Effects of Preanalytical Factors on Serum 25-hydroxyvitamin D_3 and 25-hydroxyvitamin D_2 Measurements Using LC-MS/MS for the Clinical Laboratory Testing

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Abstract

Background: Vitamin D testing is increasing worldwide. Although immunoassays are still widely used in Japan for the measurement of serum 25-hydroxyvitamin D (25OH-D) as an indicator of vitamin D status, development of a simple and high-throughput MS-based method is needed for routine use in clinical laboratories.
Materials and methods: We designed a method using a triple quadrupole mass spectrometer equipped with a two-step separation approach that used the Aria TLX-2 HPLC system in the selected reaction monitoring mode. Analytical performance of the system and effects of various preanalytical factors were tested.
Results: High-throughput quantitative analysis of 25OH-D3 and D2 at 15 samples/hour was achieved using 25µl of serum/plasma. Intra- and inter-assay CVs for 25OH-D3 were 5% and 7%, respectively. Limit of detection for 25OH-D3 was 0.31ng/ml. A good correlation (r^2=0.947) was found between the present system and the DiaSorin radioimmunoassay. Serum 25OH-D3 levels in apparently healthy Japanese subjects were 25.5±9.8 ng/ml for men and 20.9±7.1 ng/ml for women. No significant effects of clotting time, repeated freeze-thaw cycles, and the common interfering substances were found. It was noteworthy that some unexpected peaks appeared in blood specimens collected in the particular blood collection tube.
Conclusions: This high-throughput LC-MS/MS 25OH-D assay has the potential to be used as a routine clinical laboratory assay for assessing vitamin D status.

Introduction

Vitamin D is a fat-soluble steroid hormone that plays a key role in calcium homeostasis in the body. The two forms of vitamin D, vitamin D3 and D2, are obtained from the diet. However, only vitamin D3 can be formed by exposure of the skin to sunlight. Vitamin D3 and D2 are metabolized to 25-hydroxyvitamin D3 (25OH-D3) and 25-hydroxyvitamin D2 (25OH-D2) in the liver. The circulating liver metabolites 25OH-D3 and D2 can be used as markers of the vitamin D status, as they have a long half-life in the blood and their concentrations are considered to be in equilibrium with the vitamin D in the body. Both forms of vitamin D are metabolized in the kidney to 1α,25-dihydroxyvitamin D (1,25(OH)2D).

Serum 25OH-D levels have been conventionally measured by immunological methods. However, immunoassays are mainly limited by the cross-reactivity of the antibodies and the non equimolar recognition of the 25OH-D3 and 25OH-D2 forms of the 25OH-D metabolites. In countries where vitamin D2 containing supplements are available, it is desirable to report separate results for 25OH-D3 and 25OH-D2. It also has been reported that results given by immunoassays can be affected by occasional changes of the antibody, or the reformulation of reagents. Additionally, methodological differences and between-laboratory variability has also led to a lack of standardization.

In an attempt to overcome these problems, several liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods have been described for determining 25OH-D with an inter-laboratory imprecision similar to most immunoassays. However, several preanalytical steps need to be optimized to improve the throughput prior to any routine use of this LC-MS/MS system for 25OH-D measurements in clinical laboratories. This report describes a simple and high-throughput assay for 25OH-D3 and 25OH-D2 in the serum and plasma using a LC-MS/MS system that is composed of a triple quadrupole mass spectrometer equipped with an Aria TLX-2 HPLC system. This study also determined the effects of various preanalytical factors, such as interference, effect of meals, and sample collection processes, including effects associated with collection tubes and coagulation times.

Methods

1) 25OH-D measurement by LC-MS/MS

A total of 50 µL of acetonitrile with internal standard (75 ng/ml) was added to 25 µL of each sample. After vortexing the mixture for 30 s, samples were centrifuged at 15,000 x g for 10 min in order to create stable pellets of precipitated proteins. After transferring 55 µL of supernatant to the vial, 50 µL of the mixture was analyzed by LC-MS/MS.

The LC-MS/MS analysis was performed on a TSQ Vantage triple quadrupole mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA) coupled with an Aria TLX-2 HPLC system (Thermo Fisher Scientific Inc.). Samples were ionized using an atmospheric-pressure chemical-ionization (APCI) source.

To provide the multiplexing capability and increase the throughput, two HPLC systems performed parallel analyses. A TurboFlow column XL C18-P, 0.5 x 50 mm (Thermo Fisher Scientific Inc.) was used for the first dimensional separation, while a Hypersil GOLD 50 x 3 mm, 5-µm analytical column (Thermo Fisher Scientific Inc.) was used for the second dimensional separation. Mobile phases A and B consisted of water containing 0.1% formic acid and methanol containing 0.1% formic acid, respectively. The final mixture of mobile phase C contained 40% acetonitrile, 40% 2-propanol and 20% acetone.

Table 1. SRM transitions.

