D_3 to 25(OH)D_3 in human body.[23] On the basis of the regression model, the regression coefficients were calculated, and strong correlation between sunlight intensity and 25(OH)D_3 level was found (shown in Fig. 4). The higher correlations were seen in both males and females with UV-B radiation ($r^2 = 0.8477$, $P < 0.0001$ for male, and $r^2 = 0.7384$, $P = 0.0003$ for female) than those with sunshine duration ($r^2 = 0.6826$, $P = 0.0009$ for male, and $r^2 = 0.5598$, $P = 0.0051$ for female). Moreover, males showed a stronger

correlation with sunlight intensity than females, suggesting a monthly change in 25(OH)D_3 in females was less significant than that in males.

A previous study[22] that was carried out in the Tokai area (35.3°N) of Japan in 2005 reported that the seasonal change was not apparently different between the normal males and females, even though the authors reported the average serum 25(OH)D level in female was significantly lower than that in male (15.1 ± 6.0 *vs.* 18.6 ± 7.1 ng/mL). Another study[24] in the Northeast Kyusyu area (33.4 – 33.5°N) of Japan in 2011 reported the mean value of 25(OH)D in males and females were 28.0 and 26.3 ng/mL in the summer, respectively, while 22.9 and 19.4 ng/mL in the late autumn, respectively. In the current study, all of sample donors lived in Sapporo (43.4°N) and worked as clinical technicians in the hospital, for whom the sunshine was quite limited. That might be the reason why the serum 25(OH)D_3 concentration was generally lower than the normal level. Another finding, that the 25(OH)D_3 level varied less in females than in males, might be due to multiple factors, including hormone, dietary, sports, *etc.* One of the possible reasons might be cosmetics, which could help whitening the skin by reducing UV irradiation. Cosmetics and skin-whitening products are popular among in Japanese females, but there might be a side effect, as it was reported that vitamin D production decreases exponentially along with the thickness of UV-protective cosmetics.[25]

Conclusions

In this study, a simple, precise, and accurate method for serum 25(OH)D_3 determination by LC/MS/MS, was develped. Among the investigated volunteers, both males and females exhibited the level of vitamin D deficit, and 25(OH)D_3 in female was even lower than in male. In spite that the serum samples were collected from indoor workers, the serum 25(OH)D_3 concentration was strongly correlated to the intensity of sunshine, with a two-month's lag. Moreover, the monthly change in 25(OH)D_3 in males was more significant than that in females.

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Supporting Information

Supporting Information includes: Table S1, The age and gender information of volunteers; Table S2, Sunshine duration and UV-B radiation at Sapporo; Table S3, 25(OH)D_3 concentration in tested volunteers; Fig. S1, The calibration curve under the current LC/MS condition. This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

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