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Original Article

Quantitation of serum 25(OH)D2 and 25(OH)D3 concentrations by liquid chromatography tandem mass spectrometry in patients with diabetes mellitus

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ABSTRACT

Vitamin D has been considered to regulate calcium and phosphorus homeostasis and to preserve skeletal integrity. Serum 25-hydroxyvitamin D (25(OH)D) is the best indicator of vitamin D levels. The association of serum 25(OH)D deficiency with increased risk of type 1 diabetes (T1DM) and type 2 diabetes (T2DM) is controversial. We investigated serum 25(OH)D_2 and 25(OH)D_3 levels in diabetes patients by using liquid chromatography tandem mass spectrometry (LC-MS/MS). Serum 25(OH)D2 and 25(OH)D3 levels were measured with liquid chromatography tandem mass spectrometry in electrospray ionization positive mode. Chromatograms were separated using an ACE5 C18 column on a gradient of methanol. The total 25(OH)D levels were calculated as the sum of 25(OH)D3 and 25(OH)D2 levels. A total of 56 patients with T1DM and 41 patients with T2DM were enrolled in this study. There were 42 and 28 non-diabetic, age-matched volunteers who participated as the T1DM controls and the T2DM controls, respectively. The total 25(OH)D levels were lowest in the 21–40 age group. The levels of both 25(OH)D3 and the total 25(OH)D were significantly higher in the T1DM and T2DM groups than in the controls ($p < 0.01$ in T1DM and $p < 0.05$ in T2DM group, respectively). The 25(OH)D2 levels were only significantly higher in T1DM patients than in the controls. The percentages of vitamin D deficiency (total 25(OH)D less than 20 ng/mL) in

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the T1DM, T2DM, the T1DM controls and the T2DM controls were 7.1%, 0%, 14.3% and 3.6%, respectively. The percentages of vitamin D insufficiency (total 25(OH)D less than 30 ng/mL) in the T1DM, T2DM, the T1DM controls and the T2DM controls were 26.8%, 7.3%, 54.8% and 17.9%, respectively. The percentages of vitamin D deficiency and insufficiency were significantly lower in the T1DM patients than in the T1DM controls ($p < 0.01$). In the present study, both type 1 and type 2 diabetes patients had higher serum 25(OH)D levels and lower percentages of vitamin D deficiency/insufficiency.

1. Introduction

Vitamin D, which comes from exposure to sunlight as vitamin D3 and from diet or dietary supplements as vitamin D2, is considered essential for intestinal calcium absorption and for regulating calcium and phosphorus homeostasis in bone mineralization [1–3]. Vitamin D is also involved in maintaining innate immunity balance, skeletal and smooth muscle function and endothelial cell proliferation. It has been reported that vitamin D can reduce the risk of many chronic illnesses, including cancers, autoimmune diseases, infectious diseases, and cardiovascular diseases [4].

Some studies have reported that vitamin D deficiency is associated with an increased risk of diabetes mellitus [5,6]. Vitamin D deficiency increases insulin resistance, decreases insulin production and is associated with metabolic syndrome. Increasing vitamin D intake during pregnancy reduces the development of islet autoantibodies in offspring [6]. For new onset T1DM patients, mean blood levels of both 25-hydroxyvitamin D3 (25(OH)D3) and 1,25-(OH)2D3 are significantly lower than in controls [7]. A study in high latitude Finland found that daily vitamin D_3 supplementation during the first year of life could reduce the risk of T1DM approximately 80% [8]. However, in a solar-rich environment in Florida, USA, there was no significant difference in blood 25-hydroxyvitamin D (25(OH)D) levels between newly diagnosed, established T1DM patients and controls [9]. For T2DM, it also reported that a combined daily intake of calcium and vitamin D could reduce the risk of T2DM by 33% compared with a daily intake of less calcium and vitamin D [10]. However, in another study, the association between vitamin D deficiency and T2DM was not shown statistically [11].

