Analysis of Vitamin D Components in Serum of Minors by UHPLC-MS/MS

Yi-yang He¹, Yuan-meng Li², Yu-chun CHEN³, Bi-tao Wu¹, Yu-wei YANG¹ and Jia-fu FENG^{1,*}

¹Department of Laboratory Medicine, and ³Department of Pharmacy, Mianyang Central Hospital,
School of Medicine, University of Electronic Science and Technology of China,
Mianyang 621000, China

²Department of Clinical Laboratory, Nanchong Central Hospital, Nanchong 637000, China

(Received December 7, 2020)

Summary Serum 25-hydroxyvitamin D [25(OH)D] concentration represents the body's reserves of vitamin D, which is mostly used by clinicians to evaluate the storage status of vitamin D in the body. The present study aimed to investigate the serum vitamin D components in different health status of minors to correctly evaluate the vitamin D storage in vivo. A total of 2,270 minors were included in the study, which was divided into healthy group (1,204 cases) and disease group (1,066 cases, including 270 short stature, 433 respiratory infections, 175 malnutrition and 188 tic disorder subjects). The levels of 25-hydroxyvitamin D₂ [25(OH)D₂] and 25-hydroxyvitamin D₃ [25(OH)D₃] were measured by UHPLC-MS/MS in all subjects, and the 25(OH)D₃ activity equivalents [25(OH)D₃-AE] and 25(OH)D were calculated. In addition, the 3-epi-25-hydroxyvitamin D₃ [3-epi-25(OH)D₃] concentrations of 278 subjects (including 147 healthy and 131 disease subjects) were measured by random sampling. 25(OH)D₂, 25(OH)D₃, 25(OH)D and 25(OH)D₃-AE levels in disease group were significantly lower than those in healthy group ($p < 0.001$). According to the level of 25(OH)D, the sufficiency of vitamin D [25(OH)D ≥ 30 ng/mL] was 65.4% in healthy group and 50.5% in disease group. When the 25(OH)D₂ activity was converted into 25(OH)D₃-AE, 53.2% of the patients in the healthy group had sufficiency vitamin D, and 39.1% in the disease group. The 3-epi-25(OH)D₃ level in the disease group was significantly lower than that in the healthy group ($p < 0.001$). Not only the 25(OH)D, but also the both of 25(OH)D₂ and 25(OH)D₃ levels may overestimate the vitamin D status in subjects. For accurate evaluation, at least the serum levels of 25(OH)D₂, 25(OH)D₃ and 3-epi-25(OH)D₃ should be determined simultaneously.

Key Words vitamin D, UHPLC-MS/MS, minor, 25-hydroxyvitamin D, 25-hydroxyvitamin D₂, 25-hydroxyvitamin D₃, 25-hydroxyvitamin D₃ activity equivalents, 3-epi-25-hydroxyvitamin D₃

Vitamin D is a class of fat-soluble steroid derivatives. There are more than 50 forms of vitamin D metabolites in human blood circulation (1). Vitamin D has no biological activity. It has physiological functions only after it is hydroxylated to 25-hydroxy vitamin D [25(OH)D] in liver, and hydroxylated to 1,25(OH)₂D and other active substances in kidney (2). 25(OH)D is the main storage form of vitamin D in the human body, accounting for more than 95% of the total vitamin D (3). This mainly includes 25-hydroxyvitamin D₂ [25(OH)D₂] and 25-hydroxyvitamin D₃ [25(OH)D₃]. Because its half-life is as long as 2 to 3 wk, 25(OH)D is recognized internationally as the best indicator of the body's vitamin D nutritional status (4). The main physiological function

of vitamin D in the human body is to maintain the stability of blood calcium and phosphorus, and has the effect of promoting osteogenesis. However, many studies in recent years have shown that in addition to the recognized role of traditional cognition in skeletal effects, vitamin D levels in the body are closely related many common clinical diseases, such as diabetes (3, 5), chronic urticaria (6), childhood respiratory infection (7), rheumatic immune diseases (8) and tumors (9).

Although 25(OH)D₂ and 25(OH)D₃ are the main forms of existence, the sources of them are different. The former mainly comes from plants, the latter mainly comes from animals. The content and activity of 25(OH)D₃ are significantly higher than those of 25(OH)D₂ under normal physiological conditions, respectively. Therefore, clinical and laboratory experts pointed out that the vitamin D detection method should be able to detect 25(OH)D₂ and 25(OH)D₃ at the same time. This can ensure that vitamin D supplements derived from plants or animals can be detected (10). The vitamin D storage level can be calculated from the contents of 25(OH)D₂ and 25(OH)D₃, and then the

*To whom correspondence should be addressed.

E-mail: jiafufengacad@foxmail.com

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; 25(OH)D₂, 25-hydroxyvitamin D₂; 25(OH)D₃, 25-hydroxyvitamin D₃; 25(OH)D₃-AE, 25(OH)D₃ activity equivalents; 3-epi-25(OH)D₃, 3-epi-25-hydroxyvitamin D₃; UHPLC-MS/MS, Ultra-high-performance Liquid Chromatography-tandem Mass Spectrometry.

