Journal of Clinical Laboratory Analysis 27: 284–289 (2013)

Measurement of Serum Total Vitamin D (25-OH) Using Automated Immunoassay in Comparision With Liquid Chromatography Tandem-Mass Spectrometry

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Background: The associations of vitamin D deficiency with many nonskeletal diseases are still being discovered. We evaluated the use of an automated immunoassay to measure serum total vitamin D (25-OH) and assessed vitamin D status in a Korean adult population. *Methods:* We compared the Elecsys Vitamin D (25-OH) Total Assay (Roche Diagnostics) with liquid chromatography-tandem mass spectrometry (LC-MS/MS) using 300 serum samples. Total imprecision was calculated using three levels of quality control materials and serum samples. We also investigated the vitamin D status using data for 70,762 cases who had a routine health check-up in our hospitals. *Results:* The regression equation: Elecsys $=$ 0.882 $\times$ LC-MS/MS $+$ 6.814 ($r = 0.926$). Total imprecision was within 10% for all quality control materials and serum samples. The prevalence of vitamin D deficiency using cut-off values of $<$50 nmol/l ($<$20 ng/ml) were 70.3% in males and 86.4% in females, respectively. The prevalence of vitamin D deficiency was higher in younger subjects than in older subjects (P for linear-by-linear association was $<$0.001). Serum vitamin D levels were highest in September and lowest in February. *Conclusion:* The Elecsys Vitamin D (25-OH) Total Assay was comparable to LC-MS/MS and appropriate for routine clinical use. Vitamin D deficiency is common in Korean adults. J. Clin. Lab. Anal. 27:284–289, 2013.

Key words: vitamin D; LC-MS/MS ; electrochemiluminescence

INTRODUCTION

Vitamin D is an essential nutrient for human beings. Serum or plasma 25-hydroxy (25-OH) vitamin D is the major form of vitamin D and the best single indicator of vitamin D status (1). 25-OH vitamin D exists in two forms: 25-OH vitamin D_3 and 25-OH vitamin D_2. Only 25-OH vitamin D_3 is synthesized in skin and is the major form of 25-OH vitamin D; 25-OH vitamin D_2 is the minor form and is only detectable in serum or plasma when subjects take a supplement containing vitamin D_2 (2).

Vitamin D deficiency and insufficiency are associated with skeletal disease, rickets, osteomalacia, etc. An association between nonskeletal diseases (cardiovascular diseases, diabetes mellitus, cancer, etc.) and vitamin D deficiency was recently discovered (3), and the need for evaluating vitamin D status is thus increasing (4). However, there are no definite cut-off values defining vitamin D deficiency or insufficiency. Although some institutions recommend cut-off values for deficiency and insufficiency, the clinical correlations for these cut-off values are not clear (5, 6). There are many methods to measure vitamin D, including radio-immunoassay (RIA), high-performance liquid chromatography (HPLC), and liquid chromatography-tandem mass spectrometry

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Received 13 November 2012; Accepted 4 February 2013
DOI 10.1002/jcla.21598
Published online in Wiley Online Library (wileyonlinelibrary.com).

(LC-MS/MS). LC-MS/MS is considered the standard method to measure serum or plasma vitamin D. Several automated immunoassays have been introduced for clinical use. Recently, Roche Diagnostics launched the Vitamin D (25-OH) Total Assay (Roche, Basel, Switzerland) for clinical use.

We compared the Roche Vitamin D (25-OH) Total Assay with LC-MS/MS. We also assessed vitamin D status in a Korean adult population.

MATERIALS AND METHODS

Subjects

We used data for 70,762 adult subjects, aged 20–79 years, who had a routine health check-up in our hospitals between January 2011 and June 2012.

Roche Elecsys Vitamin D (25-OH) Total Assay

The Elecsys uses a competitive immunoassay for the detection of total vitamin D (25-OH). By incubating the samples with a pretreatment reagent (dithiolthretiol, sodium hydroxide), bound vitamin D (25-OH) is released from the vitamin D binding protein. By incubating the pretreated sample with ruthenium-labeled vitamin D binding protein, a complex between the vitamin D (25-OH) and the ruthenylated vitamin D binding protein is formed. After the addition of streptavidin-coated microparticles and vitamin D (25-OH) labeled with biotin, unbound ruthenium-labeled vitamin D binding proteins become bound. A complex consisting of the ruthenylated vitamin D binding protein and the biotinylated vitamin D (25-OH) is formed and becomes bound to the solid phase via interaction of biotin and streptavidin.

