Journal of Chromatography A, 1205 (2008) 186–190

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Short communication

Simultaneous determination of retinol and α-tocopherol in polymeric diets for enteral nutrition

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ARTICLE INFO

Article history:
Received 1 April 2008
Received in revised form 22 July 2008
Accepted 23 July 2008
Available online 29 July 2008

Keywords:
Retinol
α-Tocopherol
Reversed-phase HPLC
Enteral diets

ABSTRACT

Existing methods for simultaneous measurements of retinol and α-tocopherol in enteral formulas require large sample and solvent volumes and are time-consuming and costly. We have developed a simple, sensitive, cost-effective method for the determination of these vitamins in polymeric diets that can easily be applied to standard quality control of large numbers of samples. Our analytical procedure comprises deproteinization with pure ethanol, saponification with a 3.6 M KOH solution in a sonicator for 30 min at 65 °C under a nitrogen atmosphere, solubilization of samples in phosphate buffer and extraction with hexane. Vitamins are separated by reversed-phase HPLC and quantified by dual-wavelength spectrophotometry. The method gives satisfactory results, with recovery rates of 106.3 ± 1.5% for retinol and 102.3 ± 1.5% for α-tocopherol and RSDs ranging between 1.2 and 4.8% for precision. This method is suitable for the quality control of enteral formulas.

1. Introduction

Over the last decades, artificial nutrition has gained importance in the management of patients who are unable to eat for a prolonged period or who are malnourished. The composition of standard formulas has mostly been based on adult or infant nutritional requirements [1]. However, newly-found pharmacological properties of several micronutrients [2] show that these need to be supplied in amounts larger than the recommended daily allowance (RDA) set for healthy people. Of these micronutrients, vitamins are sensitive to degradation during storage and their content in enteral formulas may be affected by factors such as light, oxygen or temperature. For example, Frias and Vidal-Valverde [3], investigating the stability of thiamine, vitamin A and E in enteral diets, demonstrated that while vitamin content exceeded several times the USA RDA in freshly prepared enteral diets, it fell far below the USA RDA after 3 months of storage: vitamin deficiencies have been described [4,5] despite nominally adequate supplies. There is, therefore, a need for a reliable method to determine vitamin content in enteral diets during both manufacture and storage of these products.

Little has been reported in the literature on the simultaneous determination of vitamin A and vitamin E in polymeric diets for enteral nutrition [3,6]. Methods developed for vitamin measurement in infant formula [7–9] and dairy products [10,11] offer some data, but as vitamins in formulas are usually added as esters, total vitamin assay in enteral diets requires a preliminary saponification step to cleave ester linkages [12]. This makes determination of vitamins more difficult owing to their limited stability during sample preparation. Most methods using saponification are cumbersome as they need large sample and solvent volumes, and are time-consuming and costly. These drawbacks make them ill-suited to the quality control of large numbers of samples. The second step in vitamin determination is extraction from the saponification mixture. Extraction by diethyl ether (alone or combined with light petroleum) [10,13], dichloromethane/methanol [9] or hexane/toluene [14] has been described, but several authors have reported satisfactory results with hexane, which is certainly the most frequently used solvent [6–8]. The last step is the separation itself. Some authors have used chromatographic separation with *n*-hexane/1-octanol [6] or ethyl acetate/hexane [8], but quantification is mostly performed by RPLC using acetonitrile [10] or methanol [9], which gives better reproducibility [12]. We set out to develop a specific, reliable, cost-effective method for the simultaneous assay of retinol and α-tocopherol in polymeric diets for enteral nutrition that could be easily applied to quality control analysis.

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doi:10.1016/j.chroma.2008.07.076

2. Materials and methods

2.1. Materials

All solvents were for HPLC use. Acetonitrile, ethanol, hexane and methanol were purchased from VWR Prolabo (Fontenay-sous-Bois, France). KOH, pyrogallol, retinyl palmitate, α-tocopherol and retinol were obtained from Sigma (L'Isle d'Abeau Chesnes, France). The internal standard (tocol) was from Spiral Labs. (Couternon, France).

Since fat-soluble vitamins are light sensitive, vitamin solutions and samples were prepared away from light. In addition, to further protect vitamins from oxidation during the deproteinization and extraction steps, 1% (m/v) pyrogallol was added to ethanol and hexane [15].

Standard commercial polymeric diets (PDs) for enteral nutrition were generously provided by Nestlé Clinical Nutrition (Noisiel, France). According to the label, this polymeric diet contains 38 g/L proteins, 40 g/L lipids, 126 g/L carbohydrates, minerals and vitamins including 840 μg/L (2.9 μmol/L) retinol and 13 mg/L (30.2 μmol/L) α-tocopherol.

2.2. Preparation of standards

Standard stock solutions of retinol (3 mM) and α-tocopherol (15 mM) were prepared in ethanol and stored at −80 °C away from light. Before use, their concentrations were measured by spectrophotometry at 325 nm for retinol and at 292 nm for α-tocopherol using an extinction coefficient $\varepsilon_{(325\,nm)} = 52.480$ (ethanol) for retinol [16] and $\varepsilon_{(292\,nm)} = 3.260$ (ethanol) for α-tocopherol [16].

