Available online at www.sciencedirect.com
ScienceDirect
Clinical Biochemistry 41 (2008) 676–680
CLINICAL BIOCHEMISTRY

Inappropriate serum levels of retinol, α-tocopherol, 25 hydroxyvitamin D_3 and 24,25 dihydroxyvitamin D_3 levels in healthy Spanish adults: Simultaneous assessment by HPLC

J.M. Mata-Granados [a,b], M.D. Luque de Castro [b], J.M. Quesada Gomez [a,c,*]

[a] SANYRES (GRUPO PRASA), E-14012, Córdoba, Spain
[b] Department of Analytical Chemistry, Annex C-3, Rabanales Campus, University of Córdoba, E-14071, Córdoba, Spain
[c] Mineral Metabolism Unit, Reina Sofia Hospital, Córdoba, E-14004, Spain

Received 10 July 2007; received in revised form 30 January 2008; accepted 3 February 2008
Available online 15 February 2008

Abstract

Objective: Simultaneous assessment of the status of lipid-soluble vitamins; retinol, α-tocopherol, 25 hydroxyvitamin D_3 and 24,25 dihydroxyvitamin D_3 in serum of blood donors, paradigm of a healthy population.

Patients and methods: Serum samples were supplied by the Regional Blood Donors Center in Cordoba from 215 healthy Spanish individuals (166 males and 99 females). Target analytes were determined using liquid–liquid extraction and separation–detection by HPLC.

Results: The method was validated using standard reference material (SRM 968c, NIST). Standard errors were 1.4%, 2.1% and 1.8% for $25OHD_3$, vitamin A and vitamin E, respectively.

The ranges thus assessed were as follows: 17.1±8.0 nmol/L, for $24,25(OH)_2D_3$, 40.3±34.6 nmol/L for $25OHD_3$, 2.57±0.7 μmol/L for retinol and 22.13±8.30 μmol/L for α-tocopherol. Females showed lower serum levels of retinol ($p<0.01$), α-tocopherol ($p<0.01$) and $25OHD_3$ ($p=0.028$).

A total of 10.4% subjects showed vitamin E deficiency, 85.4% had normal levels and 4.2% had high levels of vitamin E. 65.6% of the target subjects showed normal levels of retinol, and 1.6% had moderate or severe vitamin A deficiency. High levels of vitamin A were found in 32.8% of the subjects.

Fourteen percent of the healthy subjects showed severe vitamin D deficiency (serum levels of $25OHD_3$ <25 nmol/L), 50.8% had vitamin D_3 insufficiency ($25OHD_3$ from 25 to 50 nmol/L), 17.6% of the subjects had suboptimal $25OHD_3$ serum levels ($25OHD_3$ from 50 to 75 nmol/L), only 16.8% had an adequate status of $25OHD_3$ and 0.8% had high levels of vitamin D ($25OHD_3$ >200 nmol/L). Among subjects with vitamin D below 50 nmol/L, 49.38% had high levels of retinol (over 2.4 μmol/L). This association is considered a risk factor for osteoporosis and fracture.

Conclusions: The reported data of high prevalence of lipid-soluble vitamin values outside the physiological range have important repercussions on public health. These data also uphold the need for simultaneous measurement of fat-soluble vitamins as a valuable tool in clinical practice as well as in epidemiological studies for awareness and correction.

Keywords: Fat-soluble vitamins; Vitamin D; Healthy population

Introduction

Fat-soluble vitamins are essential for human health. Classical syndromes of severe fat-soluble vitamin deficiencies are fairly unusual in technologically advanced societies, with the exception of certain risk groups such as elderly people, vegans and food faddish subjects, alcohol-dependent individuals, extremely poor individuals, patients with malabsorption and prolonged parenteral feeding [1,2]. However, suboptimal serum levels of fat-soluble vitamins are accepted risk-factors for degenerative diseases such as cardiovascular diseases [3,4], cancer [5,6] and osteoporosis [6–8].

Excessive intake of fat-soluble vitamins is not unusual. In fact, megavitamin therapy and indiscriminate use of vitamins

* Corresponding author. Mineral Metabolism Unit, Reina Sofia Hospital, Córdoba, E-14004, Spain. Fax: +34 957 204704.
E-mail address: md1qugoj@uco.es (J.M. Quesada Gomez).

0009-9120/$ - see front matter © 2008 The Canadian Society of Clinical Chemists. Published by Elsevier Inc. All rights reserved.
doi:10.1016/j.clinbiochem.2008.02.003

nowadays are potential causes of hypervitaminosis or vitamin toxicity for vitamins A, D, E [9–12], especially dangerous during pregnancy [13]. Therefore, assessment of the levels of lipid-soluble vitamins in serum for physiological, epidemiological and clinical studies is a challenge for physicians, general practitioners, specialists and researchers.

Serum retinol (vitamin A) concentrations in normal healthy humans are quite constant within a narrow, tightly regulated range that decreases only when liver vitamin A stock is near exhaustion [14].

Vitamin E (tocopherol) is a generic term for several compounds. Among them, α and γ tocopherol are the most abundant forms in food and serum, and their levels have been commonly used as a measurement of vitamin E status [15].