Liquid Chromatography-Tandem Mass Spectrometry

We analyzed vitamin D levels by LC-MS using previously described methods (7). The internal standard (IS) 26, 27-hexadeuteriumlabeled 25-OHvitamin D_3 was purchased from Medical Isotope, Inc. (Pelham, NH). Specimens were analyzed with the Acquity ultra performance liquid chromatography separation module (Waters, Milford, MA) using a BEH C18 column (2.1 × 50 mm, 1.7 μm). The Quattro Premier tandem mass spectrometer (Waters) was operated in positive electrospray mode, and the samples were examined using multiple reaction monitoring with the following (m/z) precursor/product ion transitions: 25(OH) D_3 558.35/298.1; 25(OH)D_2 570.35/298.1; and IS 564.35/298.1. Total run time for a sample was 3 min. Integration of peak area and data analysis were performed using QuanLynx 4.0 software (Waters).

Method Comparison and Imprecision Analysis

We compared Elecsys with LC-MS/MS using 300 of the 70,762 samples. In addition, we calculated linear regression and plotted a Bland–Altman graph. Total CV% was calculated using three levels of serum samples and quality control materials (Roche).

Vitamin D Status

A total of 70,762 serum samples were used to assess vitamin D status in a Korean adult population. The prevalence of vitamin D deficiency and insufficiency were calculated using generally accepted cut-off values (<20 ng/ml: <50 nmol/l and <30 ng/ml: <75 nmol/l) (5, 6). Vitamin D deficiency and insufficiency were calculated by age group, and we investigated vitamin D status according to the month of sample collection.

Statistics

Continuous data for the two groups were compared using the Student's *t*-test or Mann-Whitney test. Linear-by-linear association was used to evaluate trends in vitamin D deficiency and insufficiency according to age. A P value <0.05 was considered statistically significant. Statistical analysis was done with PASW Statistics 20.0 (IBM, Armonk, New York, USA).

RESULTS

The regression equation was: Elecsys = 0.882 (95% confidential interval: 0.841–0.923, $P < 0.05$) × LC-MS/MS + 6.814 (95% confidential interval: 4.494–9.134, $P < 0.05$) ($r = 0.926$). The Elecsys showed positive bias compared to LC-MS/MS (bias = 1.044 ± 21.62 nmol/l). The linear regression graph and Bland–Altman plot are shown in Figure 1. Total imprecisions were 4.1%, 2.7%, and 1.5%, at PreciControl 1, 2, and 3, respectively (Table 1). Total imprecisions were 5.8%, 5.0%, and 3.4%, at samples 1, 2, and 3, respectively. The characteristics of the 70,762 cases enrolled in our study are shown in Table 2. Serum vitamin D (25-OH) levels were higher in males (45 ± 15.3 nmol/l) than females (35 ± 15.5 nmol/l) ($P < 0.001$) (Table 2). The prevalence of vitamin D deficiency and insufficiency according to the cut-off values of <50 nmol/l and 50–75 nmol/l were 70.3% and 25.3% in males and 86.4% and 11.0% in females, respectively (Fig. 2). The prevalence of vitamin D insufficiency and deficiency were higher in young subjects than older subjects (P for linear-by-linear association was <0.001) (Fig. 2). Serum vitamin D levels

