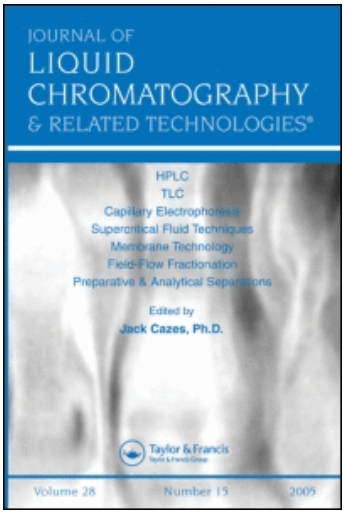

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Simona Pichini[a]; Piergiorgio Zuccaro[a]; Manuela Pellegrini[a]; Simonetta Di Carlo[a]; Antonella Bacosi[a]; Ilaria Palmi[a]; Gianna Tossini[b]; Roberta Pacifici[a]
[a] Clinical Biochemistry Department, Istituto Superiore di Sanità, Rome, Italy [b] Istituto Nazionale per le Malattie Infettive, IRCCS, Rome, Italy

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DETERMINATION OF FAT-SOLUBLE NUTRIENTS IN SERUM BY LIQUID CHROMATOGRAPHY AND MULTIWAVELENGTH DETECTION

Simona Pichini,[1] Piergiorgio Zuccaro,[1,*] Manuela Pellegrini,[1] Simonetta Di Carlo,[1] Antonella Bacosi,[1] Ilaria Palmi,[1] Gianna Tossini,[2] and Roberta Pacifici[1]

[1]Clinical Biochemistry Department, Istituto Superiore di Sanità, V.le Regina Elena 299, 00161 Rome, Italy
[2]Istituto Nazionale per le Malattie Infettive, IRCCS, L. Spallanzani, Rome, Italy

ABSTRACT

Preanalytical and analytical requirements to attain a precise and accurate determination of fat-soluble nutrients in serum by reversed-phase liquid chromatography and multiwavelength detection are described. Retinol, α-tocopherol, α and β-carotene and lycopene were extracted from serum samples with two volumes of hexane-ethanol (5 : 1 v/v). Analytical recovery of retinol and α-tocopherol ranged from 80–90%, while those of α and β-carotene and lycopene never exceed 50%.

Chromatography was performed using a reversed-phase column with a mobile phase consisting of ethanol-acetonitrile

*Corresponding author. E-mail: zuccaro@iss.it

1 : 1 with 0.1 mL diethylamine per liter of solvent. Limits of quantitation were 5 ng/mL for retinol, 200 ng/mL for α-tocopherol, and 5 ng/mL for α, β-carotene, and lycopene, respectively. This method has been used in large-scale determinations of fat-soluble nutrients, never needing dilution of samples or being under limit of quantitation.

INTRODUCTION

The simultaneous measurement of some fat soluble nutrients such as retinol, α-tocopherol, α and β-carotene, and lycopene in serum samples presents several drawbacks and technical problems, due to the chemical nature of the compounds, which might hinder a precise and accurate determination in studies regarding general health or specialized applications (cancer and noncancer disease prevention, hypo- and hypervitaminosis, etc.).(1–6)

Retinol and α-tocopherol can present a rapid photoxidation, both in serum and standard samples, while α,β-carotene, and lycopene can undergo isomerization for the effect of temperature. Furthermore, there is no unique extraction procedure from serum, which gives similar recoveries for all the analytes and blank sera for internal quality control, and method validation must be checked for their content of fat soluble nutrients before spiking with substances under investigation. Finally, while retinol and α-tocopherol have their maximum of light absorbance in the ultraviolet interval, α,β-carotene, and lycopene show maximum absorbance in the visible light interval.

We improved a pre-existent liquid chromatographic method with multi-wavelength detection (7) to simultaneously measure retinol, α-tocopherol, α and β-carotene, and lycopene in serum samples using retinyl acetate as internal standard. Particular attention was dedicated to the pre-analytical step of the assay, which is of utmost importance in obtaining reliable results in a window of time suitable for large scale determinations.

EXPERIMENTAL

Chemicals

Retinol, retinyl acetate (I.S.), α-tocopherol, α,β-carotene, lycopene, and control sera were purchased from Sigma (Milan, Italy). 0.45 μm pore filters were from Millipore (Millipore-Waters, Rome, Italy). All solvents were of analytical reagent grade.

Chromatographic Instrumentation and Conditions

The HPLC system consisted of Crystal 200 Series HPLC pump (Unicam, Cambridge, UK), a Crystal 240 diode array detector (Unicam), and a refrigerated autosampler Varian 3900 (Varian S.p.A., Torino, Italy) set at 4°C and a Crystal Integrated Control Software. The column used was an Adsorbosphere C18 (15 cm × 4.6 mm, 5 μm particle size) from Alltech (Alltech Italia S.r.l., Sedriano, Milan, Italy).

Resolution of the analytes was achieved with a mobile phase consisting of ethanol-acetonitrile 1 : 1 v/v with 0.1 mL diethylamine per liter of solvent, pumped at a flow rate of 0.9 ml/min for 7 minutes, and then changed to 1.6 mL/min until the end of the chromatographic run.

Solutions and Sample Preparation

The entire assay was conducted in darkness under low potency (40 W) gold fluorescent light.