Similarly, serum 25 hydroxyvitamin D_3 (25-hydroxycholecalciferol; $25OHD_3$) levels are the best indicator of vitamin D status. Its quantification includes the $25OHD_3$ generated mainly by hepatic 25-hydroxylase from vitamin D_3, which in turn is formed in the skin from its precursor (7-dehydrocholesterol), as well as from dietary intake and supplements [16]. Increases in serum concentrations of 25 hydroxyvitamin D_2 are observed only after ingestion of vitamin D_2 drug preparations. Traditionally, equivalent biological actions by D_2 and D_3 compounds have been accepted, but recent evidence suggests a stronger effect of D_3 compounds [17]. The physiopathological role of 24,25 dihydroxyvitamin D_3 has been less clearly established and is not usually quantified on clinical grounds [18].

Simultaneous assessment of lipid-soluble vitamins retinol, α-tocopherol, 25 hydroxyvitamin D_3 and 24,25 dihydroxyvitamin D_3 levels in serum of a healthy population has been carried out in order to evaluate the prevalence of inappropriate levels, and establish the inter-related effects of these vitamins according to their levels, as well as their potential impact on public health.

Patients and methods

Patients

Subjects were recruited between April and May from blood donors at the "Reina Sofia" Hospital in Córdoba, Spain (latitude 37.53°) [19]. The study protocol was approved by the Ethical Investigation Committee of the "Reina Sofia" University Hospital. Once informed consent had been given, after an overnight fast blood samples were drawn by venipuncture into evacuated tubes from 215 healthy people from Córdoba (116 males and 99 females), aged between 18 and 65 years (mean: males 38.1 and females 39.5 years old). The serum samples were separated by centrifugation, transferred into capped polypropylene tubes, immediately stored at −80 °C and protected from light until analysis.

Table 1
Validation of the method by CRM 968c, at two concentration levels

	μg/mL	$25(OH)D_3$	Vitamin A	Vitamin E
Level I	Certified value	0.015±0.002	0.841±0.027	7.47±0.47
	Found value (n=4)	0.016±0.002	0.844±0.032	7.51±0.42
Level II	Certified value	0.016±0.002	0.484±0.001	16.79±0.76
	Found value (n=4)	0.015±0.002	0.479±0.001	16.81±0.54

Table 2
Mean values of metabolites of vitamin D_3, retinol and α-tocopherol in serum (mean±standard deviation) and comparison between genders

	Total (n=215)	Reference interval	Males (n=166)	Females (n=99)
Age (years)	38±11.7	18–65	37.3±12.2	38.7±11.2^{ns}
$24,25(OH)_2D_3$ (nmol/L)	17.1±8.0	0.5–27.2	18.8±8.2	16.7±7.4^{ns}
$25(OH)D_3$ (nmol/L)	40.3±34.6	12.1–126.5	44.8±26.2	37±23.1*
Vitamin A (μmol/L)	2.57±0.7	0.98–4.17	2.96±0.6	2.23±0.55**
Vitamin E (μmol/L)	22.13±8.3	3.8–40.5	22.8±9.3	20.6±6.7**

ns: non significant; *p=0.028; **p<0.01.

Bioanalytical measurements

Vitamins A, E, 25 hydroxyvitamin D_3 and 24,25 dihydroxyvitamin D_3 levels were measured by an automated HPLC method [20]. This method consists of two steps: first, a three-step liquid–liquid extraction; a protein precipitation with ethanol, two extractions with hexane and evaporation to dryness with a nitrogen stream. Then, the residue was reconstituted in methanol and injected into the HPLC system for individual separation and detection of the analytes. The variation coefficients of the method for $24,25(OH)_2D_3$, $25OHD_3$, vitamin A and vitamin E intra-assay were 2.25%, 4.3%, 2.95%, 2.02% and those of inter-assay were 9.5%, 9.2%, 5.3%, 2.8%, respectively.

The method was validated using standard reference material (SRM 968c, National Institute of Standards and Technology, Gaithersburg MD, USA). SRM 968c is used to validate methods that determine fat-soluble vitamins, carotenoids and cholesterol in human serum or plasma. SRM 968c provides certified concentration values for all-*trans* retinol (vitamin A), α-tocopherol (vitamin E) and reference concentration values for 25 hydroxyvitamin D_3. The results (n=4) of the method validation by 968c standard reference material are shown in Table 1.

Fat-soluble vitamins deficiency and insufficiency and excess thresholds

Recently, the consensus of vitamin D nutritional experts present at the 13th Vitamin D Workshop established that the $25OHD_3$ minimum desirable serum concentration should exceed 50 nmol/L [20]. Thus, there is a severe deficiency for $25OHD_3$ serum levels lower than 25 nmol/L, a moderate deficiency (or insufficiency) from 25 to 50 nmol/L and suboptimal $25OHD_3$ serum levels correspond to the 50–75 range nmol/L. A sufficient or adequate status of $25OHD_3$ serum level is above 75 nmol/L, and above 200 nmol/L is considered high [20–23].

Normal reported serum retinol levels seem to be highly regulated within a range of 0.7 to 2.8 μmol/L, with severe vitamin A deficiency below 0.35 μmol/L, moderate deficiency from 0.35 to <0.7 μmol/L, and, as regards fracture risk, serum levels above 2.4 μmol/L of vitamin A are considered high risk [24–26].

Adequate serum levels of vitamin E lie between 11.6 and 46.4 μmol/L, being deficient when plasma alpha tocopherol