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Improved sensitivity of serum/plasma 1α,25-dihydroxyvitamin D quantification by DAPTAD derivatization

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ABSTRACT

Background: Although immunoassays have several limitations such as the cross-reactivities of antibodies, such techniques are widely used for serum/plasma 1,25$(OH)_2$D quantification. An accurate method is required for the determination of the 1,25$(OH)_2$D status.

Methods: We designed a serum/plasma 1,25$(OH)_2$D quantification method using LC-MS/MS. Immunoaffinity extraction (IE) and the recently developed Cookson-type reagent 4-(4′-dimethylaminophenyl)-1,2,4-triazoline-3,5-dione (DAPTAD) were used for sample preparation and derivatization, respectively. Analytical and pre-analytical validations were performed. Serum 1,25$(OH)_2D_3$ concentrations were determined in 232 healthy Japanese individuals.

Results: The intra- and inter-assay CVs for 1,25$(OH)_2D_3$ were 5.2% and 7.0%, respectively. The limit of quantification for 1,25$(OH)_2D_3$ was 7.1 pg/ml. Rheumatoid factor (RF) at concentrations below 517 IU/ml did not affect serum 1,25$(OH)_2$D analysis. No significant differences were observed for various blood collection tubes, repeated freeze–thaw cycles, whole blood standing time, or serum storage time. A strong correlation between LC-MS/MS and radioimmunoassay (RIA) was observed ($r = 0.786$), but serum 1,25$(OH)_2$D concentrations obtained from RIA were 2-fold higher than those obtained from LC-MS/MS. Serum 1,25$(OH)_2D_3$ concentrations by LC-MS/MS were 18.7–53.9 pg/ml.

Conclusion: A highly sensitive and selective LC-MS/MS-based serum/plasma 1,25$(OH)_2$D quantification method was developed using IE and DAPTAD derivatization. This method will enable the accurate determination of serum/plasma 1,25$(OH)_2$D concentrations in the clinical setting.

1. Introduction

Vitamin D is a pro-hormone with the following two forms: cholecalciferol (D_3) and ergocalciferol (D_2). Both can be obtained from the diet. D_3 can also be synthesized from 7-dehydrocholesterol in the skin upon ultraviolet irradiation. Vitamin D is metabolized in the liver to 25-hydroxyvitamin D [25(OH)D], which is the best indicator of vitamin D status, by 25-hydroxylase. Subsequently, 1α,25-dihydroxyvitamin D [1,25$(OH)_2$D], which is a hormonally active form of vitamin D, is produced in the kidney by 1α-hydroxylation of 25(OH)D [1,2]. 1,25$(OH)_2$D has important roles in regulating calcium and phosphorus metabolism [1,2]. The measurement of serum/plasma 1,25$(OH)_2$D concentrations is useful in the evaluation of rickets, osteomalacia, chronic renal failure, and hyperparathyroidism [3,4].

Conventionally, serum/plasma 1,25$(OH)_2$D has been measured in most clinical laboratories using immunological methods such as radioimmunoassay (RIA) and enzyme immunoassay (EIA) [5]. However, immunoassays have several limitations: 1) the antibodies exhibit cross-reactivities with other blood components; 2) immunoassays are unable to distinguish 1,25$(OH)_2D_3$ and 1,25$(OH)_2D_2$; and 3) immunoassays require extensive sample pretreatment to minimize the contribution from cross-reactive vitamin D metabolites and non-specific reactive substances [4,6,7]. Thus, a highly specific and more convenient method is required for the accurate determination of the serum/plasma 1,25$(OH)_2$D status.

Liquid chromatography–tandem mass spectrometry (LC-MS/MS) has been used for analyzing serum/plasma vitamin D metabolites, and this technique is considered to be the gold standard method because of

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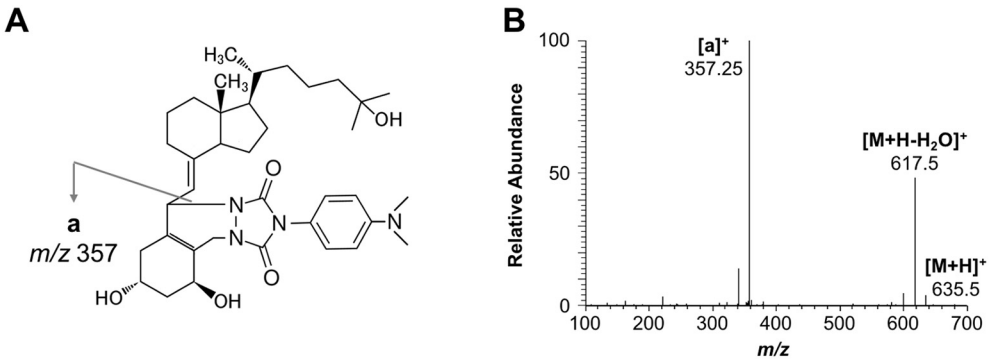

Fig. 1. The structure (A) and the MS/MS spectrum (B) of $1,25(OH)_2D_3$-DAPTAD.