Serum 25(OH)D is the best indicator of vitamin D because it is the most plentiful and stable metabolite of vitamin D [12]. Of the circulating vitamin D metabolites, 25(OH)D is the most abundant form and has the longest half-life (approximately 1–2 weeks) [13]. Currently, the measurement of 25(OH)D is universally accepted as one of the best biomarkers for measuring the vitamin D status of patients or populations [12,14]. In addition to radioimmunoassay and high-performance liquid chromatography, it is also possible to determine the concentrations of serum 25(OH)D2 and 25(OH)D3 using liquid chromatography-tandem mass spectrometry (LC-MS/MS) [13,15–18]. As technology has advanced, LC-MS/MS has been considered the standard method for serum or plasma 25(OH)D measurement [19]. The National Health and Nutrition Examination Survey (NHANES) of the United States and the UK Food Standards Agency also recommend LC-MS/MS for their future National Diet and Nutrition Survey [16]. The evidence for serum levels of 25(OH)D and its subtypes in T1DM and T2DM patients compared to the general population is still controversial. There are very few studies on serum vitamin D levels in diabetes patients who use LC-MS/MS. The present study aimed to compare the concentrations of serum 25(OH)D2 and 25(OH)D3 levels among T1DM patients, T2DM patients and age-matched controls using LC-MS/MS and to determine the percentages of vitamin D deficiency and insufficiency in T1DM and T2DM patients in Taiwan.

2. Methods

2.1. Study participants

Fifty-six Individuals with T1DM and forty-one individuals with T2DM were enrolled from the outpatient departments of Kaohsiung Medical University Hospital. Forty-two and twenty-eight non-diabetic, age-matched volunteers who participated as the T1DM controls and the T2DM controls, respectively. Total 167 samples were measured in this study. The study protocol was approved by the Institutional Review Board of the study hospital (KMUH-IRB-990495). After informed consent was provided by all the participants and their legal guardians, peripheral blood samples were obtained from DM patients and the controls. Case-control comparisons were performed depending on the availability of the samples at the time of analysis. The study was conducted over a period of a year during the spring and early summer in Southern Taiwan.

2.2. Sample preparation

The 25(OH)D_3 and 25(OH)D_2 reference compounds were obtained from Sigma–Aldrich (St. Louis, MO, USA). The isotopically labeled internal standard d6-25(OH)D_3 was purchased from Cerilliant (Round Rock, TX, USA). Venous blood samples were collected at random with a BD vacutainer. After centrifugation, the serum was frozen and stored at −20 °C until analysis. We added 7.5 μL 1000 ng/mL internal standard of d6-25-hydroxyvitamin D3 (d6-25(OH)D3) to 500 μL-serum sample in each test tube and then added 4 mL ethyl acetate for

liquid–liquid extraction. Each sample was mixed on a suspension mixer for 30 min and then centrifugated for 3 min at 2330 g. After centrifugation, the supernatants were decanted and then dried under nitrogen gas. The residues were than redissolved in 300 μL solvent B (0.005% formic acid in methanol). The mixture was vortex mixed for 10 s and filtered through a 0.22 μm polyvinylidene difluoride filter for mass spectrometry. Then 100 μl was injected into LC–MS/MS system. All control and fortified samples were prepared in the same manner.

2.3. Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis

The 25(OH)D2 and 25(OH)D3 levels were measured by high-performance liquid chromatography (Agilent Technologies, Palo Alto, CA, USA) coupled with triple-quadrupole mass spectrometry (API 4000QTrap, Applied Biosystems/MDS SCIEX, Concord, Canada) and an electrospray ionization (ESI) source in the positive ion mode. A 100 μL sample was injected into an ACE5 C18 column (250 mm × 4.6 mm, i.d., 5 μm, Aberdeen, Scotland) at a flow rate of 800 μL/min in gradient mode. The mobile phase was use solvent A (0.005% formic acid in water) and solvent B (0.005% formic acid in methanol) (Table 1). The multiple reaction monitoring mode was used with the characteristic fragmentation transitions m/z 413.4 → 355.2 of 25(OH) D2 and m/z 401.4 → 365.2 of 25(OH) D3 for quantification with the dwell time set at 150 ms and with unit resolution. The m/z 413.4 → 395.4 of 25(OH) D2, m/z 401.4 → 383.3 of 25(OH) D3 and m/z 407.4 → 389.4 of the internal standard isotope-labeled d6-25(OH)D3 were used to confirm quantitative analysis.