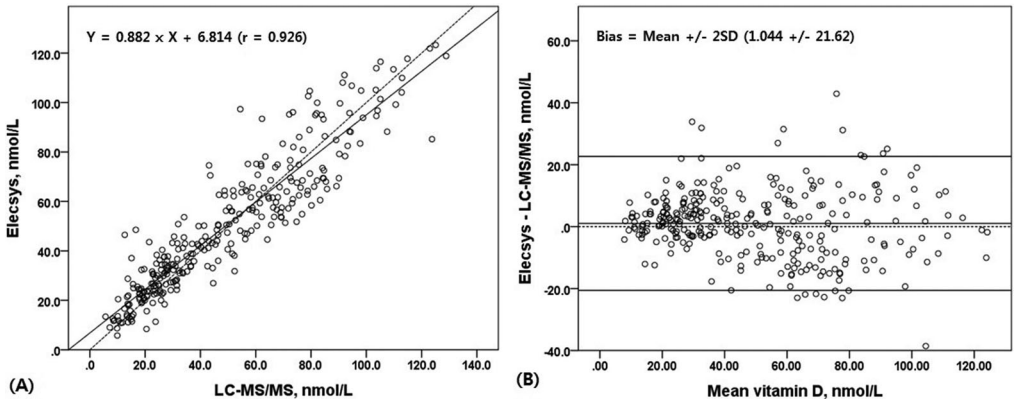

Fig. 1. Comparison of Elecsys with LC-MS/MS for vitamin D (25-OH) total assay. Scatter plot (A) and bias plot (B) are shown. The dotted line indicates standard line (A) and zero bias line (B).

TABLE 1. Imprecision Analysis Using Control Materials and Serum Samples

	Mean ± SD, nmol/l	Total imprecision, CV%
PreciControl Bone[a] 1	42.2 ± 2.04	4.1
PreciControl Bone[a] 2	106.8 ± 2.92	2.7
PreciControl Bone[a] 3	126.5 ± 1.95	1.5
Serum sample 1	38.4 ± 2.25	5.8
Serum sample 2	59.2 ± 2.95	5.0
Serum sample 3	98.1 ± 3.34	3.4

[a]PreciControl Bone (Roche, Basel, Switzerland).
CV, coefficient of variance; SD, standard deviation.

TABLE 2. Characteristics of 70,762 Study Cases

	Male	Female
Age, years		
20–29	388 (51.8)	361 (48.2)
30–39	18,811 (57.1)	14,154 (42.9)
40–49	17,695 (62.5)	10,602 (37.5)
50–59	3,850 (61.9)	2,371 (38.1)
60–69	1,090 (53.0)	965 (47.0)
70–79	270 (56.8)	205 (43.2)
Thyroid stimulating hormone (mIU/l), mean ± SD	2.1 ± 1.7	2.4 ± 2.6
Serum calcium (mmol/l), mean ± SD	2.35 ± 0.08	2.30 ± 0.08
Total vitamin D (25-OH) (nmol/l), mean ± SD[a]	45 ± 15.3	35 ± 15.5

Note: The data are presented as number (%).
[a]Total vitamin D (25-OH) level was significantly higher in males than females, $P < 0.001$.

peaked in samples collected in September in both males and females (Fig. 3).

DISCUSSION

Our data show that the Roche Elecsys Vitamin D (25-OH) Total Assay has good correlation with LC-MS/MS. Several studies have been conducted to evaluate Roche vitamin D_3 assays, a previous version of vitamin D assay. Some studies showed that Roche vitamin D_3 assays are in good agreement with RIA or LC-MS/MS, with correlation coefficients (r) ranging from 0.836 to 0.871 (8, 9). However, some studies showed that the Roche vitamin D_3 assay has poor correlation with RIA or LC-MS/MS (10, 11). The Roche vitamin D_3 assay can detect vitamin D_3 and a small percentage of vitamin D_2. According to the manufacturer's instructions, the Roche vitamin D_3 assay detects only 10% of vitamin D_2. Vitamin D_3 is generated in the skin by aid of sunlight or through dietary intake. Dietary intake is the only source of vitamin D_2. Many vitamin D supplements contain mainly vitamin D_2. Therefore, the Roche vitamin D_3 assay cannot accurately evaluate vitamin D status in subjects taking vitamin D supplements. Moreover, the cross-reactivity to vitamin D_2 or D_3 or other metabolites, as well as the matrix effect, and interfering antibodies are considered causes of analytical uncertainty in automated immunoassays (12, 13). Roche replaced their preexisting vitamin D_3 assays with vitamin D (25-OH) total assays in 2011. Several studies have shown that the Roche Vitamin D (25-OH) Total Assay has good correlation with LC-MS/MS (7, 12). Although the LC-MS/MS method is considered the gold standard method, it needs a special instrument and personnel and is thus expensive. RIA methods are comparable with LC-MS/MS (8), but a major limitation of RIA methods is the generation of radioactive waste. Therefore, Roche's automated immunoassays for vitamin D (25-OH) total assay is more suitable for evaluating vitamin D status, especially for subjects taking supplements that contain vitamin D_2.

