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Single-step preparation of selected biological fluids for the high performance liquid chromatographic analysis of fat-soluble vitamins and antioxidants

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ABSTRACT

Fat-soluble vitamins and antioxidants are of relevance in health and disease. Current methods to extract these compounds from biological fluids mainly need use of multi-steps and multi organic solvents. They are time-consuming and difficult to apply to treat simultaneously large sample number.

We here describe a single-step, one solvent extraction of fat-soluble vitamins and antioxidants from biological fluids, and the chromatographic separation of *all-trans*-retinoic acid, 25-hydroxycholecalciferol, *all-trans*-retinol, astaxanthin, lutein, zeaxanthin, *trans*-β-apo-8′-carotenal, γ-tocopherol, β-cryptoxanthin, α-tocopherol, phylloquinone, lycopene, α-carotene, β-carotene and coenzyme Q_{10}. Extraction is obtained by adding one volume of biological fluid to two acetonitrile volumes, vortexing for 60 s and incubating for 60 min at 37 °C under agitation. HPLC separation occurs in 30 min using Hypersil C18, 100 × 4.6 mm, 5 μm particle size column, gradient from 70% methanol + 30% H_2O to 100% acetonitrile, flow rate of 1.0 ml/min and 37 °C column temperature. Compounds are revealed using highly sensitive UV-VIS diode array detector.

The HPLC method suitability was assessed in terms of sensitivity, reproducibility and recovery. Using the present extraction and chromatographic conditions we obtained values of the fat-soluble vitamins and antioxidants in serum from 50 healthy controls similar to those found in literature. Additionally, the profile of these compounds was also measured in seminal plasma from 20 healthy fertile donors.

Results indicate that this simple, rapid and low cost sample processing is suitable to extract fat-soluble vitamins and antioxidants from biological fluids and can be applied in clinical and nutritional studies.

1. Introduction

Fat-soluble vitamins are compounds with multiple biological roles and are essential to ensure correct cell functioning that strictly depends on their circulating levels. With the exception of vitamin D_3, humans are not able to synthesize fat-soluble vitamins (A, E and K) that are generally present in different amounts as pro-vitamins in various types of foods, mostly of vegetal origin. After their ingestion, pro-vitamins usually need one or more biotransformation step to generate the corresponding final active vitamin forms [1,2]. To ensure adequate levels within the body, a con-

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stant daily ingestion of pro-vitamin/vitamin-reach foods with diet is necessary. In this, the so called Mediterranean diet is considered one of the best dietary regimen, ensuring the consumption of a large variety of fruits, vegetables and plant-derived products (such as extra-virgin olive oil) containing relevant amounts of most fat-soluble pro-vitamins/vitamins [3].

It is important to stress that the Mediterranean diet also allows the intake of fat-soluble antioxidants, among which carotenoids, such as lutein, zeaxanthin, astaxanthin, α-carotene, β-carotene, lycopene, etc., are certainly one of the most well represented groups in foods of vegetal origin [4]. Several studies indicated that these compounds, due to their capacity to scavenge reactive oxygen species (ROS) [5], may exert beneficial effects in different pathological states characterized by increased ROS production, such as neurodegenerations [6], myocardial ischemia [7], diabetic retinopathy [8].

In the last decades, a renovated scientific interest has been developed not only towards fat-soluble antioxidants, but also towards fat-soluble vitamins. It was discovered that specific fat-soluble vitamins are either useful adjuvant in the pharmacological therapies of several pathologies [9,10] or possibly implicated in some chronic neurodegenerations [11–13]. These facts strongly increased the importance in the clinic setting of a correct identification and quantification of fat-soluble vitamins and antioxidants, often present in very low concentrations in body fluids [14].

Several methods have been proposed for the separation of hydrophobic vitamins and antioxidants in different biological matrices, mainly based on reversed-phase HPLC and using various highly sensitive detectors to quantify their concentrations at the nanomolar levels (fluorometric, electrochemical, MS detectors) [15–19]. Due to the chemical nature (moderately to highly hydrophobic) of fat-soluble vitamins and antioxidants, and to the needs of their quantification in highly polar and complex biological samples (serum, plasma, seminal plasma), specific and efficient sample processing are required. To date, sample treatment for the extraction of fat-soluble vitamins and antioxidants are mainly based on complex multi-steps procedures [20]. Besides using large amounts of highly toxic organic solvents (chloroform, *n*-hexane), these methods have the disadvantage to be time-consuming and expensive, rendering problematic their application for the routine preparation of high number of samples in one shot.