Analyte	Parent Mass	Product Mass	Collision energy	Notes
25OH-D3	383.29	91.15	45	For check of background interference
		105.08	40	For check of background interference
		365.17	10	*For calculation*
25OH-D2	395.28	209.19	25	For check of background interference
		211.12	42	For check of background interference
		269.30	18	For check of background interference
		377.42	10	*For calculation*
25OH-D3-d6 (Internal standard)	389.35	91.21	46	For check of background interference
		105.18	40	For check of background interference
		263.16	14	*For calculation*

Table 2. LC method.

Time (sec.)	Turbo flow column				Analytical column		
	Flow rate (ml/min.)	%A	%B	%C	Flow rate (ml/min.)	%A	%B
30	2	100	-	-	0.7	70	30
5	2	100	-	-	0.5	70	30
90	0.2	100	-	-	0.5	70	30
20	2	-	-	100	0.7	65	35
30	2	-	-	100	0.7	40	60
30	2	100	-	-	0.7	10	90
195	2	20	80	-	0.7	10	90
50	2	100	-	-	0.7	70	30

2) LC-MS/MS method validation

Low (10.7 ng/ml) and medium (17.5 ng/ml) serum control pools and a standard solution (SRM972) were used for assessing assay imprecision. Over 20 consecutive days, 25OH-D3 and 25OH-D2 were measured with three replicates per day (inter-day), while 25OH-D3 was measured with 20 replicates per day (intra-day) using low and medium serum control pools for 25OH-D3 and a standard solution for the 25OH-D2 analysis.

The limit of detection and the limit of quantification were estimated by using a serial dilution of a low serum control pool for 25OH-D3 and a low standard solution for the 25OH-D2. The limit of detection and the limit of quantification were defined as the peaks that gave a signal to noise ratio of 3:1 and 10:1, respectively.

Dilution linearity was evaluated by measuring the duplicates of 5 dilutions of the low and medium serum control pools, along with a standard solution for the 25OH-D3 and a standard solution for the 25OH-D2 analysis.

Accuracy was estimated by 4 different concentrations of standard solutions that contained 25OH-D3 and a standard solution for the 25OH-D2 analysis. Recovery was determined in duplicate after spiking low and medium serum control pools with two different concentrations of standard solutions that contained 25OH-D3.

The method was assessed for potential interference by spiking a separate medium serum control pool with bilirubin F, bilirubin C, hemolytic hemoglobin, chyle, and rheumatoid factor (IgM-RF) via the use of Interference Check A Plus and Interference Check RF Plus (Sysmex Co., Hyogo, Japan).

3) Assessment of preanalytical factors of 25OH-D

Serum and plasma sampling, and the handling procedures used to evaluate the preanalytical factors were as follows.

(1) To assess the effects of time intervals between the venipuncture and sample separation, serum samples were collected at room temperature at 0.25, 0.5, 1, 2, 4 and 6 h after the venipuncture. Serum samples used in this experiment were collected on different days from the same subjects that were examined in experiment (1).

(2) To assess the effects of leaving serum samples at room temperature prior to storage at –80°C, serum samples were left at 0.25, 0.5, 1, 2, 4 and 6 h.

(3) To test the effects of the freeze-thaw cycles, serum samples were frozen at –80°C in a deep freezer and then thawed at room temperature for 15 min. Samples underwent 1 to 5 freeze-thaw cycles.

(4) To estimate the effects of the routinely used anti-coagulants or separating gel on 25OH-D, samples were collected in Vacutainer blood collection tubes that contained anti-coagulants (EDTA, heparin) or separating gel. To examine these differences, we investigated 9 types of blood collection tubes.
(A) serum plain tubes (VP-P070K, no additive)
(B) serum tubes (4S1001, separating gel and silica, accelerating clotting agent used as the additive)
(C) serum tubes for rapid coagulation (4Q1060, separating gel and thrombin, accelerating clotting agent as the additive)
(D) serum tubes for neonates (365967, separating gel and thrombin, accelerating clotting agent as the additive)
(E) plasma tubes (4F2618, dipotassium EDTA (1.5 mg/ml) and sodium fluoride (1 mg/ml) as the additive)
(F) plasma tubes (4E2034, disodium EDTA (1.5 mg/ml) as the additive)
(G) plasma tubes (4E4090, dipotassium EDTA (1.5 mg/ml) as the additive)
(H) plasma tubes for neonates (365974, dipotassium EDTA (1 mg/ml) as the additive)
(I) plasma tubes (4H2011, sodium heparin (15 IU/ml) as the additive)

(A); VENOjectII blood collection tubes (Terumo Co., Ltd., Tokyo, Japan)
(B), (C), (E)-(G), (I); Insepack™ blood collection tubes (Sekisui Medical Co., Ltd.)
(D), (H); Microtainer® blood collection tubes (Becton, Dickinson and Company, Franklin Lakes, NJ)

Results

LC-MS/MS method evaluation

Table 3. LC-MS/MS assay performance.

		25OH-D3	25OH-D2
Precision (%CV)	Intra-day (n=20)	4.0-5.2	10.6
	Inter-day (n=20)	6.6-7.2	-
Limit of detection (ng/ml)		0.31	0.88
Limit of quantification (ng/ml)		0.86	1.41
Accuracy (%)		94.8-104.5	125.5
Linear range (ng/ml)		70.7	26.4
Extraction recovery (%)		102.6-106.0	-

Table 4. Effect of interference substances.