The best way to define optimal serum or plasma level of vitamin D is unclear. As Viljoen et al. reported, the index of individuality (IoI, ratio of within-subject to between-subject variation) for vitamin D is 0.3 (14). A low IoI (<0.6) indicates that individual results reflect only a small

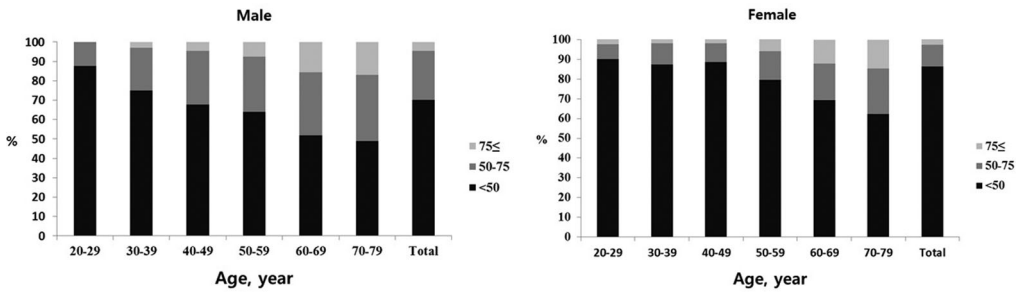

Fig. 2. Prevalence of vitamin D insufficiency (50–75 nmol/l) and deficiency (<50 nmol/l) according to age group. The proportions of males and females with vitamin D level below 75 nmol/l were significantly decreased with age ($P = 0.01$ analyzed by linear-by-linear association).

part of a population-based reference interval (15). Therefore, a population-based reference interval—such as mean ± 2SD or median 95 percentile—is not recommended in the case of serum vitamin D (14,16). Recently, the US Institute of Medicine (IOM) recommended reference intakes for vitamin D and optimal levels of serum vitamin D (25-OH) (>50 nmol/l) (5). The 50 nmol/l recommendations is derived based on recommended dietary allowances, covering the requirements of at least 97.5% of normal, healthy Americans. The US Endocrine Society guideline defined vitamin D deficiency as less than 50 nmol/l (<20 ng/ml) and insufficiency as less than 72.5 nmol/l (≤29 ng/ml) (6). The Society suggests 50 nmol/l as the vitamin D deficiency cut-off level based on studies that show an inverse relationship between parathyroid hormone (PTH) or risk of bone fracture and vitamin D (25-OH) (17,18). They define vitamin D insufficiency as less than 72.5 nmol/l based on a study that shows that calcium absorption increases with serum vitamin D level (19).

The prevalence of vitamin D deficiency and insufficiency vary by region, age, disease group, season, cut-off level, etc. In the United States, the prevalence of vitamin D deficiency (<50 nmol/l) increased from 22% to 36% during 1988–2004 (20). Decreased sun exposure time, increased obesity, and decreased outdoor activity were considered the primary causes of the increasing prevalence of vitamin D deficiency (20). Our data show that vitamin D deficiency and insufficiency rates (based on <50 nmol/l and 50–75 nmol/l, respectively) were 70.3% and 25.3% in Korean males and 86.4% and 11.0% in Korean females, respectively. The deficiency rate (<50 nmol/l) was higher than a previous study that used data from the Korea National Health and Nutrition Examination Survey (KNHANES) 2008 (<50 nmol/l: male 47.3%, female 64.5%; 50–72.5 nmol/l: male 37.5%, female 28.8%) (21). The KNHANES selected participants according to age, region, occupation, etc., using a proportional allocation-systematic sampling method similar to that of the US NHANES (22). However, in the current study, 86.6% of participants were 30–49 years of age, and the discrepancy in results may be caused by the different age distribution of the participants. In addition, our laboratory is located in a downtown area of the capital city, and thus participants are mainly people with indoor jobs; spending too much time indoors is considered a possible cause of vitamin D deficiency and insufficiency.