In this work, we describe a novel procedure for the efficient fat-soluble vitamin and antioxidant extraction from biological fluids based on a single-step, single-organic solvent treatment of samples, coupled to a simple and fast reversed phase HPLC method for the simultaneous separation of *all-trans*-retinoic acid, 25(OH)-D_3, *all-trans*-retinol, astaxanthin, lutein, zeaxanthin, *trans*-β-apo-8′-carotenal, γ-tocopherol, β-cryptoxanthin, α-tocopherol, lycopene, α-carotene, β-carotene, phylloquinone and coenzyme Q_{10}. The method was validated in terms of sensitivity, reproducibility and recovery and was successfully applied to measure the aforementioned compounds in two biological matrices, serum and seminal plasma, obtained from two groups of healthy donors.

2. Materials and methods

2.1. Chemicals

Standards of *all-trans*-retinoic acid, 25(OH)-D_3, *all-trans*-retinol, astaxanthin, lutein, zeaxanthin, *trans*-β-apo-8′-carotenal, γ-tocopherol, β-cryptoxanthin, α-tocopherol, phylloquinone, lycopene, α-carotene, β-carotene and coenzyme Q_{10} were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA) at the highest purity available. HPLC-grade solvents (methanol, acetonitrile, chloroform and ethanol) were supplied from Carlo Erba Reagenti (Milano, Italy). Ultrapure water was obtained from a Milli-Q system (Millipore, Millford, MA, USA).

2.2. Sampling of serum and seminal plasma

Peripheral venous blood samples were obtained from 50 healthy volunteers (33 males, 27 females; 48.6 ± 19.3 years of age) recruited among the personnel of the Catholic University of Rome. Assumption of dietary supplements, rich in fat-soluble vitamins or antioxidants, was used as the only exclusion criterion. Whole blood was collected from the antecubital vein into a VACUETTE® polypropylene tube containing serum separator and clot activator (Greiner-Bio One GmbH, Kremsmunster, Austria) and immediately protected from light. After 30 min at room temperature in the dark, samples were centrifuged at 1890*g* for 10 min at 10 °C and 250 μl of the separated sera were immediately withdrawn and processed for the extraction of fat-soluble vitamins and antioxidants.

Seminal plasma was prepared from ejaculates obtained from 20 fertile healthy volunteers (44.3 ± 12.6 years of age) recruited among the personnel of the University of Catania. The ascertained fertility (presence of offspring) was used as the only inclusion criterion. Within 60 min from ejaculation, the light-protected liquefied semen samples were centrifuged at 1480*g* for 10 min at 10 °C and the upper seminal plasma was immediately withdrawn and processed for fat-soluble vitamin and antioxidant extraction.

2.3. Single-step extraction of fat-soluble vitamins and antioxidants

An aliquot of each serum or seminal plasma sample (250 μl) was added to 500 μl of HPLC-grade CH_3CN. After vigorous vortexing for 60 s, these mixtures were incubated at 37 °C for 1 h in a water bath under agitation (to allow full extraction of lipid soluble compounds) and then centrifuged at 20,690*g* for 15 min at 4 °C to precipitate proteins. Clear supernatants were directly used for the reversed phase HPLC analysis of fat-soluble vitamins. All the aforementioned procedures were carried out by protecting samples from light, in order to avoid degradation of photo-sensitive molecules.

To assess recovery, aliquots of 5 randomly selected serum samples were either extracted with no addition or spiked with low (five samples) or high (five samples) concentrations of standard mixtures of fat-soluble vitamins and antioxidants, extracted and deproteinized according to what afore described and then analysed by HPLC.

Additionally, in order to compare efficiency of this new sample processing with a previously well established method (using a multi-step, multi-organic solvent treatment of samples), five randomly selected serum samples were treated for fat-soluble vitamin and antioxidant extraction according to Granado-Lorencio (Granado-Lorencio [15]).

2.4. Reversed phase HPLC assay of fat-soluble vitamins and antioxidants

Stock solutions of *all*-trans-retinoic acid, all-*trans*-retinoic, 25(OH)-D_3, α–tocopherol, γ-tocopherol and phylloquinone were prepared in ethanol to get a 1 mM final concentration. Lutein, zeaxanthin, astaxanthin, β-cryptoxanthin, α-carotene, β-carotene, *trans*-β-apo-8′-carotenal, lycopene and coenzyme Q_{10} were dissolved as 1 mM stock solutions in chloroform. All standards were carefully protected from light.

Stock solutions were stored at −80 °C for up to one month with a loss ≤ 1%. Proper standard mixtures with known concentrations used to validate the HPLC method (linearity, sensitivity, reproducibility) were prepared daily by diluting stock solutions with HPLC-grade CH_3CN. Before HPLC analysis, mixtures were vortexed

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