Table 1. Serum vitamin D component of subject. [M (P₂₅, P₇₅), unit: ng/mL]

Indicators	Healthy group	Disease group	χ^2 or z value	p value
Cases/male	1,204/662	1,066/689	21.86*	<0.001
25(OH)D ₂	1.91 (0.80, 8.09)	1.48 (0.70, 4.34)	4.49*	<0.001
25(OH)D ₃	29.48 (22.31, 37.19)	26.23 (21.71, 32.79)	6.02*	<0.001
25(OH)D	34.29 (27.13, 42.46)	30.06 (24.71, 37.29)	9.17*	<0.001
25(OH)D ₃ -AE	30.91 (24.02, 38.92)	27.46 (23.12, 33.54)	7.27*	<0.001
25(OH)D ₂ /25(OH)D ₃	0.07 (0.03, 0.26)	0.06 (0.03, 0.15)	2.51*	0.012

25(OH)D₃-AE=25(OH)D₂/3+25(OH)D₃; * χ^2 value; * z value.

nutritional status of vitamin D can be accurately evaluated. However, both 25(OH)D₂ and 25(OH)D₃ exist in two forms of binding or non-binding to proteins, and epimers formed under the action of corresponding enzymes. Therefore, the evaluation of vitamin D storage in the body cannot be accurately evaluated by the detection of only the total serum 25(OH)D level, or the separate detection of 25(OH)D₂ and 25(OH)D₃, especially for minor subjects. The traditional methods used by clinical laboratories to detect serum vitamin D content are mainly immunological methods, such as radioimmunoassay, immunoluminescence, and enzyme-linked immunosorbent assay (11). At present, various chemiluminescence methods are the most widely used. Most of them can only detect total 25(OH)D. Few can detect the levels of 25(OH)D₂ and 25(OH)D₃ separately, nor can they detect other metabolite forms of vitamin D. With the development of medical technology, Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS) gradually entered the clinical laboratory, and is the "gold standard" for detecting 25(OH)D₂ and 25(OH)D₃ (12). At present, some studies have previously reported the detection of vitamin D metabolites by Ultra-high-performance LC-MS/MS (UHPLC-MS/MS) technique (13–15). However, there are few clinical laboratories that use 3-epi-25-hydroxyvitamin D₃ [3-epi-25(OH)D₃] as a routine detection and report it to the clinic. In this paper, an UHPLC-MS/MS detection system was presented for detecting vitamin D metabolites, including 25(OH)D₂, 25(OH)D₃ and 3-epi-25(OH)D₃. Furthermore, the effect of different vitamin D metabolites activities on the result judgment was analyzed. This provides a reliable evidence-based basis for laboratory examinations and clinical evaluations of minor vitamin D-related diseases and their internal storage. The results are now reported as follows.

SUBJECTS AND METHODS

Objects and groups. We selected 2,270 cases of minors (<18 y old) who came to Mianyang City Central Hospital for medical treatment or health checkup in the sunny summer (June to August) in 2019, aged 12.0 y (1.0 m–16.0 y). According to the purpose of going to the hospital, they were divided into a healthy group ($n=1,204$) and a disease group ($n=1,066$). The disease group included 270 cases of short stature, 433 cases of respiratory infections, 175 cases of malnutrition and

188 cases of tic disorders.

Written informed consent were obtained from every patient and the study was approved by the Human Ethics Committee of Mianyang Central Hospital (S2018085).

Sample testing. The subject fasted overnight. Before 10:00 am, a fasting venous blood (about 5 mL) was collected with a disposable blood collection tube containing coagulant/fibrinogen coagulant (US BD Company). After 0.5 to 1.0 h, the blood was centrifuged at 3,000 rpm ($1,761 \times g$) for 10 min to separate the serum. If the serum is not used in time, the separated serum samples can be frozen at -80°C for 1 mo.

A JasperTM HPLC liquid chromatograph was connected in series with an AB SCIEX Triple QuadTM 4500MD mass spectrometer to detect the content of vitamin D components in serum. Standards and instrument parameter settings used in the experiment are:

a. Standard products. 25(OH)D₂ (isotopic doping $\geq 98\%$, purity $\geq 95\%$), 25(OH)D₃ (isotopic doping $\geq 99\%$, purity $\geq 95\%$) and 3-epi-25(OH)D₃-d₃ (isotopic doping $\geq 98\%$, purity $\geq 98\%$) were purchased from IsoSciences MPD Chemical Company (IsoSciences, USA). 25(OH)D₂-d₆ and 25(OH)D₃-d₆ were purchased from Medical Isotopes Inc (Medical Isotopes, USA). Both their abundance and chemical purity are >98 atom% D and $>99\%$ respectively. 3-epi-25(OH)D₃ was purchased from Toronto Research Chemicals (Toronto, Canada) with purity of 95%.

b. Sample processing. We added 10 μL of internal standard solution (the concentrations of 25(OH)D₂-d₆, 25(OH)D₃-d₆ and 3-epi-25(OH)D₃-d₃ were 500 ng/mL, 1.00 $\mu\text{g/mL}$ and 100 ng/mL, respectively) to 200 μL of serum, well-mixed, and added 1.0 mL of tert-butyl methyl ether. Then, the solution was centrifuged at 13,000 rpm for 5 min. Eight hundred microliters of supernatant was drawn into a clean Eppendorf tube and blow dry. Then, 100 μL of a 65% methanol solution containing 0.1% formic acid was added to the residue to reconstitute it, and the solution was mixed thoroughly for the test.

c. Chromatographic conditions. C18 chromatography column (Phenomenex, USA) was used for sample separation. The column temperature was 40°C , and the flow rate was 0.6 mL/min. The mobile phase A is an aqueous solution containing 0.1% formic acid, and mobile phase B is a methanol solution containing 0.1%

formic acid, gradient elution.

d. Mass spectrometry conditions. APCI+ ion source was used for mass spectrometry. Dwell time is 40 ms. The scanning mode of positive ion Multiple Reaction Monitoring (MRM) was used, and the expected ion structure uses the dehydration and hydrogenation peak $[M-H_2O+H]$. The ion pairs (precursor ion/quantitative product ion) of 25(OH)D₂, 25(OH)D₂-d₆, 25(OH)D₃, 25(OH)D₃-d₆, 3-epi-25-(OH)D₃ and 3-epi-25(OH)D₃-d₃ were 395.3/269.2 *m/z*, 419.2/229.3 *m/z*, 383.3/365.3 *m/z*, 389.3/263.3 *m/z*, 383.2/365.2 *m/z* and 386.4/368.2 *m/z*, respectively. The declustering voltages used to monitor 25(OH)D₂, 25(OH)D₂-d₆, 25(OH)D₃, 25(OH)D₃-d₆, 3-epi-25-(OH)D₃ and 3-epi-25(OH)D₃-d₃ were 10 V, 10 V, 130 V, 130 V, 130 V and 130 V, respectively. The collision energies were 25 eV, 22 eV, 16 eV, 19 eV, 16 eV and 19 eV, respectively.

e. Data collection. Analyst[®] MD software (version number: 1.6.3) was used for chromatogram output, Multiquant[™] MD software (version number: 3.0.2) was used for data processing. Both of them were AB SCIEX Triple Quad[™] 4500MD mass spectrometers (ABI, United States) supporting operating system.