In general, old age is considered a risk factor for vitamin D deficiency (20, 23–26) due to a decrease in vitamin D_3 synthesis in the skin with age and lower intakes of dietary vitamin D. For example, data from the US

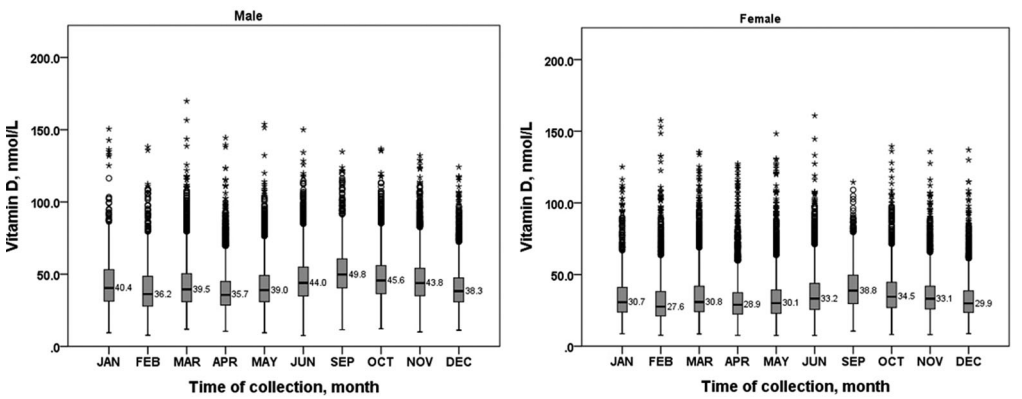

Fig. 3. Box and whisker plots of total vitamin D (25-OH) concentration according to month of collection. Boxes show interquartile range and median values. Samples were not collected in July and August.

NHANES III (1988–1994) show an increasing prevalence of vitamin D deficiency and insufficiency with age (20). However, several studies have shown that the prevalence of vitamin D deficiency and insufficiency are higher in younger people than those of an older age (27–29). Our data also show that vitamin D deficiency and insufficiency are most prevalent in 20- to 29-year-olds and decrease in prevalence with age. This result is consistent with another Korean study that used data from the KNHANES 2008 (21). The cause of the unexpectedly high prevalence of vitamin D deficiency in young people is unclear, but the higher proportion of indoor jobs resulting from industrial changes in Korea (from agriculture and fisheries to manufacturing and white-collar jobs) is a possible explanation (21). In addition, other behavioral factors such as indoor lifestyles, sunscreen overuse, less vitamin D supplementation, and lower intakes of vitamin D–rich foods were considered as causes of the high prevalence of vitamin D insufficiency or deficiency in young people (21,30). Further investigation is needed to explain the relatively high prevalence of vitamin D deficiency in young Koreans.

Serum vitamin D levels vary by season; vitamin D levels are lower in winter and spring than in summer and falls (31,32). Our data show that vitamin D levels were highest in September and lowest in February. Although our data were not collected in July and August, an overall seasonal variation pattern was observed.

The major limitation of our study is that we did not quantitate the vitamin D_2 and D_3 separately by LC-MS/MS. Therefore, comparability of the Roche vitamin D (25-OH) Total assay for fractionated D_2 and D_3 versus LC-MS/MS could not be assessed.

In conclusion, vitamin D plays an important role not only in musculoskeletal disease, but in cancer and cardiovascular and immunologic diseases. Level of 25-OH vitamin D is the generally accepted standard marker of vitamin D status. The newly launched Roche Elecsys Vitamin D (25-OH) Total Assay correlates well with LC-MS/MS and could be used in clinical settings. The high prevalence of vitamin D deficiency and its association with many diseases underscore the importance of performing accurate vitamin D assays.

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