2.4. Validation of the analytical method

The precision was calculated by the percent coefficient of variation (CV, %). Intra-day precision of the proposed method was evaluated by analyzing five copies of spiked quality control (QC) samples at 5 concentration levels (0.5, 1, 2.5, 5 and 10 ng/ml for 25(OH)D2, and 5, 10, 25, 50 and 100 ng/ml for 25(OH)D3) in the same day. Inter-day precision was evaluated by analyzing five copies of 5 QC samples on each of five different days within a 5-day period. Accuracy was expressed by (mean of measured concentration/spiked concentration) × 100%. The recoveries of the proposed method were determined by comparing the response of the analytes from the pre-extracted standard samples with the response of analytes from post-extracted standard samples at the equivalent concentrations. The linearity of the method was investigated by calculating the regression line by the method of least squares and expressed by the correlation coefficient. The concentrations of each calibrator were within ±20% of the target concentration. The sensitivity of the method was evaluated by determining the limit of detection (LOD) and limit of quantitation (LOQ). The LOD was defined by a signal to noise (S/N) of 3 from the chromatograms of sample-spiked standard, and the LOQ was defined by a signal-to-noise (S/N) of 10.

Table 1 – High-performance liquid chromatography (HPLC) gradient conditions for the chromatographic separation procedure.

	Total time (min)	Flow rate (μL/min)	Mobile Phase	
			Solvent A (%)	Solvent B (%)
0	0.0	800	0	100
1	1.0	800	0	100
2	8.0	800	50	50
3	8.1	800	0	100
4	11.0	800	0	100

Solvent A: 0.005% formic acid in water. Solvent B: 0.005% formic acid in methanol.

2.5. Statistical analysis

The statistical analysis was performed using IBM SPSS Statistics (Version 19, IBM, Armonk, NY, USA) and GraphPad Prism (Version 5, GraphPad Prism Software, Los Angeles, CA, USA). When the results were below the limit of detection (LOD), the data used in the analysis contained an imputed value (LOD divided by the square root of 2). The differences in vitamin D concentrations between the DM patients and controls were analyzed by one-way ANOVA. The Chi-square test was used to determine differences in the percentages of vitamin D deficiency or insufficiency among the T1DM patients, the T2DM patients, and the controls. A p-value <0.05 indicated a statistically significant difference between groups.

3. Results

3.1. Validation study

For 25(OH)D2, the intra-day precision results of different control levels ranged from 2.66% to 7.07%, and the inter-day precision ranged from 7.60% to 15.5%. The intra-day accuracy ranged from 96.3% to 109.7%, and the inter-day accuracy ranged from 95.7% to 106.7%. For 25(OH)D3, the intra-day precision ranged from 0.58 to 2.49%, and the inter-day precision ranged from 5.68% to 7.63%. The intra-day accuracy ranged from 97.8% to 106.4%, and the inter-day accuracy ranged from 95.4% to 102.2%. The recovery study ranged from 82.1% to 86.0% for 25(OH)D2 and from 76.4% to 80.4% for 25(OH)D3. The accuracy, precision, and recovery of LC-MS/MS analysis are shown in Table 2. The upper limit of quantification for 25(OH)D2 was 100 ng/mL, and the LOD and LOQ of 25(OH)D2 were 0.1 and 0.2 ng/mL, respectively. The upper limit of quantification for 25(OH)D3 was 200 ng/mL, and the LOD and LOQ of 25(OH)D3 were 0.25 and 0.5 ng/mL, respectively.

