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Rapid and simultaneous determination of 25-OH-vitamin D_2 and D_3 in human serum by LC/MS/MS: Validation and uncertainty assessment

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Abstract: 25-OH Vitamin D_3 and 25-OH Vitamin D_2 were determined by rapid, simple and validated LC/MS/MS method without using SPE and isotopic standard. The relative standard deviations (RSD) were found to be 3.1 and 2.8 % for 25-OH Vitamin D_3 and 25-OH Vitamin D_2, respectively. The correlation coefficient was grater than 0.99 for both analytes in the calibration range. The recoveries at 100 µg L^{-1} concentration averaged to 92 % for 25-OH vitamin D_3 and 94 % for 25-OH-vitamin D_2. The uncertainty of the measurements for 25-OH vitamin D_3 and for 25-OH-vitamin D_2 was 7.77 % and 7.24 %, respectively.

Keywords: 25-OH Vitamin D_3, 25-OH Vitamin D_2, LC/MS/MS, method validation, uncertainty

1. Introduction

"Vitamin D_3, commonly known as the "sunshine vitamin," is essential for calcium metabolism and for bone health. The major physiologic function of Vitamin D is to maintain the blood calcium and phosphorus levels within the normal range, metabolic functions are essential for most of the life processes. Vitamin D exists in two forms, which are vitamin D_2 and D_3. Vitamin D_2 originates from yeast and plant sterol ergosterol, while vitamin D_3 is synthesized from 7-dehydrocholesterol in skin by the action of sunlight. Under UV ray between 290-315 nm, 7-hydrocholesterol and ergostreol are converted to pre-vitamins D_3 and D_2, respectively. However, as they are thermodinamically unstable, easily reaarange to more stable forms, vitamin D_3 and vitamin D_2" [1-3].

25-OHase enzyme converts the Vitamins D_3 and D_2 to 25-OH-Vitamin Ds in liver. enzyme. 24, 25 and 1, 25 OH derivatives are also syntheses by the corresponding hydroxylation enzymes [1]. "Quantification of these metabolites is widely applied as a means of assessing vitamin D status because of their clinical significance in a variety of disorders, which lead to alterations in the concentration of circulating hormones. The measurement of the serum

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concentrations of 25-OH vitamin D_3 and D_2 have been performed for differential diagnosis of hypo- and hypercalcemic disorders in metabolic bone disorders" [1].

In previous studies, protein binding and radioimmuno assays and direct determination by HPLC/UV, Gas chromatography-mass spectrometry technique, have been reported in the literature [4-12]. However, these reported procedures are time consuming and can not solve the interference problem during analysis. "Recently, a candidate reference method for the quantification of circulating 25-OH-vitamin D_3 in serum, which uses column switching for online SPE, manual cartridge extraction before LC/MS/MS analysis [13]" Also, routine isotope dilution ID-LC/MS/MS assay for the measurement of 25 OH vitamin D_2 and D_3 have been reported and [3]. However, both SPE and ID-LC/MS/MS techniques are not cost effective techniques for the routine test laboratories.

The purpose of this work were to develop simple, efficient, reliable determination method of 25-OH-vitamin D_3 and D_2 in plasma and serum by LC/MS/MS without isotopic internal standards and to validate the method by calculating measurement uncertainties using the bottom-up method.

2. Experimental

2.1. Chemicals

2.1.1. Vitamin standards and solutions

25-OH-vitamin D_2 (99 %), 25-OH-vitamin D_2 (99 %) and 1α-OH vitamin D_3 (99 %) were purchased from Calbiochem, Darmstadt, Germany. 25-OH Vitamin D_2 and D_3 stock solutions (1 mg L^{-1}) were prepared in ethanol. 1α-OH vitamin D_3, as an internal standard, was also prepared in ethanol at 1 mg L^{-1} concentration. 25-OH-vitamin D_3 liyophilesed serum control Clincheck® (108 μg L^{-1}, Recipe, Munich, Germany) was used as an internal quality standard. Working calibrators containing 25-OH Vitamin D_2 and D_3 were prepared in ethanol-water (40:60, v/v) in the range of 10-500 μg L^{-1} (based on sample volume). Dilutions were performed using automatic pipettes and glass volumetric flasks (A class). They were stored in a refrigerator at –20 ^{o}C in glass containers. In all experiments, 100 μL of 1 mg L^{-1} Internal standard (IS) was added.