	25OH-D3	25OH-D2
Bilirubin F (mg/dL)	18.7	-
Bilirubin C (mg/dL)	19.7	-
Hemoglobin (mg/dL)	498	-
Chyle (FTU)	1440	-
Rheumatoid factor (IU/mL)	500	-

Bilirubin F, bilirubin C, hemoglobin, chyle, and rheumatoid factor did not interfere with measurement of 25OH-D up to the above described concentration.

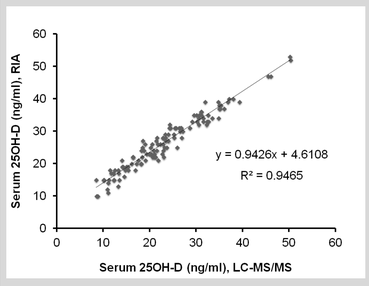

Figure 1. Linear regression analysis of the 120 clinical samples examined by the RIA and LC-MS/MS assays.

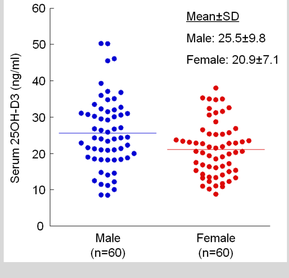

Figure 2. Concentrations of 25OH-D3 determined in 120 healthy Japanese subjects (60 men and 60 women) by the LC-MS/MS assay. Subject age (mean±SD): male, 49.3±14.9; female, 49.5±15.7.

Pre-analytical evaluation

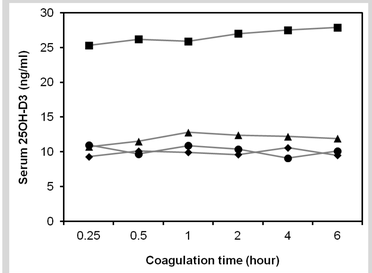

Figure 3. Time courses of the 25OH-D3 concentrations between the venipuncture and separation in blood collection tubes. The diamond, square, triangle and circle marks correspond to the 25OH-D3 concentrations in serum of 4 healthy volunteers.

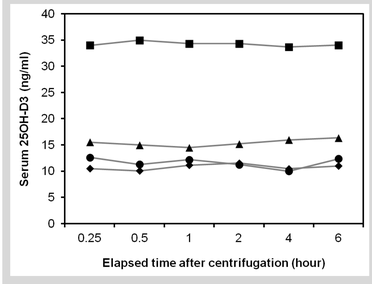

Figure 4. Time courses of the 25OH-D3 concentration between serum separation and storage. The diamond, square, triangle and circle marks indicate 25OH-D3 concentrations in the serum of 4 healthy volunteers.

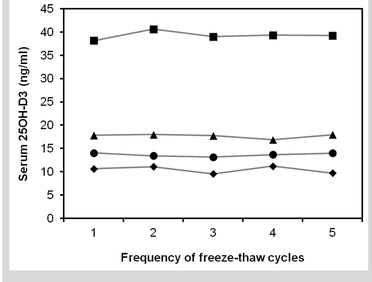

Figure 5. The effect of repeated freeze-thaw cycles on 25OH-D3 concentration of serum. The diamond, square, triangle and circle marks indicate 25OH-D3 concentrations in serum of 4 healthy volunteers.

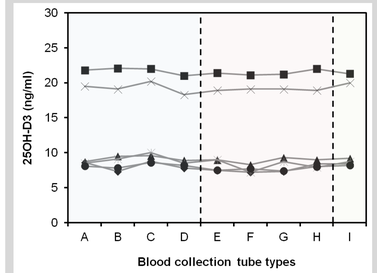

Figure 6. The effects of anticoagulants or separating gel on 25OH-D3 concentrations. Nine types of blood collection tubes were investigated. The each mark indicates the 25OH-D3 concentration in the serum of six healthy volunteers. Blood collection tube types: A-D, serum; E-H, plasma (EDTA); I, plasma (haparin).

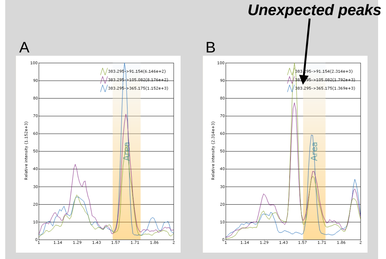

Figure 7. SRM Chromatogram of 25OH-D3. A, InsepackTM blood collection tube (4S1001). B, VENOjectII blood collection tube (VP-AS076K50). In the VENOjectII blood collection tube, unexpected peaks were observed in 2 transitions to check background interference(**arrow**). Orange shadow: 25OH-D3 peaks.

Conclusion

- ✓ We developed a LC-MS/MS method coupled with the TLX-2 HPLC system that can be used to perform separate quantifications of serum 25OH-D3 and 25OH-D2.
- ✓ The results obtained were in good agreement with those obtained by a widely used commercial radioimmunoassay.
- ✓ This LC-MS/MS method may be applicable for routine determination of serum/plasma 25OH-D levels in clinical laboratories.