3.2. Sample characteristics

A total of 56 patients with T1DM and 41 patients with T2DM were enrolled in the present study. The demographic characteristics of the patient and control groups are shown in Table 3. In DM patients, twenty-three (41.1%) T1DM and twenty-three (56.1%) T2DM patients were male. The mean

Table 2 – Accuracy, precision, and recovery of liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis.

Compound & Concentration (ng/mL)	Precision (CV%) (n = 5)		Accuracy (%) (n = 5)		Recovery (%) (n = 5)
	Intra-day	Inter-day	Intra-day	Inter-day	
25(OH)D2					
0.5	7.07	15.5	101.6 ± 14.4	97.4 ± 30.2	–
1	4.36	11.8	109.7 ± 9.55	100.0 ± 23.6	–
2.5	3.91	7.6	96.5 ± 7.54	95.7 ± 14.5	82.1
5	2.66	9.65	96.4 ± 5.13	100.8 ± 19.5	86.0
10	2.87	10.8	96.3 ± 5.52	106.7 ± 23.1	82.8
25(OH)D3					
5	2.49	5.75	99.9 ± 4.97	101.9 ± 11.7	–
10	0.98	7.63	98.1 ± 1.92	97.2 ± 14.8	76.9
25	1.85	5.68	104.7 ± 3.88	95.4 ± 10.8	76.4
50	1.95	5.94	106.4 ± 4.15	99.2 ± 11.8	80.4
100	0.58	6.03	97.8 ± 1.14	102.2 ± 12.3	79.2

CV: coefficient of variation. —: the recovery study did not performed.

ages and standard deviations in T1DM and T2DM patients were 19.32 ± 4.59 years and 60.71 ± 16.26 years, respectively. There were 42 and 28 non-diabetes age-matched volunteers who participated in the present study as the T1DM controls and the T2DM controls, respectively. Seventeen (40.5%) T1DM controls and eleven (39.3%) T2DM controls were male. The mean ages and standard deviations of the T1DM controls and the T2DM controls were 20.79 ± 3.29 years and 54.07 ± 11.04 years, respectively. The gender, mean age and age group distributions between the DM patients and the controls were not significantly different.

3.3. *Serum 25(OH)D and metabolite concentrations grouped by demographic variables*

Serum total 25(OH)D was calculated as the sum of 25(OH)D3 and 25(OH)D2 in the present study. The 25(OH)D3 and 25(OH)D2 were above LOD in 100% of samples, but 25(OH)D2 concentrations were measured below LOQ (0.2 ng/mL) in thirty-one (18.6%) samples in this study. Visual inspection revealed similar demographic patterns of mean total 25(OH)D (Fig. 1A) and mean 25(OH)D3 (Fig. 1B), such as a U-shaped age pattern. The mean concentrations of 25(OH)D and 25(OH)D3 were significantly lower in those aged 21–40 years than the other age groups (Fig. 1A and B). Additionally, mean concentrations of 25(OH)D, 25(OH)D3, and 25(OH)D2 were similar between male and female groups (Fig. 1C).

3.4. *The concentrations of total 25(OH)D in T1DM patients, T2DM patients and controls*

Among all the samples, the minimum and maximum concentrations of total 25(OH)D were 11.14 ng/mL and 75.87 ng/mL, respectively. As shown in Table 4, the mean total 25(OH)D concentrations for T1DM patients and T1DM controls were 36.01 ng/mL and 29.38 ng/mL, respectively. The mean total 25(OH)D concentrations for T2DM patients and T2DM controls were 46.49 ng/mL and 39.21 ng/mL, respectively. The mean concentrations of total serum 25(OH)D in all T1DM patients were significantly higher than in T1DM controls ($p < 0.01$). The mean concentrations of total serum 25(OH)D for all the T2DM patients were also significantly higher than in the T2DM controls ($p < 0.05$). Among males, the T2DM patients had significantly higher mean concentrations of total 25(OH)D than the T2DM controls (mean concentration: 49.10 ng/mL for T2DM patients and 34.77 ng/mL for T2DM controls; $p < 0.01$). Among females, the T1DM patients had significantly higher mean total 25(OH)D concentrations than the T1DM controls (mean concentration: 34.93 ng/mL for T1DM patients and 26.98 ng/mL for T1DM controls; $p < 0.01$).