3-epi-25(OH)D₃ analysis. Two hundred seventy-eight samples were randomly selected, including 147 healthy cases and 131 disease cases with different diseases. 3-epi-25(OH)D₃ was performed using isotope dilution UHPLC-MS/MS. The detection procedure for 3-epi-25(OH)D₃ is the same as in "Sample testing," except that the column used for 3-epi-25(OH)D₃ is replaced with an F5 chromatographic column, the column temperature is modified to 35°C, the flow rate is changed to 0.65 mL/min, and the dwell time of 3-epi-25(OH)D₃ is adjusted to 45 ms and that of 3-epi-25(OH)D₃-d₃ to 60 ms.

Statistical analysis. The results of this experiment were statistically analyzed with SPSS 25.0. Because the levels of vitamin D components in this study did not obey the normal distribution, the measured results were described by the median (interquartile range) [M (P₂₅, P₇₅)]. The difference in indicators between the healthy group and the disease group was compared, and we selected the two-sample Mann-Whitney *U* rank-sum test. The difference in the indicators among each disease group was compared, and we selected multiple independent samples Kruskal-Wallis *H* rank-sum test. Then, each pair was compared by Bonferroni method (adjust the alpha level method). In all statistical analysis, the difference between two sides with $p < 0.05$ was considered as significant.

RESULTS

Serum vitamin D component level of subject

The serum levels of 25(OH)D₂, 25(OH)D₃, 25(OH)D and 25(OH)D₃ activity equivalents [25(OH)D₃-AE] are shown in Table 1. Since the data in this group were not normally distributed, the Mann-Whitney *U* test was used to analyze the differences between the healthy group and the disease group. The results showed that the serum 25(OH)D₂ level ($z=4.49$, $p < 0.001$) and

Table 2. Serum vitamin D components of common diseases of minors. [M (P₂₅, P₇₅), unit: ng/mL]

Indicators	Healthy group	Short stature	Respiratory infections	Malnutrition	Tic disorder	H value, <i>p</i> value
Cases/male	1,204/662	270/167	433/263	175/99	188/160	62.87, <0.001
25(OH)D ₂	1.91 (0.80, 8.09)	1.44 (0.70, 4.16)*	1.61 (0.81, 5.68)	0.99 (0.57, 2.27)**	1.91 (0.93, 4.62)	48.83, <0.001
25(OH)D ₃	29.48 (22.31, 37.19)	25.44 (21.10, 30.26)**	28.22 (22.88, 36.01)	25.63 (21.41, 32.90)*	24.41 (20.73, 29.55)**	70.67, <0.001
25(OH)D	34.29 (27.13, 42.46)	28.08 (23.93, 33.50)**	32.64 (25.64, 39.97)	27.01 (23.18, 37.11)**	29.46 (25.10, 34.08)**	124.23, <0.001
25(OH)D ₃ -AE	30.91 (24.02, 38.92)	26.36 (22.34, 31.09)**	30.14 (23.90, 36.96)	25.92 (22.33, 34.38)**	26.38 (22.66, 31.08)**	89.65, <0.001
25(OH)D ₂ /25(OH)D ₃	0.07 (0.03, 0.26)	0.05 (0.03, 0.15)	0.06 (0.03, 0.19)	0.04 (0.02, 0.11)**	0.09 (0.04, 0.18)	29.41, 0.020

25(OH)D₃-AE = 25(OH)D₂/3 + 25(OH)D₃; * $p < 0.05$ compared with the healthy group; ** $p < 0.05$ compared with the respiratory infection group; # $p < 0.05$ compared with the tic disorder group.

Fig. 1. UHPLC-MS/MS spectra of typical serum 25(OH)D₃ metabolites. The mass spectrum peak of 3-epi-25(OH)D₃ is closely related to 25(OH)D₃. 25(OH)D₃ and 3-epi-25(OH)D₃ elute at 3.39 min and 3.47 min, respectively, which was very close.

serum 25(OH)D₃ level ($z=6.02$, $p<0.001$), 25(OH)D level ($z=9.17$, $p<0.001$) and 25(OH)D₃-AE level ($z=7.27$, $p<0.001$) in the disease group were significantly lower than those in the healthy group, respectively.

Serum 25(OH)D₂ and 25(OH)D₃ levels of subject

Among the subjects, the serum 25(OH)D₂ levels from high to low were the healthy group, tic disorder group, respiratory tract infection group, short stature group and malnutrition group, as shown in Table 2. The median levels of them were 1.91 (0.80, 8.09) ng/mL, 1.91 (0.93, 4.62) ng/mL, 1.61 (0.81, 5.68) ng/mL, 1.44 (0.70, 4.16) ng/mL and 0.99 (0.57, 2.27) ng/mL, respectively. There were significant statistical differences among groups ($H=48.83$, $p<0.001$). By Bonferroni method, the serum 25(OH)D₂ level in the malnourished group was significantly lower than that in the both healthy group ($z=6.33$, $p<0.001$), respiratory tract infection group ($z=4.57$, $p<0.001$) and the tic disorder group ($z=4.82$, $p<0.001$); the serum 25(OH)D₂ level in the short stature group was significantly lower than that in the healthy group ($z=3.64$, $p=0.003$). Among the subjects, the serum 25(OH)D₃ levels from high to low were the healthy group, respiratory infection group, malnutrition group, short stature group, and tic disorder group. The median levels of them were 29.48 (22.31, 37.19) ng/mL, 28.22 (22.88, 36.01) ng/mL, 25.63 (21.41, 32.90) ng/mL, 25.44 (21.10, 30.26) ng/mL and 24.41 (20.73, 29.55) ng/mL, respectively. There were statistical differences among groups ($H=70.67$, $p<0.001$). By Bonferroni method, the serum 25(OH)D₃ level in both the short stature group and the tic disorder group was significantly lower than that in both the healthy group ($z=5.88$ and 6.44 , all $p<0.001$) and respiratory infection group ($z=4.40$ and 5.15 , all $p<0.001$); the serum 25(OH)D₃ level in the malnutri-

tion group was significantly lower than that in the healthy group ($z=3.14$, $p=0.017$).