Calibration samples were prepared by adding increasing amounts of a working standard solution to 100 μL of PD (final concentrations: 0.35, 0.88, 1.8, 3.5, 7.0 μM for retinol and 7.7, 19.3, 38.3, 76.6, 153.2 μM for α-tocopherol). Thereafter, calibration samples underwent the same analytical procedure as PD samples (see below Section 2.4).

2.3. Preparation of PD samples

2.3.1. Saponification

A 50-μL volume of internal standard (tocol 64 μM) was added to 100 μL of PD and samples were then deproteinized by adding 900 μL of pure ethanol (containing 1% pyrogallol [15]). Saponification was then performed by adding 200 μL of KOH (see concentration below) to 100 μL deproteinized PD samples.

Optimization of sample saponification required determining the KOH concentration for complete hydrolysis of vitamin esters. Eight samples of the same deproteinized PD were mixed with 200 μL of different KOH solutions ranging from 0.18 M to 9.0 M. Samples were homogenized and incubated in a sonicator at 65 °C for 30 min. Hydrolysis was followed by the progressive disappearance of the vitamin ester peak and the parallel increase in the retinol peak by HPLC quantification.

To further improve this saponification step, the influence of oxygen and nitrogen on vitamin stability during the saponification process was investigated.

2.3.2. Vitamin extraction

Since vitamin extraction can be affected by ionic strength and pH [17], extraction was carried out after addition of either 500 μL of water or 500 μL of phosphate buffer (0.2 M, 0.1% EDTA, pH 3.9). Vitamins were then extracted with 2 mL of hexane [6–8] (containing 1% pyrogallol [15]), agitated for 5 min and centrifuged for 10 min at 4000 × *g*. Extraction was repeated three times. Extracts were then desiccated by freeze-drying and reconstituted with 200 μL of an acetonitrile/methanol solution (1:1, v/v) under a nitrogen atmosphere. To evaluate extraction efficiency, each hexane extract was treated as a different sample. Recovery was established for each extraction condition.

2.4. Chromatographic conditions and quantification

The HPLC separation methods and the quantification conditions are based on a standard method for the simultaneous determination of retinol, tocopherol and carotene in biological fluids [18]. Briefly, vitamins were separated and quantified on a Dionex (Voisin le Bretonneux, France) HPLC system consisting of a P680 pump, an ASI-20 autosampler injector and a PDA-100 photodiode-array detector. Separation was performed on a C_8 column (25 cm × 0.46 cm; particle diameter: 5 μm). The column temperature was maintained at 24 °C. The eluent was a gradient of acetonitrile/methanol/water (from 75:15:10 (v/v) at t_0 to 85:12:3 (v/v) at t = 24 min). Flow rate was 1 mL/min up to t = 9 min and 0.8 mL/min thereafter. UV detection was performed at 325 nm for retinol and 292 nm for α-tocopherol. Injection volume was 20 μL with a running time of 30 min between each routine injection. HPLC data were analyzed with the Dionex Chromeleon software. Vitamins were identified by their retention time and UV spectral data compared with those of corresponding standards. Vitamin concentration was calculated from peak areas by the internal standard method, the ratio of the peak area of retinol or α-tocopherol to the peak area of tocol being inserted into the standard curve equation.

Quality of peak separation was assessed by the calculation of resolution (Rs) and peak symmetry (Ss).

A resolution of Rs > 1 and a peak symmetry Ss between 0.8 and 1.2 were considered satisfactory [19].

2.5. Method validation

2.5.1. Linearity

Linearity was assessed using nine vitamin-enriched PD samples with concentrations ranging from 0.2 to 7.6 μM for retinyl palmitate and 2.6 to 102.5 μM for α-tocopherol, a range of concentration largely covering that found in enteral diets.

2.5.2. Sensitivity

Sensitivity of the method was assessed by determining detection limit (DL) and quantification limit (QL) [7].

2.5.3. Precision

The precision of the technique was evaluated by the inter- and intra-serial repetition method. Intra-assay or within-day repeatability was assessed by 10 successive replicate determinations of two PD samples at concentrations corresponding to 0.7 and 2.9 μM of retinol and 7.5 and 30.2 μM of α-tocopherol, respectively.

Inter-assay or between-day reproducibility of the technique was assessed by analyzing, on four distinct occurrences, five replicates of two PD samples at concentrations of 0.7 and 2.9 μM of retinol and 7.5 and 30.2 μM of α-tocopherol, respectively.

2.5.4. Recovery

Recovery was evaluated by analyzing PD samples after adding known amounts of retinol and α-tocopherol to 100 μL PD samples. Recovery was tested for three vitamin levels (1.3, 2.6 and 5.2 μM of retinol and 38.1, 76.3 and 152.5 μM of α-tocopherol) and replicated five times for each level. Average recovery was calculated by comparing mean values of replicates with theoretical concentrations of each replicate.