Table 1
Comparison of sensitivity.

Compound	SRM transition			Increasing sensitivity[a]
$1,25(OH)_2D_3$	399.35 $[M + H\text{-}H_2O]^+$	>	381.30 $[M + H\text{-}2H_2O]^+$	1.0
$1,25(OH)_2D_3$-PTAD	574.40 $[M + H\text{-}H_2O]^+$	>	314.10 $[a]^+$	6.2
$1,25(OH)_2D_3$-DAPTAD	635.45 $[M + H]^+$	>	357.24 $[a]^+$	46.2

[a] Increasing sensitivities were calculated from their peak areas. The peak area of $1,25(OH)_2D_3$ was used as a base of 1.0.

its sensitivity and selectivity [8]. We recently reported an LC-MS/MS method for the quantification of serum vitamin D metabolites using supported liquid extraction (SLE) and the Cookson-type reagent 4-(4′-dimethylaminophenyl)-1,2,4-triazoline-3,5-dione (DAPTAD) [9]. Although this LC-MS/MS technique is an accurate method for analyzing 25(OH)D and $24,25(OH)_2D$, the technique still lacks sensitivity for quantification of serum $1,25(OH)_2D$, which is present at concentrations 1000-fold lower than those of 25(OH)D. To overcome this limitation, we applied immunoaffinity extraction (IE) of $1,25(OH)_2D$ to improve the signal-to-noise ratio.

2. Materials and methods

2.1. Study subjects

Serum samples were obtained from 232 apparently healthy individuals. The ethics committee of our institute approved the study protocol, and a written informed consent was obtained from each individual prior to their enrollment in the study. No formal exclusion criteria were defined aside from the need for informed consent.

Table 2
Assay performance.

	$1,25(OH)_2D_3$	$1,25(OH)_2D_2$
CV (%)		
Intra-assay (N = 20)	5.2	7.4
Inter-assay (N = 20)	7.0	8.2
Recovery (%)	78.4	76.0
LOQ[a] (pg/ml)	7.1	7.6

[a] LOQ, limit of quantification.

2.2. Reagents and standard materials

Purified $1,25(OH)_2D_3$ (≥ 99.0%) and $1,25(OH)_2D_2$ (≥ 97.0%) were from Sigma Aldrich. Deuterium-labeled $1,25(OH)_2D$ [d_6-$1,25(OH)_2D_3$ (99%) and d_6-$1,25(OH)_2D_2$ (99%)] were from Medical Isotopes, Inc. The Cookson-type reagents, 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) and DAPTAD, were obtained from Sigma Aldrich and synthesized by the previously described method, respectively [10]. The synthesis and quality management of DAPTAD are described in Supplementary method S1.1. Distilled water (for HPLC) was purchased from Nacalai Tesque Inc. Acetonitrile (for LC/MS), hexane (for vitamin determination), and ethyl acetate (for spectrochemical analysis) were from Wako.

2.3. Sample preparation

2.3.1. SLE

Internal standards were generated by combining 200 µl of serum with 20 µl of 2500 pg/ml d_6-$1,25(OH)_2D_3$ in acetonitrile. A total volume of 220 µl of each diluted sample was loaded into ISOLUTE SLE + (Biotage) and held for 10 min at room temperature (RT). Elution was performed 3 times with 700 µl of hexane-ethyl acetate (50:50) solution.

2.3.2. IE

Internal standards were generated by combining 200 µL of the sample (serum or plasma) with 300 µL of Dulbecco's phosphate-

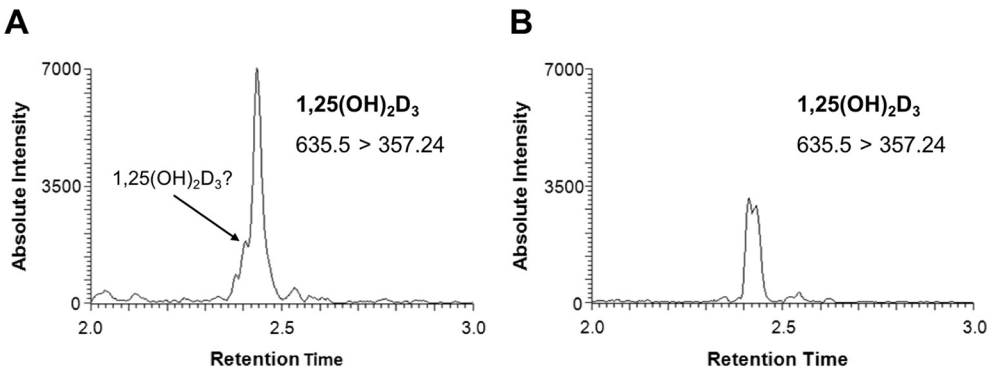

Fig. 2. Comparison of different serum/plasma preparation methods: SLE (A) and IE (B).