Serum 25(OH)D and 25(OH)D₃-AE levels of subject

Among the subjects, the levels of 25(OH)D from high to low were the healthy group, respiratory tract infection group, tic disorder group, short stature group and malnutrition group, as shown in Table 2. The median levels of them were 34.29 (27.13, 42.46) ng/mL, 32.64 (25.64, 39.97) ng/mL, 29.46 (25.10, 34.08) ng/mL, 28.08 (23.93, 33.50) ng/mL and 27.01 (23.18, 37.11) ng/mL, respectively. There were statistical differences among groups ($H=124.23$, $p<0.001$). By Bonferroni method, the serum 25(OH)D level in the short stature group ($z=8.96$, $p<0.001$), malnutrition group ($z=8.96$, $p<0.001$) and tic disorder group ($z=5.93$, $p<0.001$) were significantly lower than that in the healthy group. In addition, the serum 25(OH)D level in the short stature group ($z=5.75$, $p<0.001$), malnutrition group ($z=4.15$, $p<0.001$), and tic disorder group ($z=3.53$, $p=0.004$) was significantly lower than that in the respiratory infection group.

According to some previous reports (16–20), the activity of 25(OH)D₃ is 2–3 times higher than that of 25(OH)D₂. In order to investigate the storage level of vitamin D more strictly, the maximum difference between 25(OH)D₂ and 25(OH)D₃ activity was assumed to be three times. It is converted into 25(OH)D₃ activity that 25(OH)D₂ activity was divided by 3, and then 25(OH)D₃ activity was added. The two were combined to form 25(OH)D₃ activity equivalents [25(OH)D₃-AE], namely 25(OH)D₃-AE=25(OH)D₂/3+25(OH)D₃. The results are shown in Table 2. Among the subjects, 25(OH)D₃-AE levels were from high to low in the healthy group, respiratory tract infection group, tic disorder group, short stature group and malnutrition

Table 3. The detection range and estimation interval of the subject's serum vitamin D components.

Indicators	Healthy group (n=147)		Disease group (n=131)		z value	p value
	R (Min, Max)	M (P ₂₅ , P ₇₅)	R (Min, Max)	M (P ₂₅ , P ₇₅)		
25(OH)D ₂	16.54 (0.23, 16.77)	1.36 (0.62, 6.15)	40.64 (0.06, 40.70)	1.17 (0.48, 5.19)	1.07	0.286
25(OH)D ₃	28.26 (9.38, 37.64)	27.09 (20.46, 31.14)	47.25 (5.59, 52.84)	24.87 (15.74, 30.23)	1.56	0.118
25(OH)D	25.99 (17.97, 43.96)	30.34 (23.47, 34.60)	61.62 (7.79, 69.40)	28.56 (20.82, 37.43)	1.51	0.132
3-epi-25(OH)D ₃	10.72 (0.17, 10.89)	2.39 (1.27, 3.79)	14.59 (0.18, 14.77)	1.31 (0.77, 2.68)	4.49	<0.001
3-epi-25(OH)D ₃ /25(OH)D ₃	0.32 (0.01, 0.33)	0.11 (0.06, 0.16)	0.71 (0.01, 0.71)	0.07 (0.03, 0.13)	3.60	<0.001
3-epi-25(OH)D ₃ /25(OH)D	0.32 (0.01, 0.33)	0.08 (0.06, 0.11)	0.33 (0.01, 0.34)	0.06 (0.03, 0.10)	3.80	<0.001
25(OH)D ₂ /25(OH)D ₃	1.11 (0.01, 1.12)	0.05 (0.02, 0.26)	3.74 (0.00, 3.74)	0.06 (0.02, 0.32)	0.16	0.873

M: median, R: range, Min: minimum, Max: maximum, P: percentiles.

group. The median levels of them were 30.91 (24.02, 38.92) ng/mL, 30.14 (23.90, 36.96) ng/mL, 26.38 (22.66, 31.08) ng/mL, 26.36 (22.34, 31.09) ng/mL and 25.92 (22.33, 34.38) ng/mL, respectively. There were statistical differences among the groups ($H=89.65$, $p<0.001$). By Bonferroni method, the serum 25(OH)D₃-AE level in both the short stature group ($z=7.16$, $p<0.001$), malnutrition group ($z=4.47$, $p<0.001$) and the tic disorder group ($z=6.41$, $p<0.001$) was significantly lower than that in the healthy group. In addition, the serum 25(OH)D₃-AE level in the short stature group ($z=5.08$, $p<0.001$), malnutrition group ($z=3.06$, $p=0.022$), tic disorder group ($z=4.75$, $p<0.001$) was significantly lower than that in the respiratory infection group.