3.5. *The concentrations of 25(OH)D3 in the T1DM patients, T2DM patients and controls*

The patterns for 25(OH)D3 concentrations in the T1DM patients, T2DM patients and controls were similar to total 25(OH)D (Table 4). In all the samples, the minimum and maximum concentrations of 25(OH)D3 were 10.9 ng/mL and 74.5 ng/mL, respectively. The serum 25(OH)D3 concentrations were significantly higher for the T1DM patients than the T1DM controls (mean concentration: 35.61 ng/mL for T1DM patients and 29.01 ng/mL for controls; $p < 0.01$). The mean serum 25(OH)D3 concentrations for the T2DM patients (46.11 ng/mL) were also significantly higher than the T2DM controls ($p < 0.05$). Among males, the T2DM patients had significantly higher mean total 25(OH)D concentrations than the T2DM controls (mean concentration: 48.71 ng/mL for T2DM patients and 34.18 ng/mL for T2DM controls; $p < 0.01$). Among females, the T1DM patients had significantly higher mean total 25(OH)D concentrations than the T1DM controls (mean concentration: 34.52 ng/mL for T1DM patients and 26.59 ng/mL for T1DM controls; $p < 0.01$).

3.6. *The concentrations of 25(OH)D2 in T1DM patients, T2DM patients and controls*

The concentrations of 25(OH)D2 among the different groups are shown in Table 4. In all the samples, the minimum and maximum concentrations of 25(OH)D2 were less than LOQ (0.2 ng/mL) and 2.76 ng/mL, respectively. The mean 25(OH)D2 concentrations in the T1DM patients and the female T1DM patients were 0.402 ng/mL and 0.414 ng/mL, respectively. The mean 25(OH)D2 concentrations in the T1DM patients and the female T1DM patients were both significantly higher than all

Table 3 – Characteristics of DM patients (including T1DM and T2DM) and the controls.

Covariate	Levels	T1DM patients		T1DM Controls		*P* value	T2DM patients		T2DM Controls		*P* value
		n	(%)	n	(%)		n	(%)	n	(%)	
Gender	Male	23	41.1	17	40.5	0.953	23	56.1	11	39.3	0.170
	Female	33	58.9	25	59.5		18	43.9	17	60.7	
Age, years	(Mean ± SD)	19.32 ± 4.59		20.79 ± 3.29		0.069	60.71 ± 16.26		54.07 ± 11.04		0.064

the T1DM controls and the male T1DM controls ($p < 0.01$). The mean 25(OH)D2 concentration for the T2DM patients was 0.373 ng/mL, which was lower than that of the T2DM controls (0.446 ng/mL). However, there were no significant differences in 25(OH)D2 concentrations between the T2DM patients and the T2DM controls.

3.7. *Percentages of vitamin D deficiency and insufficiency in DM patients and controls*

According to the literature review and to the recommendations of the experts [4,20], we defined total 25(OH)D concentrations less than 20 ng/mL (50 nmol/L) as vitamin D deficiency and total 25(OH)D concentrations between 21 and 29 ng/mL (52–72 nmol/L) as relative insufficiency in the present study.

As shown in Table 5, the percentages of the concentrations of total 25(OH)D less than 20 ng/mL (vitamin D deficiency) in the T1DM patients, T1DM controls, T2DM patients and T2DM controls were 7.1%, 14.3%, 0% and 3.6%, respectively. The percentages of vitamin D deficiency were not significantly different among the T1DM patients, T2DM patients and their age-matched controls. The percentages of the concentrations of total 25(OH)D less than 30 ng/mL (vitamin D insufficiency) in the T1DM patients, T1DM controls, T2DM patients and T2DM controls were 26.8%, 54.8%, 7.3% and 17.9%, respectively. The percentages of vitamin D insufficiency were significantly lower in all the T1DM patients and the female T1DM patients than in their age-matched controls ($p < 0.01$). However, the percentages of vitamin D insufficiency were not significantly different between the T2DM patients and the T2DM controls, even when stratified by gender (Table 5).