2.1.3. Organic solvents and reagents

Ethanol (99.0 %, Merck, HPLC grade), methanol (99.5%, Merck, HPLC grade), acetonitrile (99.5%, Merck, HPLC grade), isopropyl alcohol (99 % Merck, HPLC grade) heptane (99 %, Labkim, GC grade) were the organic solvents used throughout the study.

2.2. Instruments

2.2.1. LC/MS/MS

A Thermo Finnigan Surveyor (Thermo Electron, USA) equipped with a ZivakDs reversed phase column 100 x 4.6 mm i.d., 3 μm particle size was purchased from Zivak company, Turkey. The mobile phase was composed of methanol and 0.5 mL formic acid in water (95:5, v/v). The flow rate of mobile phase was 0.5 mL min^{-1}, the column temperature was 35 ^{0}C, and the injection volume was 25 μL.

A Thermo Finnigan LCQ Deca ion trap tandem mass spectrometry (Thermo Electron, USA) with APCI and ESI sources were used. The best results were obtained with APCI source.

Quantitative analysis was performed by MS/MS mode for both vitamins. The parent ions of 25-OH vitamin D_3, 25-OH vitamin D_2 and internal standard 1α-OH vitamin D_3 were selected as *m/z* 383, 395 and SRM *m/z* 401 for 1α-OH vitamin D_3. Daughter ions were observed as *m/z* 365 and 377 for 25-OH vitamin D_3 and 25-OH vitamin D_2, respectively. The collision energies were applied as 30, 30 and 25. (Table 1). The sample chromatograms at a concentration level between the 50 μg L^{-1} and 100 50 μg L^{-1} values of the vitamins is shown in Figure 2.

2.2.2. Other Equipments

Vortex: Heidolph Reax top (100-2400 L min^{-1}), Germany, Ultrasonic bath: Bandelin Sonorex, Germany, Centrifuge: Beckman Coulter, USA.

2.3. Sample preparation

Proteins were precipitated by mixing 500 μL of serum/plasma with a 200 μL mixture of methanol:acetonitrile (70:30) and 100 μL of internal standard solution in 2 mL eppendorf tube. The mixture was vortexed for 15 seconds, 1000 μL of heptane was added and revortexed for 45 seconds and centrifuged for 5 minutes at 8000 rpm. The 900 μL of heptane layer was transferred to a auto sampler vial and the solvent was evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 250 μL of mobile phase and 25 μL was injected to the LC/MS/MS.

2.4. Optimization of extraction methods and LC/MS/MS procedure

Composition of the solvent, applied for precipitation and extraction, was found to be the crucial parameter of the method. Methanol, ethanol, acetonitrile and isopropyl alcohol were used for the precipitation of proteins from plasma and serum. The best precipitation was obtained with methanol:acetonitrile (70:30) mixture in this assay. For extraction, the best choice was $CHCl_3$. However, if chloroform is used for extraction, transfer procedure of organic phase becomes very difficult. That's why we used heptane was found to be the best solvent for this purpose.

Ion trap mass spectrometry system is suitable for the analysis. Since ionization of compounds with ESI (+) or ESI (-) techniques was poor, APCI (+) was applied for determination of 25-OH Vitamin Ds. Collision energies and mass fragmentation of procedure were described in Table 1.

3. Results and discussion

3.1. Validation

All validation procedures were performed using patient sera for 25-OH Vitamin D_2 and D_3. 1α-OH vitamin D_3 was used as an internal standard.

3.1.1. Linearity

Calibration curves were obtained from matrix-matching calibration solutions. The lowest concentration level in the calibration curve was established as a practical determination limit. Linearity of method was assayed by analyzing the calculation of a five-point linear plot in the range of 10-500 μg L^{-1} with 6 replicates, based on linear regression and squared correlation coefficient, r^2, which should be 0.9980 and 0.9971 for the 25-OH Vitamin D_2 and D_3, respectively. Linear regression equation was $y = 0.1146 + 3.2494\ x$ for 25-OH Vitamin D_2 and $y = 0.1243 + 4.3316\ x$ for 25-OH Vitamin D_3.