3-epi-25(OH)D₃ analysis

In this experiment, the 3-epi-25(OH)D₃ level of 278 serum samples was evaluated by isotope dilution mass spectrometry. We adjusted the mass spectrometry conditions and replaced the column to achieve the effective separation of 3-epi-25(OH)D₃ and 25(OH)D₃ peaks (Fig. 1). The results showed that 25(OH)D₃ and 3-epi-25(OH)D₃ eluted at 3.39 min and 3.47 min, respectively, which was very close. In addition, the mass spectrum peak of 3-epi-25(OH)D₃ was closely related to 25(OH)D₃. Then, we used MRM to quantify the 3-epi-25(OH)D₃ after introducing the 3-epi-25(OH)D₃ standard and the isotope internal standard (Table 3). Because the data in this group were not normally distributed, Mann-Whitney U test was used to analyze the differences between the healthy and disease groups. The 25(OH)D₂ ($z=1.07$, $p=0.286$), 25(OH)D₃ ($z=1.56$, $p=0.118$), 25(OH)D₂/25(OH)D₃ ($z=0.16$, $p=0.873$) and 25(OH)D ($z=1.51$, $p=0.132$) levels of the disease group for the randomly sampled serum samples were not statistically different from that of the healthy group, and these changes were inconsistent with the changes in the overall serum samples. However, the serum 3-epi-25(OH)D₃ ($z=4.49$, $p<0.001$) level in the disease group was significantly lower than that in the healthy group, its ratio to 25(OH)D₃ (i.e., 3-epi-25(OH)D₃/25(OH)D₃) ($z=3.60$, $p<0.001$) and its ratio to 25(OH)D [i.e., 3-epi-25(OH)D₃/25(OH)D] ($z=3.80$, $p<0.001$) in the disease group is lower than that in the healthy group.

Vitamin D nutritional status

According to the recommendation of the Institute of Medicine (IOM) (21), the nutritional status of vitamin D is evaluated by the serum 25(OH)D level [equal to the sum of 25(OH)D₂ and 25(OH)D₃ levels]. The standard is: 25(OH)D $\leq$ 20 ng/mL is deficiency; 25(OH)D between 21 and 29 ng/mL is insufficient; 25(OH)D $\geq$ 30 ng/mL is sufficient.

We used 25(OH)D level to analyze the vitamin D storage status of the subject in this group. Children may consciously take vitamin D, and the season was summer. Thus, as shown in Fig. 2A, 65.4% of healthy subjects and 50.5% of disease subjects were sufficient in vitamin D. The proportion of patients with sufficient vitamin D in the disease group that less than 50% are patients with short stature (40.0%), malnutrition

Fig. 2. Vitamin D nutritional status. Deficiency: ≤ 20 ng/mL, insufficient: 21–29 ng/mL, sufficient: ≥ 30 ng/mL. A: nutritional status judged by 25(OH)D, B: nutritional status judged according to 25(OH)D₃-AE. 25(OH)D₃-AE = 25(OH)D₂/3 + 25(OH)D₃.

(43.4%) and tic disorder (48.4%). However, the activity of 25(OH)D₂ is only 1/3 to 1/2 that of 25(OH)D₃. After the conversion of 25(OH)D₂ activity to 25(OH)D₃-AE, only the healthy group (53.2%) and respiratory infection group (50.1%) had just over half of the subjects were sufficient in vitamin D (Fig. 2B). Less than one third of the patients in short stature group (29.6%) and tic disorder group (29.3%) had sufficient vitamin D. In addition, 70.4% of the patients in short stature group and 70.7% of the patients in tic disorder group were insufficiency of vitamin D.

DISCUSSION

Vitamin D deficiency has become a global public health problem. More and more researchers pay attention to serum vitamin D level. This is limited to not only their nutritional status, but also the occurrence devel-

opment, efficacy monitoring, and prognostic evaluation of many diseases, such as heart disease, kidney disease, lung disease, cancer, diabetes, hypertension, schizophrenia and autoimmunity diseases (22–24). However, the medical in-depth research on vitamin D detection technology was insufficient in the past. Those studies focused on the relationship between a certain disease and 25(OH)D level. However, there are few studies on the composition and determination of vitamin D components and their correlation with disease development.

In this study, UHPLC-MS/MS technology was used to analyze the serum vitamin D components in minor healthy subjects and patients with certain diseases. The levels of serum 25(OH)D₂, 25(OH)D₃ and 25(OH)D in the minor disease group were lower than those in the healthy group, respectively. It means that minors may cause the decrease of vitamin D levels after the disease

occurs, and vice versa. Although it has not been proven that vitamin D is an antioxidant, some studies have found that vitamin D can regulate multiple cellular pathways to synthesize antioxidant substances. This helps us to resist the oxidation of reactive oxygen species (ROS) and nitric oxide, and prevent oxidative damage to tissue cells (25). If vitamin D is at a low level in the body for a long time, the individual's ability to resist ROS will decrease. Then, the possibility of oxidative damage to tissue cells will increase, leading to an increased probability of disease occurrence and development. This may be one of the important mechanisms discovered in recent years that vitamin D is related to the occurrence and development of diseases. Of course, it is well known that vitamin D is involved in bone formation, so vitamin D is consumed in the process of human growth and development. In addition, malnutrition, endocrine disorders and bone metabolism disorders can lead to the decrease of vitamin D level (26–28). Because the sampling of this study was only a certain time point of patients, the follow-up observation was left out, the results can only generally speculate on the possible reasons for the decrease of vitamin D level. It is necessary to set up separate disease controls and conduct long-term follow-up study to understand which cause the decrease of vitamin D level in patients.

25(OH)D₂ and its metabolites have a lower ability to bind with vitamin D binding proteins than 25(OH)D₃. In addition, 25(OH)D₂ has a non-physiological metabolic form, and its half-life is shorter than 25(OH)D₃. The biological efficacy of 25(OH)D₂ is significantly lower than that of 25(OH)D₃. Thus, 25(OH)D₃'s ability to store vitamin D are two to three times higher than that of 25(OH)D₂ (29, 30). Therefore, the 25(OH)D₃ level in the body is much higher than the 25(OH)D₂ level, and the 25(OH)D₂/25(OH)D₃ ratio is extremely low under normal physiological conditions. If the patients have excessive 25(OH)D₂ levels but 25(OH)D₃ is significantly reduced due to some unknown reasons, it may lead to the illusion of sufficient storage of vitamin D. The study showed that after the activity of 25(OH)D₂ is converted to 25(OH)D₃, that is, when the activity vitamin D nutritional status is evaluated at the level of 25(OH)D₃-AE, the median level of vitamin D are decreased regardless of whether they are healthy or not. This may be the reason why the components of serum vitamin D need to be tested. This is the only way to correctly assess the vitamin D nutritional level. However, almost all existing clinical laboratories use immunolabeling technology (i.e., chemiluminescence) to detect the total serum 25(OH)D level for the evaluation of vitamin D in the body. This results in not only equating the biological activity of 25(OH)D₂ to 25(OH)D₃, but also ignoring the presence of 3-epi-25(OH)D₃ in the sample. This kind of evaluation method is extremely improper or even wrong. The index that can evaluate the level of vitamin D is the 25(OH)D₃-AE in this paper. This study shows that more than two-thirds of patients are deficiency or insufficient in vitamin D, after various vitamin D components are converted to 25(OH)D₃-AE.