4. Discussion

In the present study, we found that T1DM patients and T2DM patients had higher 25(OH)D and 25(OH)D3 levels than their age-matched controls. The mean 25(OH)D2 levels were significantly higher in T1DM patients, but not in T2DM

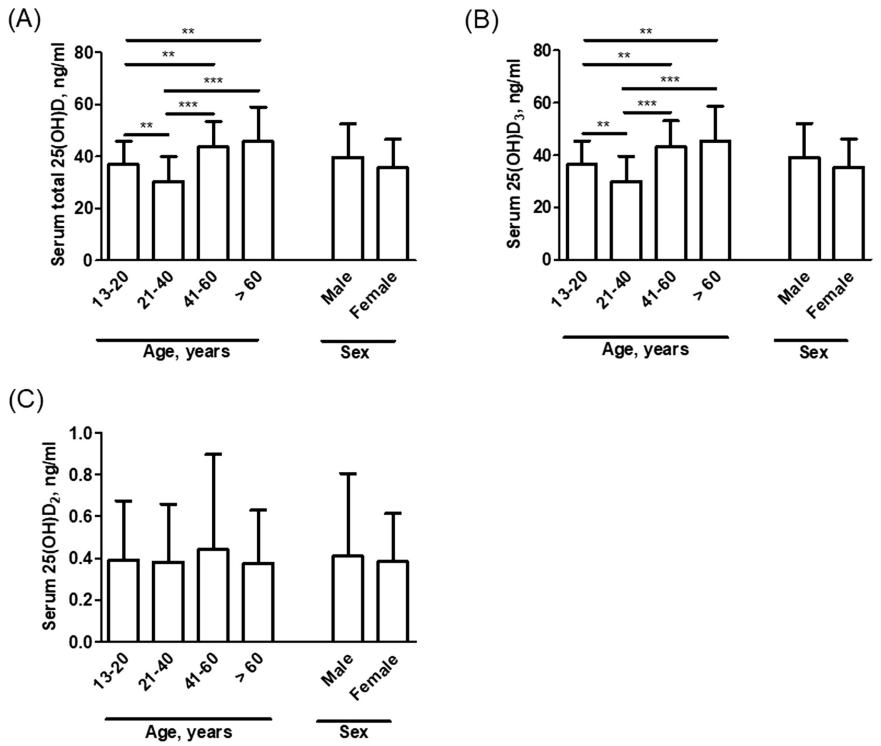

Fig. 1 – Serum total 25(OH)D and metabolite concentrations grouped by demographic variables. Demographic patterns of (A) total 25(OH)D, (B) 25(OH)D3, and (C) 25(OH)D2 for all subjects. The bars represent the means ± standard deviations. $^{**}p < 0.01$; $^{***}p < 0.001$. 25(OH)D: 25-hydroxyvitamin D.

Table 4 – Serum total 25(OH)D, 25(OH)D3 and 25(OH)D2 between DM patients and the controls.