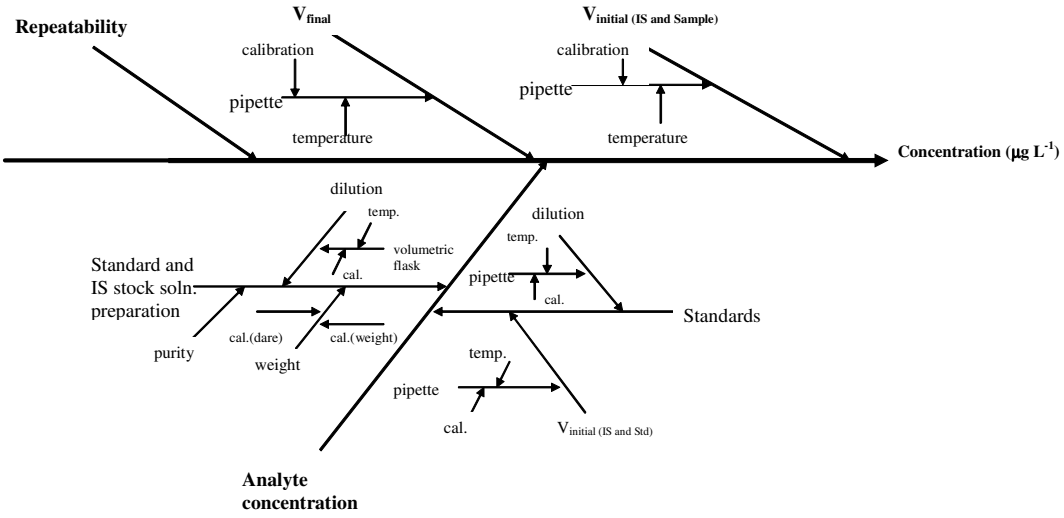

Figure 1. Cause and effect diagram for the determination of 25-OH vitamin D_3 and D_2

3.1.2. LOD and LOQ

The limit of detection (LODs) of the method was found to be 2 µg L^{-1} for 25-OH Vitamin D_3 and 1 µg L^{-1} for 25-OH Vitamin D_2 determined by considering value, which was three times the signal to background noise (S/N) ratio using 10 µg L^{-1} and 50 µg L^{-1} of 25-OH Vitamin D_3 and D_2, respectively. The limits of quantification (LOQs), which was µg L^{-1} and 10 µg L^{-1}, respectively (10 times the S/N for the above concentrations).

3.1.3. Recovery, repeatability and intermediate precision

According to the expected levels of real concentrations, the plasma was divided into 3 portions and the spiking was performed at three fortification levels (50, 100 and 250 µg L^{-1}), which are the values of the lowest, middle and the highest parts of the linear range. An unspiked serum was also analyzed to determine the 25-OH vitamin D_2 and D_3 concentrations in the blank sample. The recovery of each 25-OH vitamin at each fortification levels was evaluated. Recovery was calculated according to the following formula (equation 1).

$$Recovery(100\%) = \frac{Measured\ conncentration - endegeneus\ concentration}{spiked\ concentration} x100 \quad (1)$$

The recoveries were ranged from 89 to 94 % for 25-OH vitamin D_2 and 92 to 102 % for 25-OH vitamin D_3. Mean relative standard deviations (RSD) were found to be 2.8 and 3.1 for 100 µg L^{-1}, respectively (Table 2).

The repeatability was assessed at three concentration levels of the recovery studies. For the intermediate (reproducibility) precision, a set of spiked samples having three concentration levels were analyzed twice a week for a period of 3 weeks (Table 2). Repeatability of method was found to be 3.14 ad 2.8 % (pooled RSD).

3.2. Estimation of Uncertainty

3.2.1. Identification of uncertainty sources

The analyte concentration in the sample, expressed in μg L^{-1}, was obtained by equation 2:

$$Concentration = \frac{\text{CA x } V_{final}}{V_{initial}} \quad (2)$$

Where CA is the analyte concentration obtained by calibration (in μg L^{-1}); V_{final} is the final diluted volume (0.25 mL) before the analysis; $V_{initial}$ is the initial sample volume (0.35 mL).

3.2.2. Identification of standard uncertainties associated with each step

For the identification of uncertainty sources, use of so-called "Cause and Effect diagram" was drawn. The This fishbone diagram (Figure1) helps to prevent an uncertainty contribution incorporated into the budget more than once. Subsequently, it is possible to decide the significant and negligible ones. The concentration results of vitamins were mainly affected by the following sources.