3-epi-25(OH)D₃ can be formed by 25-hydroxyvitamin D₃-3-epimerase, which reverses the position of the hydroxyl group on the third carbon atom of 25(OH)D₃ from 3 β to 3 α (31). 3-epi-25(OH)D₃ and 25(OH)D₃ have the same molecular weight and structural formula, only the spatial conformations of them are different, which has less biological activity (30). Therefore, at the same 25(OH)D₃ level, the high 3-epi-25(OH)D₃ content results in decreased vitamin D. Because the metabolic pathway of vitamin D is immature in children, especially infants under 1 y old, C-3 isomerization may be one of the major metabolic pathways for 25(OH)D. Thus, 3-epi-25(OH)D₃ level in infants under 1 y old is high (32). The analysis results of the healthy group can further corroborate this conclusion. However, some studies have found that with the increase of infants' age, if the serum 3-epi-25(OH)D₃ concentration remains high, it indicates that the vitamin D metabolic pathway may mature slowly (32, 33). When a disease status, the median value and interval of 3-epi-25(OH)D₃ of disease group are usually higher than those of healthy group, respectively (this may vary according to the disease). This indicates that certain diseases may affect the normal metabolism of vitamin D in the body, thus affecting the body's vitamin D storage level (34). Actually, there were several reasons that might cause the lower serum 3-epi-25(OH)D₃ level in the disease group in our study. Firstly, serum 25(OH)D₃ level in the disease group were found lower than those in the healthy group. Since 3-epi-25(OH)D₃ is produced from 25(OH)D₃ by 25(OH)D₃-3-epimerase (31), and several studies have reported the positive association between 25(OH)D₃ and 3-epi-25(OH)D₃ (35, 36). It can be deduced that the decrease of 3-epi-25(OH)D₃ level may be due to the decrease of 25(OH)D₃ concentration. Secondly, this may be attributable to sampling. From the perspective of age distribution, although individuals in two groups were all below 18 y old, the age structure of the disease group was older than the healthy group in this study. It has been known that, with age increased in childhood, 3-epi-25(OH)D₃ level usually decreased (36). Therefore, depending on the older age structure, the serum 3-epi-25(OH)D₃ level in the disease group was reduced. Thirdly, the "disease group" in this study was composed of multiple diseases (respiratory infections, tic disorders, short stature and malnutrition), rather than a specific disease. Therefore, we speculated that lower serum level of 3-epi-25(OH)D₃ in the disease group might be related to the population of subjects included, compensatory regulatory mechanisms of various disease, and the disease progression status. In addition, 25(OH)D₂ has epimerization like 25(OH)D₃ (37). However, the content of 25(OH)D₂ in the human body is less than that of 25(OH)D₃ under normal physiological conditions and even in most disease conditions (34, 38, 39). In addition to research, the clinical detection of the epimer of 25(OH)D₂ is of minor significance for the evaluation of vitamin D storage. This is why we did not analyze 25(OH)D₂ epimers in this paper.

Due to different sources, many clinical and labora-

tory experts jointly recommend that vitamin D detection method should be able to detect 25(OH)D₂ and 25(OH)D₃ at the same time, so as to accurately confirm the source of vitamin D storage form in the subjects, and then vitamin D can be sufficient and reasonably supplemented (10). Furthermore, both of 25(OH)D₂ and 25(OH)D₃ can also be transformed into inactive or very low active C3 epimers under the action of 3-epimerase, especially the metabolic pathway of vitamin D is not mature in infancy (32), and the metabolic pathway of vitamin D may change to C3 epimers under the disease condition (34). Therefore, the simultaneous determination of 25(OH)D₂, 25(OH)D₃ and their C3 epimers is very important.

At present, no medical institution or research unit has provided reference ranges for 25(OH)D₂, 25(OH)D₃ and their ratios [25(OH)D₂/25(OH)D₃], because existing clinicians only evaluate the vitamin D storage in the body based on the 25(OH)D level. Reasons are as follows. First, the immunological detection methods used in existing clinical laboratories cannot yet detect the levels of 25(OH)D₂ and 25(OH)D₃, respectively. Second, most researchers think that the 25(OH)D₂ level in the body is much lower than the 25(OH)D₃ level, and the change of 25(OH)D₂ has little effect on the total 25(OH)D level. Third, few researchers can pay attention to the effect of 3-epi-25(OH)D₃ on 25(OH)D levels. However, the same 25(OH)D concentration shows different vitamin D storage levels resulting from the different composition of 25(OH)D₂, 25(OH)D₃ and 3-epi-25(OH)D₃. Therefore, it is necessary to determine the vitamin D components including 25(OH)D₂, 25(OH)D₃ and 3-epi-25(OH)D₃ for accurately assessing the vitamin D nutritional status. The correct selection of vitamin D detection method and the necessary component detection are essential for accurate understanding and evaluation of the vitamin D concentration.

Authorship

HYY and LYM performed the experiments. CYC and WBT participated in experiment preparation. LYM and YYW performed the data analyses. FJF participated in the study design and final review of the manuscript. HYY, LYM and FJF wrote and edited the manuscript. All authors read and approved the final manuscript for publish.

HYY and LYM contributed equally to this work.