Group	T1DM patients (N = 56)		T1DM Control (N = 42)		T2DM patients (N = 41)		T2DM Controls (N = 28)	
	n	Mean (range), ng/mL	n	Mean (range), ng/mL	n	Mean (range), ng/mL	n	Mean (range), ng/mL
Total 25(OH)D								
All	56	36.01 (33.30, 38.72)**	42	29.38 (26.92, 31.84)	41	46.49 (42.47, 50.50)*	28	39.21 (35.38, 43.04)
Male	23	37.55 (32.98, 42.13)	17	32.92 (29.45, 36.39)	23	49.10 (42.39, 55.81)**	11	34.77 (30.64, 38.90)
Female	33	34.93 (31.45, 38.42)**	25	26.98 (23.84, 30.21)	18	43.15 (39.70, 46.61)	17	42.01 (36.48, 47.67)
25(OH)D3								
All	56	35.61 (32.89, 38.33)**	42	29.01 (26.55, 31.46)	41	46.11 (42.11, 50.12)*	28	38.76 (34.93, 42.60)
Male	23	37.17 (32.57, 41.77)	17	32.56 (29.13, 35.99)	23	48.71 (42.03, 55.39)**	11	34.18 (30.17, 38.19)
Female	33	34.52 (31.04, 38.00)**	25	26.59 (23.36, 29.83)	18	42.79 (39.34, 46.25)	17	41.73 (36.12, 47.33)
25(OH)D2								
All	56	0.402 (0.326, 0.478)**	42	0.376 (0.284, 0.467)	41	0.373 (0.298, 0.448)	28	0.446 (0.262, 0.630)
Male	23	0.385 (0.262, 0.509)	17	0.362 (<0.2, 0.537)	23	0.385 (0.253, 0.518)	11	0.584 (<0.2, 1.076)
Female	33	0.414 (0.313, 0.514)**	25	0.385 (0.276, 0.494)	18	0.358 (0.306, 0.410)	17	0.356 (0.277, 0.435)

Values are shown as mean (95% confidence intervals). 25(OH)D: 25-hydroxyvitamin D; DM: diabetes mellitus; T1DM: type 1 diabetes mellitus; T2DM: type 2 diabetes mellitus; n: numbers; *: $p < 0.05$, **: $p < 0.01$, compare with the subgroup controls.

Table 5 – Percentages of serum total 25(OH)D concentrations at various cutoff values in DM patients and the controls.

Cutoff value and group	T1DM patients (N = 56)	T1DM Controls (N = 42)	P value	T2DM patients (N = 41)	T2DM Controls (N = 28)	P value
	% (case/total)	% (case/total)		% (case/total)	% (case/total)	
Less than 20 ng/mL						
All	7.1 (4/56)	14.3 (6/42)	0.256	0 (0/41)	3.6 (1/28)	0.223
Male	4.3 (1/23)	0 (0/17)	0.384	0 (0/23)	0 (0/11)	
Female	9.1 (3/33)	24 (6/25)	0.120	0 (0/18)	5.9 (1/17)	0.296
Less than 30 ng/mL						
All	26.8 (15/56)	54.8 (23/42)	0.005**	7.3 (3/41)	17.9 (5/28)	0.179
Male	21.7 (5/23)	29.4 (5/17)	0.580	8.7 (2/23)	27.3 (3/11)	0.152
Female	30.3 (10/33)	72.0 (18/25)	0.002**	5.6 (1/18)	11.8 (2/17)	0.512

25(OH)D: 25-hydroxyvitamin D; DM: diabetes mellitus; T1DM: type 1 diabetes mellitus; T2DM: type 2 diabetes mellitus; N: numbers. **: $p < 0.01$, among the DM and the subgroup controls.

patients, than their age-matched controls. Factors that influence 25(OH)D levels include race, vitamin D supplementation, sun exposure, age and physical activity [21]. Most of the individual variation in 25(OH)D levels is difficult to explain, even when all possible factors are taken into account. It has been reported that outdoor sun exposure and time spent outdoors are better predictors of serum 25(OH)D levels than dietary vitamin D intake [22]. The mean serum levels of 25(OH)D in our controls were higher than that in previous studies [23–25]. In our study, the mean serum 25(OH) levels were 33.31 ng/ml (83.28 nmol/L) in the non-diabetes cohort. In the United States, the mean serum 25(OH) levels were approximately 29.8 ng/mL (74.4 nmol/L) in September and 22.36 ng/mL (55.9 nmol/L) in March [24]. In northern India, the mean serum 25(OH) levels were 11.8 ng/mL in healthy school children [25]. The reasons for these differences in 25(OH)D levels were most likely the geographical environment and the age of the subjects. The non-diabetes controls enrolled in our study all lived in a sunshine-rich area (Kaohsiung city latitude 22.6°), and most of them were young adults (mean age 34.1 years).