1. Standard preparation and repeatability also including calibration curve (CA)
2. Final volume of the sample extract (V_{final})
3. Sample and IS volumes ($V_{initial}$)

The combined uncertainty (in terms of relative uncertainty) can be calculated using equation 3:

$$u_{rel}(CON) = \sqrt{u^2{}_{rel}(CA) + u^2{}_{rel}(V_{final}) + u^2{}_{rel}(V_{initial})} \quad (3)$$

3.2.2.1. Estimation of the uncertainty derived from the dilution of the sample extract, u(V_{final})

The volumetric calibration standard uncertainty of 1 mL automatic pipette, u_{vcal}, was calculated by the manufacturer reported CV value of 0.06 (i.e. u_{vcal}=(0.06/100)*0.25= 0.00015 mL). The volumetric repeatability was not included since total repeatability covers all repeatability items.

The temperature effect describes the dispersion was produced by variation of liquid temperature through a rectangular distribution within ± 3°C around the calibration temperature.

$$u_{vtemp} = \frac{3xVxQ}{1.73} \quad (4)$$

Where u_{vtemp} represents the standard uncertainty of the temperature effect, V is the measured volume (0.25 mL) and Q is the coefficient of volume expansion of methanol; Q_{met}= 0.00149 $^oC^{-1}$. These sources are combined in equation 5:

$$u_V = \sqrt{(u_{vcal})^2 + (u_{vtemp})^2} \quad (5)$$

3.2.2.2. Estimation of the uncertainty derived from the initial volume of the sample, u($V_{initial}$)

Internal standard solution (0.1 mL) was added to all standards and samples. Uncertainty from automatic pipette, u_{vcal}, was calculated by the manufacturer reported CV value of 0.06 (i. e. u_{vcal}=(0.06/100)*0.1= 0.00006 mL). The volumetric repeatability was not included.

The temperature effect describes the dispersion produced by a variation of liquid temperature, through a rectangular distribution within ± 3 ^oC around the calibration temperature.

$$u_{vtemp} = \frac{3xVxQ}{1.73} \quad (6)$$

Where u_{vtemp} represents the standard uncertainty of the temperature effect, V is the measured volume (0.1 mL for IS, 0.3 mL for sample) and Q is the coefficient of volume expansion of water (solvent of the serum matrix was accepted as water); Q_{water}= 0.00021 $^oC^{-1}$. These sources are combined in the equation 7:

$$u_V = \sqrt{(u_{vcal})^2 + (u_{vtemp})^2} \quad (7)$$

3.2.2.2. Estimation of the uncertainty derived from the estimation of the analyte concentration, u(CA)

This is a combination of the uncertainties associated with the preparation of the calibration standard solutions with the addition of internal standards, u_{std} and u_{is}. It includes the uncertainty of the calibration curve. However, it was observed that the uncertainty comşing from the standards was higher than that of the calibration curve. Thus, the uncertainty of coming from the calibration curve was neglected as it was reported previously [14].

The uncertainty of stock solution is a combination of purity uncertainties of vitamins and internal standard given in the certificate, $u_{purity(std)}$ and $u_{purity(is)}$, the weighing uncertainties, $u_{m(std)}$ and $u_{m(is)}$ and uncertainty coming from the automatic pipettes used for dilution, u_V (equation 8). The solvent used for the dilution was ethanol. The u_v is calculated by using equation 7. The balance uncertainty was obtained via the certificate given by TUBITAK UME with the formula ±0.02+1.54x10$^-$5xW at 95% confidence level. It was calculated as 0.02002 mg for a 1 mg sample. Standard uncertainty, $u_{m(std)}$) was 0.02002/2. This source of uncertainty was considered twice more as the weighing process involves a difference.

The uncertainty components of the standards were formed by initial volumes of standard and internal standard and final dilution volume (equation 9).

For each calibration point, the relative combined uncertainty was calculated and their uncertainties were summed.

$$u_{std(stock)} = \sqrt{(u_V)^2 + (u_{purity(std)})^2 + (u_{m(std)})^2} \quad (8)$$

$$u_{std} = \sqrt{(u_{V_{dil}})^2 + (u_{Vinitialstd})^2 + (u_{VinitialIS})^2} \quad (9)$$

The example of calculated expanded relative uncertainties U (k=2) of the method for vitamin D_3 (140 ± 10.87 µg L^{-1}) was given in Table 3, which is 140 ± 10.13 for vitamin D_2. It was observed that the contributions from the stock solutions and initial volumes were insignificant; first the repeatability and then the standard preparation using automatic pipettes significantly contributed to uncertainty. In order to decrease the uncertainty of the analytical methods, it would be convenient to increase repeatability values and to use syringes or pipettes suitable for organic solvents especially for small volumes.