Disclosure of state of COI

The authors declare that there is no conflict of interest.

Data availability statement

The data used to support the findings of this study are available from the corresponding author upon request.

Acknowledgments

We give our sincere gratitude to support and assistance of all laboratory personnel.

Funding

This work was supported by Science and Technology Department of Sichuan Province (2015SZ0117).

REFERENCES

- 1) Jones G, Kaufmann M. 2019. Update on pharmacologically-relevant vitamin D analogues. *Br J Clin Pharmacol* **85**: 1095–1102.
- 2) Charoenngam N, Shirvani A, Holick MF. 2019. Vitamin D for skeletal and non-skeletal health: What we should know. *J Clin Orthop Trauma* **10**: 1082–1093.
- 3) Alcubierre N, Castelblanco E, Martínez-Alonso M, Granado-Casas M, Esquerda A, Traveset A, Martinez-Gonzalez D, Franch-Nadal J, Mauricio D. 2018. Vitamin D deficiency is associated with poorer satisfaction with diabetes-related treatment and quality of life in patients with type 2 diabetes: a cross-sectional study. *Health Qual Life Outcomes* **16**: 44.
- 4) Lips P, Cashman KD, Lamberg-Allardt C, Bischoff-Ferrari HA, Obermayer-Pietsch B, Bianchi ML, Stepan J, El-Hajj Fuleihan G, Bouillon R. 2019. Current vitamin D status in European and Middle East countries and strategies to prevent vitamin D deficiency: a position statement of the European Calcified Tissue Society. *Eur J Endocrinol* **180**: 23–54.
- 5) Miettinen ME, Niinistö S, Erlund I, Cuthbertson D, Nucci AM, Honkanen J, Vaarala O, Hyöty H, Krischer JP, Knip M, Virtanen SM; TRIGR Investigators. 2020. Serum 25-hydroxyvitamin D concentration in childhood and risk of islet autoimmunity and type 1 diabetes: the TRIGR nested case-control ancillary study. *Diabetologia* **63**: 780–787.
- 6) Tsai TY, Huang YC. 2018. Vitamin D deficiency in patients with chronic and acute urticaria: A systematic review and meta-analysis. *J Am Acad Dermatol* **79**: 573–575.
- 7) Cepeda SJ, Zenteno AD, Fuentes SC, Bustos BR. 2019. Vitamin D and pediatric respiratory diseases. *Rev Chil Pediatr* **90**: 94–101.
- 8) Vasile M, Corinaldesi C, Antinozzi C, Crescioli C. 2017. Vitamin D in autoimmune rheumatic diseases: a view inside gender differences. *Pharmacol Res* **117**: 228–241.
- 9) Xu DM, Liang JH, Wang L, Zhu HY, Xia Y, Fan L, Li JY, Xu W. 2020. 25-Hydroxy vitamin D deficiency predicts inferior prognosis in mantle cell lymphoma. *J Cancer Res Clin Oncol* **146**: 1003–1009.
- 10) Souberbielle JC, Body JJ, Lappe JM, Plebani M, Shoenfeld Y, Wang TJ, Bischoff-Ferrari HA, Cavalier E, Ebeling PR, Fardellone P, Gandini S, Gruson D, Guérin AP, Heikendorff L, Hollis BW, Ish-Shalom S, Jean G, von Landenberg P, Largura A, Olsson T, Pierrot-Deseilligny C, Pilz S, Tincani A, Valcour A, Zittermann A. 2010. Vitamin D and musculoskeletal health, cardiovascular disease, autoimmunity and cancer: Recommendations for clinical practice. *Autoimmun Rev* **9**: 709–715.
- 11) Makris K, Sempos C, Cavalier E. 2020. The measurement of vitamin D metabolites: part I—metabolism of vitamin D and the measurement of 25-hydroxyvitamin D. *Hormones (Athens)* **19**: 81–96.
- 12) de la Hunt A, Wallace AM, Gibson S, Viljakainen H, Lamberg-Allardt C, Ashwell M. 2010. UK Food Standards Agency Workshop Consensus Report: the choice of method for measuring 25-hydroxyvitamin D to estimate vitamin D status for the UK National Diet and Nutrition Survey. *Br J Nutr* **104**: 612–619.

