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Exogenous versus endogenous recovery of 25-hydroxyvitamins D_2 and D_3 in human samples using high-performance liquid chromatography and the DiaSorin LIAISON Total-D Assay☆

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ABSTRACT

Demand for circulating 25-hydroxyvitamin D [25(OH)D] measurements has exploded due to its relationship with many serious health problems. The present study was designed to investigate the validity of samples "spiked" with 25-hydroxyvitamin D_2 [25(OH)D_2] or 25-hydroxyvitamin D_3 [25(OH)D_3] to determine their analytical recovery by the DiaSorin LIAISON 25 OH Vitamin D Total Assay (DiaSorin Assay) and high-performance liquid chromatography (HPLC). 25(OH)D was measured in nine volunteers taking large daily doses of vitamin D_2 for 2 weeks. Samples were obtained pre-supplementation and 1 week following vitamin D_2. Pre-supplementation samples were used for exogenous recovery studies by adding 25(OH)D_2 or 25(OH)D_3. Endogenous 25(OH)D [25(OH)D_2 plus 25(OH)D_3] concentrations reported by the DiaSorin Assay or detected by HPLC were in excellent agreement. However, exogenously added 25(OH)D_2 and 25(OH)D_3 were under-recovered by the DiaSorin Assay. NIST vitamin D standards containing serum from another species (horse) or exogenous 25(OH)D_2 were similarly affected when using the DiaSorin Assay. Exogenous 25(OH)D_2, 25(OH)D_3 or serum from other species added to human samples is inappropriate in determining the analytical recovery of vitamin D compounds when using the DiaSorin Assay. Only endogenous 25(OH)D_2 and/or 25(OH)D_3 contained in human blood samples should be utilized for this purpose.

1. Introduction

Assessment of circulating 25(OH)D has become an important tool in the management of many acute and chronic diseases in the past 5 years [1]. At Heartland Assays I am involved in many experimental clinical studies involving the measurement of circulating 25(OH)D and have chosen the DiaSorin Assay as our method of choice. This system was selected because it is FDA-approved, has high sample throughput and purported equal recognition of circulating 25(OH)D_2 and 25(OH)D_3 to provide a total 25(OH)D value which is recognized as the clinically relevant value [2,3]. However, questions have arisen regarding this assay's ability to detect these two 25-hydroxylated compounds equally in human serum [4]. Heartland Assays utilizes the DiaSorin Assay for a majority of the 25(OH)D analyses requested by our research customer. Therefore, it became important to determine if this methodology did recover total 25(OH)D [25(OH)D_2 plus 25(OH)D_3] as claimed by the distributors. Therefore, the current study was designed to assess the ability of this relatively new assay system to detect exogenously added 25(OH)D_2 or 25(OH)D_3, as well as endogenous 25(OH)D_2 and/or 25(OH)D_3 naturally contained in human samples. This new method was compared with the "gold standard" of 25(OH)D detection, HPLC separation followed by UV detection [5].

2. Materials and methods

2.1. Volunteer study

The study was conducted with the written consent of each volunteer. Baseline serum or plasma samples were obtained from nine volunteers. Six of these volunteers had been consuming various amounts of vitamin D_3 for up to 4 years to maintain circulating 25(OH)D. The remaining three subjects did not report significant vitamin D supplement usage. Each of the nine subjects then consumed 50,000 IU/day of vitamin D_2 added to a glass of milk for 14 consecutive days. Seven days following the final dose serum was obtained from each volunteer and stored at −30 °C until assayed. For exogenous *in vitro* recovery experiments 32 ng/ml of either 25(OH)D_2 or 25(OH)D_3 was added, in a small volume of ethanol, to each baseline serum sample. The samples were then incu-

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bated overnight at room temperature, prepared and subjected to HPLC quantitation to determine individual levels of 25(OH)D_2 and 25(OH)D_3 (5) and to the DiaSorin Assay for total 25(OH)D.

2.2. NIST study

Samples were provided by Karen Phinney from the National Institute for Standards and Technology (NIST). NIST describes the samples as Level 1 "normal" human serum; Level 2 "normal" human serum diluted 1/1 with horse serum; and Level 3 "normal" human serum spiked with an 25(OH)D_2 attempting to be equivalent to the amount of endogenous 25(OH)D_3. Horse serum (Lot # 074K8407) for "in house" testing was purchased from Sigma, St. Louis, MO.