3.3 Internal quality criteria

In order to be sure about the quality of the results when the proposed methods are applied to routine analyses, various internal criterias have been established. The first one is a blank extract that eliminates the contamination during the extraction and clean-up processes. One blank sample was processed in each set of experiments. The second one is to check the extraction efficiency. Recoveries at the second concentration level (100 µg L^{-1}) will be accepted if the majority of recoveries are within 80-105 % range. The third one is the addition of materials on to the blank matrix and making double-checking in recovery for 25-OH vitamin Ds. Finally, 25-OH-vitamin D_3 liyophilesed serum control Clincheck® was analyzed to check the calibration curve before starting routine analysis.

4. Conclusion

This developed and fully validated method is user friendly and cheaper than SPE extraction and isotope dilution LC/MS/MS techniques. The results indicate that the methodologies developed could be important alternative of the current methods in use by clinical laboratories.

Measurement uncertainty was estimated using the data obtained from method validation. Expanded uncertainty for each compound using covering factor 2 was found as 7.77 % for vitamin D_3 and 7.24 % for vitamin D_2 ,having the concentration of 140 µg L^{-1}. The recoveries at 100 µg L^{-1} concentration averaged 92 % for 25-OH vitamin D_3 and 94 % for 25-OH-vitamin D_2.

The effectiveness of the proposed method was evaluated by analyzing the reference serum obtained from recipe (Clincheck®) and spiked patient sera. Good correlations were found between the assays and the certified values. In conclusion, the results of this study indicate that the reported method could be used as a routine procedure in clinical laboratories.

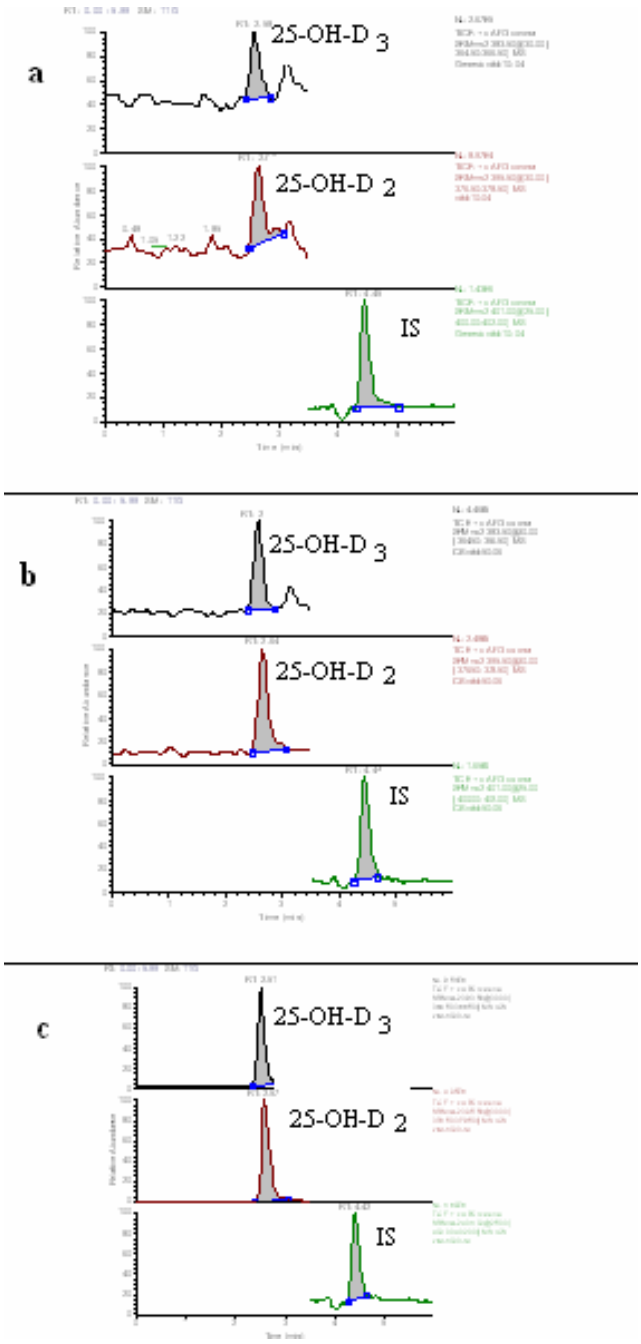

Figure 2. Sample chromatograms of 25-OH Vitamin D_3 and D_2 from spiked serum with ion trap LC/MS/MS a) 50 µg L^{-1} b) 100 µg L^{-1} c) 100 µg L^{-1}

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