Another important finding in the present study is that the percentages of vitamin D deficiency/insufficiency were lower in T1DM patients and T2DM patients than in age-matched controls. In T1DM patients, the percentages of vitamin D insufficiency (serum 25(OH)D less than 30 ng/mL (75 nmol/L) were significantly lower than in T1DM controls. These findings in this study were different from other studies for patients with new onset or established T1DM or T2DM. Pozzilli et al. reported that mean levels of both 25(OH)D3 and 1,25-(OH) 2D3 were significantly lower in patients with newly diagnosed T1DM compared to controls in Finland, and there was no correlation between 1,25-(OH)2D3 plasma levels and metabolic control status by disease diagnosis, age, or gender [7]. Another study in a Swiss sample reported that 60.5% of T1DM patients were 25(OH)D-deficient and became more deficient in winter (84.1%) [26]. In sun-rich Indonesia, the percentage of 25(OH)D deficiency was 78.2% in T2DM elderly patients, and the percentage was higher in female (56%) than in male (19%) patients ($p = 0.012$) [11]. These differences between our findings and previous studies might be explained by the variability in the geographical environment, and physicians usually advise diabetes patients to get more outside exercise in Taiwan.

In total, 14.3% of our T1DM controls and 3.6% of the T2DM controls had vitamin D deficiency. Holick et al. noted that children and young adults are potentially at high risk for vitamin D deficiency [4]. Fifty-two percent of Hispanic and black adolescents and forty-eight percentage of white

preadolescent girls had 25(OH)D levels below 20 ng/mL [23,24]. Even in the sunniest areas, vitamin D deficiency is common when most of the skin is shielded from the sun. In studies in Saudi Arabia, the United Arab Emirates, Australia, Turkey, India, and Lebanon, 30–50% of children and adults had 25(OH)D deficiency [25].

The accuracy of 25(OH)D measurement varies widely between individual laboratories and between different assay methods. Total 25(OH)D, which is the sum of 25(OH)D2 and 25(OH)D3, is used to evaluate vitamin D status. Serum 25(OH)D2 and 25(OH)D3 measurement by LC-MS/MS have been reported previously [27–30]. Although the sensitivity of this present method is equal to the previous studies, the limit of quantification of 25(OH)D2 (0.2 ng/mL) and 25(OH)D3 (0.5 ng/mL) in our study were lower than that in previous studies [27–29]. The precision data for 25(OH)D3 in our study was similar or superior than that of previous studies [27–30]. Our intra-day and inter-day precision (CV%) for 25(OH)D2 ranged 2.66–7.07% and 7.6–15.5%, respectively. The CV data for 25(OH)D2 in our study were higher than previous methods, that might because the concentrations of 25(OH)D2 used in our precision and accuracy studies were lower than the previous studies [27,28, 30]. The matrix effects which cause ion suppression or ion enhancement are problems for LC–MS/MS-based determinations. Because normal serum has endogenous 25(OH)D2 and 25(OH)D3, performing the matrix effects test is not an easy task. In this study, we used liquid–liquid extraction and stable isotope-labeled d6-25(OH)D3 to eliminate the matrix effect. Phospholipids in biological fluid is a major cause of the matrix effect in ESI, and liquid–liquid extraction can reduce this interference in the final extract by efficiently removing phospholipids and other interfering components. Stable isotope-labeled d6-25(OH)D3 was used as an internal standard and could eliminate relative matrix effect.

There are few studies for measurement of 25(OH)D2 and 25(OH)D3 concentrations in patients with T1DM and T2DM measured by LC-MS/MS. In this study, we used LC/MS/MS to measure serum 25(OH)D2 and 25(OH)D3 levels in diabetes patients and non-diabetes controls and found that T1DM and T2DM patients had higher serum 25(OH)D levels and lower percentages of vitamin D insufficiency than their age-matched controls.

Conflicts of interest

The authors have no conflicts of interest to disclose.

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