- 13) Ding S, Schoenmakers I, Jones K, Koulman A, Prentice A, Volmer DA. 2010. Quantitative determination of vitamin D metabolites in plasma using UHPLC-MS/MS. *Anal Bioanal Chem* **398**: 779–789.
- 14) Hu T, Li H, Liu H, Cong L, Liu L, An Z. 2020. High throughput UHPLC-MS/MS method for the simultaneous quantification of six vitamin D metabolites: application for vitamin D determination in patients after liver or kidney transplantation. *Anal Methods* **12**: 5591–5600.
- 15) Abouzid M, Karaźniewicz-Lada M, Pawlak K, Burchardt P, Kruszyna L, Głowska E. 2020. Measurement of plasma 25-hydroxyvitamin D₂, 25-hydroxyvitamin D₃ and 3-epi-25-hydroxyvitamin D₃ in population of patients with cardiovascular disease by UPLC-MS/MS method. *J Chromatogr B Analyt Technol Biomed Life Sci* **1159**: 122350.
- 16) Armas LA, Hollis BW, Heaney RP. 2004. Vitamin D₂ is much less effective than vitamin D₃ in humans. *J Clin Endocrinol Metab* **89**: 5387–5391.
- 17) Glendenning P, Chew GT, Seymour HM, Gillett MJ, Goldswain PR, Inderjeeth CA, Vasikaran SD, Taranto M, Musk AA, Fraser WD. 2009. Serum 25-hydroxyvitamin D levels in vitamin D-insufficient hip fracture patients after supplementation with ergocalciferol and cholecalciferol. *Bone* **45**: 870–875.
- 18) Binkley N, Gemar D, Engelke J, Gangnon R, Ramamurthy R, Krueger D, Drezner MK. 2011. Evaluation of ergocalciferol or cholecalciferol dosing, 1,600 IU daily or 50,000 IU monthly in older adults. *J Clin Endocrinol Metab* **96**: 981–988.
- 19) Heaney RP, Recker RR, Grote J, Horst RL, Armas LA. 2011. Vitamin D₃ is more potent than vitamin D₂ in humans. *J Clin Endocrinol Metab* **96**: E447–E452.
- 20) Tripkovic L, Wilson LR, Hart K, Johnsen S, de Lusignan S, Smith CP, Bucca G, Penson S, Chope G, Elliott R, Hippönen E, Berry JL, Lanham-New SA. 2017. Daily supplementation with 15 µg vitamin D₂ compared with vitamin D₃ to increase wintertime 25-hydroxyvitamin D status in healthy South Asian and white European women: a 12-wk randomized, placebo-controlled food-fortification trial. *Am J Clin Nutr* **106**: 481–490.
- 21) Aloia JE. 2011. The 2011 report on dietary reference intake for vitamin D: where do we go from here. *J Clin Endocrinol Metab* **96**: 2987–2996.
- 22) Osorio Landa HK, Perez Diaz I, Laguna Barcenás SDC, López Navarro JM, Abella Roa ME, Corral Orozco M, Mancilla Ortega JP, Martínez Duarte DA, Morales Montalvo SI, Múzquiz Aguirre S, Sánchez Landa E. 2020. Association of serum vitamin D levels with chronic disease and mortality. *Nutr Hosp* **37**: 335–342.
- 23) L'Espérance K, Datta GD, Qureshi S, Koushik A. 2020. Vitamin D exposure and ovarian cancer risk and prognosis. *Int J Environ Res Public Health* **17**: 1168.
- 24) Yamamoto EA, Jørgensen TN. 2020. Relationships between vitamin D, gut microbiome, and systemic autoimmunity. *Front Immunol* **10**: 3141.
- 25) Câmara AB, Brandão IA. 2021. The relationship between vitamin D deficiency and oxidative stress can be independent of age and gender. *Int J Vitam Nutr Res* **91**: 108–123.
- 26) Jones KDJ, Hachmeister CU, Khasira M, Cox L, Schoenmakers I, Munyi C, Nassir HS, Hüntner-Kirsch B, Prentice A, Berkley JA. 2018. Vitamin D deficiency causes rickets in an urban informal settlement in Kenya and is associated with malnutrition. *Matern Child Nutr* **14**: e12452.
- 27) Mokhtar RR, Holick MF, Sempértegui F, Griffiths JK, Estrella B, Moore LL, Fox MP, Hamer DH. 2018. Vitamin D status is associated with underweight and stunting in children aged 6–36 months residing in the Ecuadorian Andes. *Public Health Nutr* **21**: 1974–1985.
- 28) Jawa A, Riaz SH, Khan Assir MZ, Afreen B, Riaz A, Akram J. 2016. Causes of short stature in Pakistani children found at an Endocrine Center. *Pak J Med Sci* **32**: 1321–1325.
- 29) Callejo M, Mondejar-Parreño G, Esquivel-Ruiz S, Olivenza MA, Moreno L, Blanco I, Escribano-Subías P, Cogolludo A, Barbera JA, Perez-Vizcaino E. 2020. Total, bioavailable, and free vitamin D levels and their prognostic value in pulmonary arterial hypertension. *J Clin Med* **9**: 448.
- 30) Charoenngam N, Shirvani A, Holick MF. 2019. Vitamin D for skeletal and non-skeletal health: What we should know. *J Clin Orthop Trauma* **10**: 1082–1093.
- 31) Al-Zohily B, Al-Menhali A, Gariballa S, Haq A, Shah I. 2020. Epimers of vitamin D: A review. *Int J Mol Sci* **21**: 470.
- 32) Yazdanpanah M, Bailey D, Walsh W, Wan B, Adeli K. 2013. Analytical measurement of serum 25-OH-vitamin D₃, 25-OH-vitamin D₂ and their C₃-epimers by LC-MS/MS in infant and pediatric specimens. *Clin Biochem* **46**: 1264–1271.
- 33) Bailey D, Veljkovic K, Yazdanpanah M, Adeli K. 2013. Analytical measurement and clinical relevance of vitamin D(3) C₃-epimer. *Clin Biochem* **46**: 190–196.
- 34) Li D, Jeffery LE, Jenkinson C, Harrison SR, Chun RE, Adams JS, Raza K, Hewison M. 2019. Serum and synovial fluid vitamin D metabolites and rheumatoid arthritis. *J Steroid Biochem Mol Biol* **187**: 1–8.
- 35) Gjerde J, Kjellevold M, Dahl L, Berg T, Bøkevoll A, Markhus MW. 2020. Validation and determination of 25(OH) vitamin D and 3-Epi25(OH)D₃ in breastmilk and maternal- and infant plasma during breastfeeding. *Nutrients* **12**: 2271.
- 36) Berger SE, Van Rompay MI, Gordon CM, Goodman E, Eliasziw M, Holick MF, Sackeck JM. 2018. Investigation of the C-3-epi-25(OH)D₃ of 25-hydroxyvitamin D₃ in urban schoolchildren. *Appl Physiol Nutr Metab* **43**: 259–265.
- 37) Tuckey RC, Cheng CYS, Slominski AT. 2019. The serum vitamin D metabolome: What we know and what is still to discover. *J Steroid Biochem Mol Biol* **186**: 4–21.
- 38) Harvey SM, Murphy VE, Gibson PG, Clarke M, Jensen ME. 2020. The impact of sample type on vitamin D quantification and clinical classification during pregnancy. *Nutrients* **12**: 3872.
- 39) Lin YC, Lee HH, Tseng SC, Lin KD, Tseng LP, Lee JF, Lee YH, Chen BH. 2019. Quantitation of serum 25(OH)D₂ and 25(OH)D₃ concentrations by liquid chromatography tandem mass spectrometry in patients with diabetes mellitus. *J Food Drug Anal* **27**: 510–517.