3. Results

3.1. Volunteer study

In this group of volunteers the baseline total 25(OH)D was 48.3 ± 19.0 and 43.7 ± 16.8 ng/ml ($\bar{x} \pm$ SD) by HPLC and the DiaSorin Assay, respectively. In these baseline samples HPLC analysis demonstrated 99% of the circulating 25(OH)D to be of the D_3 form, only two of nine subjects had detectable (>1.0 ng/ml) 25(OH)D_2. Following 14 days of oral vitamin D_2 supplementation, total 25(OH)D levels were determined to be 81.1 ± 21.9 and 80.0 ± 25.5 ng/ml by HPLC and the DiaSorin Assay, respectively. By HPLC analysis the elevations in 25(OH)D_2 ranged from 25 to 88 ng/ml. Reciprocal declines in 25(OH)D_3 ranged from 0.0 to 27.0 ng/ml. In these post-supplementation samples, HPLC analysis also revealed 25(OH)D_3 to be 43.5% of the total while the remaining 56.5% was 25(OH)D_2. The regression relationship of pre- and-post samples between HPLC and DiaSorin Assay was DiaSorin Assay = 1.04 HPLC − 5.27, $r^2 = 0.95$ (Fig. 1). The recovery of exogenously added 25(OH)D_2 or 25(OH)D_3 to baseline samples was 98.3 ± 5.7 and 99.0 ± 6.7%, respectively by HPLC analysis, and 22.8 ± 19.7 and 62.7 ± 24.8%, respectively by DiaSorin Assay analysis (Table 1).

3.2. NIST study

Table 1 summarizes values obtained from the DiaSorin Assay and HPLC analyses. Values provided by NIST are included in the table. We obtained horse serum from Sigma in order to determine any potential matrix problems since one of the NIST samples (Level #2) contained 50% horse serum. NIST Level 1 concentrations measured by the DiaSorin Assay compared favorable with HPLC results. However, NIST Level 2 was higher (DiaSorin Assay versus HPLC) and Level 3 was lower than the DiaSorin versus HPLC. The higher concentration in the NIST Level 2 can be attributed to the impact of the horse serum matrix (we have observed similar issues with other species particularly porcine serum) and lower levels in NIST Level 3 were attribute to the lack of recovery of exogenous material by the DiaSorin Assay.

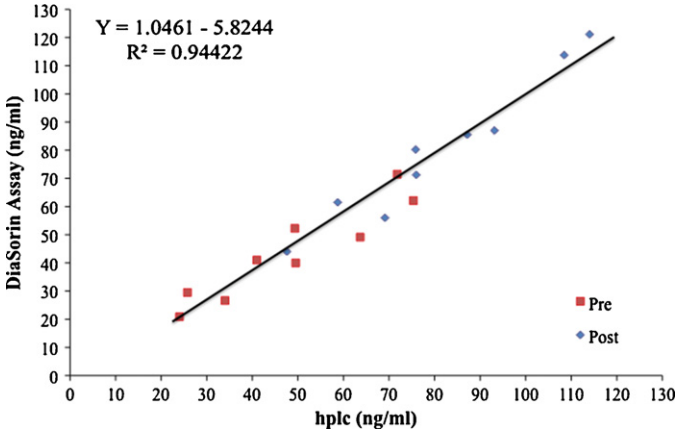

Fig. 1. Elevations in plasma total 25(OH)D in volunteers following supplementation with vitamin D_2 as measured by the DiaSorin LIAISON 25 OH Vitamin D Total Assay versus high-performance liquid chromatography. Volunteers were given vitamin D_2 as described in Section 2. Pre-supplementation concentrations are represented by the closed boxes and post-supplementation by the closed diamonds.

Table 1
Comparison of 25(OH)D concentrations measured by the DiaSorin Assay and HPLC as a result of various exogenous and endogenous treatments.

Sample ID	Total 25(OH)D (ng/ml)	
	DiaSorin Assay	HPLC
Baseline	43.7 ± 16.8	48.3 ± 19.0
Diet vitamin D_2	81.1 ± 21.9	80.0 ± 23.5
Baseline + 25(OH)D_2[a]	51.0 ± 16.8 (22.8%)	79.7 ± 19.0 (98.3%)
Baseline + 25(OH)D_3[a]	63.7 ± 20.4 (62.7%)	80.0 ± 18.5 (99.0%)
Horse serum	12.7 ± 1.0	4.7 ± 0.2
NIST Level 1 [22–24][b]	24.4 ± 0.8 (106%)	26.0 ± 1.1 (113%)
NIST Level 2 [12–14][b]	19.8 ± 0.5 (152%)	15.9 ± 0.7 (122%)
NIST Level 3 [42–46][b]	27.2 ± 1.0 (61.8%)	48.1 ± 3.0 (109%)

[a] 32 ng/ml was added to each of nine samples. Values in parentheses represent the amount of 25(OH)D recovered as a % of mean values.
[b] Values in brackets are expected values provided by NIST. Values in parentheses represent amount of 25(OH)D recovered as a % of mean values.

4. Discussion

This brief report reveals an important artifact that could lead to false conclusions about the ability of direct competitive antibody-based chemiluminescence assays to quantitatively detect 25(OH)D_2 and/or 25(OH)D_3 in patient samples. It has proven to be difficult to produce an antibody that is co-specific for the detection of 25(OH)D_2 and 25(OH)D_3 in human serum. In fact, only one such antibody has been reported and that is the antibody utilized in the DiaSorin 25(OH)D assays [6].

In the U.S. it is imperative that any 25(OH)D assay used for clinical diagnosis has the ability to detect total 25(OH)D, i.e. the sum of 25(OH)D_2 and 25(OH)D_3. With a single exception, all competitive protein binding assays introduced commercially have discriminated against 25(OH)D_2 including the now defunct Nichols Advantage 25(OH)D assay system [4]. It is also a fact that approximately 90% of the U.S. population has no detectable 25(OH)D_2 in their circulation. This is because vitamin D_2 is rarely used as a supplement anymore and patients only receive it when being treated for vitamin D deficiency by a physician. Since blood samples in the general population rarely contain significant amounts of 25(OH)D_2, and because the compound is usually discriminated against by most antibody-based assays, it is the compound most often added exogenously to human serum to assess cross-reactivity and determine analytical recovery.

We have assumed since the early 1970s that when one adds exogenous 25(OH)D to a blood sample it rapidly binds to its carrier protein, the vitamin D-binding protein (DBP), with little interaction to other blood components [7]. Up to this point in vitamin D assay technology, exogenous addition of 25(OH)D_2 or 25(OH)D_3 has served us well in our testing of quantitative analytical recoveries of these compounds [5]. Problems were never encountered because extraction procedures were based on organic solvents of one kind or another and they all destroyed the DBP and liberated the 25(OH)D into solution. The direct serum or plasma assays emerging today do not destroy the carrier proteins. Instead they rely on pH changes and/or blocking agents that liberate the 25(OH)D from its carrier protein but do not affect the ability of the steroid to bind

to a specific antibody. This later disruption method is the method employed in the DiaSorin Assay [8].

5. Conclusion

The results clearly demonstrate that exogenously added 25(OH)D_2 or 25(OH)D_3 do not distribute themselves on the DBP as occurs when assembled *in vivo*. A similar problem was reported by Carter et al. at the 2006 Vitamin D Workshop [9]. Exogenously added 25(OH)D therefore, likely distributes to moieties other than the DBP. This is suggested by the clear linear relationship observed from *in vivo* human samples containing elevated amounts of 25(OH)D_2 when assayed by the DiaSorin Assay versus HPLC (Fig. 1). On the other hand the failure of quantitative recovery is apparent from exogenously added 25(OH)D_2 or 25(OH)D_3 to the same samples and the assay methods compared (Table 1).

This publication describes an *in vitro* anomaly that really has no physiological relevance but could result in erroneous conclusions about 25(OH)D assay performance when comparing sample destruction methods such as HPLC versus the newer sample disruption method such as the Liaison assay. Caution is warranted when preparing samples for such comparisons as is being done by vitamin D External Quality Assessment Scheme (DEQAS) and NIST.

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