Stock standard solutions of all the analytes (0.5 mg/mL) were prepared by dissolving retinol and α-tocopherol in absolute ethanol and α, β-carotene and lycopene in chloroform and stored in amber glass vials (500 μL) under nitrogen at −80°C. An aliquot of vial content was used to prepare daily working solutions and the remaining portion was discarded. Freeze-thaw stability was determined by thawing, assaying, and then refreezing dilutions (10 μg/mL and 1 μg/mL) of stock standard solutions of all the analytes over three consecutive days. The concentrations chosen were those, which allowed an accurate determination of percentages of degradation.

Serum standards were prepared daily by adding known amounts of working solutions to control human serum. Before use, each control serum lot was analyzed for the presence of fat-soluble nutrients under examination to be taken into account, in the preparation of spiked samples used throughout the entire sample preparation to create calibration curves, to determine analytical recovery and intra- and interday variabilities.

Blood samples were obtained from women enrolled in a National Nutrition Examination Survey. Blood was collected from fasting subjects, immediately centrifuged, transferred to polypropylene tubes, and kept frozen at −80°C until analysis.

Samples (800 μL), with 6 μL of retinyl acetate as internal standard (0.5 mg/mL) added, were extracted with two volumes (1.5 mL) of hexane-ethanol (2.5 : 1 v/v). Aqueous and organic phases were separated for 5 min at 1500 g in a refrigerated centrifuge (4°C). The organic phase was evaporated, redissolved in

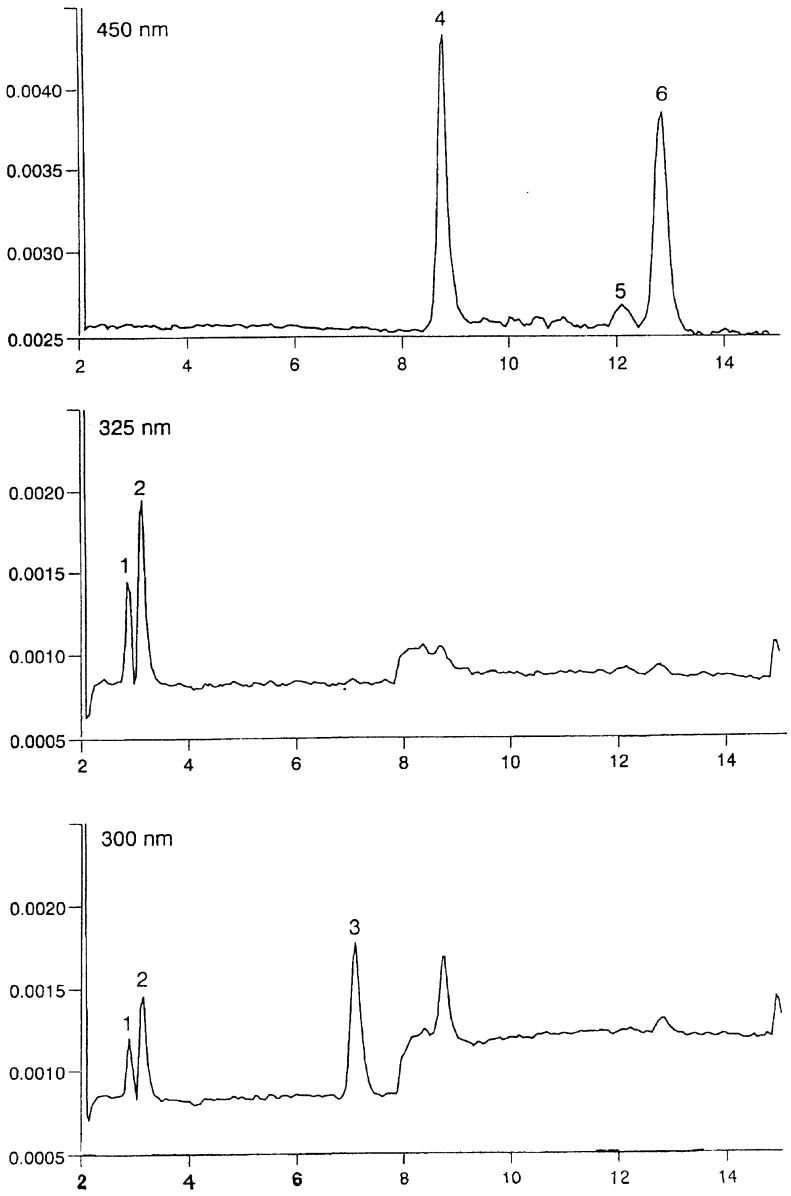

Figure 1. Chromatogram of an extract of serum sample containing (1) 221.7 ng/mL retinol, (2) 0.5 mg/nl retinil acetate (I.S.), (3) 11.0 α-tocopherol, (4) 1.70 μg/mL lycopene, (5) 38.1 ng/mL α-carotene, and (6) 3.98 μg/mL β-carotene.

ethanol-acetonitrile 1 : 1 v/v, filtered through a micropore filter into injection vials, and immediately put in the refrigerated (4°C) autosampler.

RESULTS AND DISCUSSION

Shown in Figure 1 is a typical chromatogram of a serum sample containing 221.7 ng/mL retinol, 11.0 α-tocopherol, 1.70 μg/mL lycopene, 38.1 ng/mL α-carotene, and 3.98 μg/mL β-carotene, respectively. Light absorbance was measured at 325 nm for retinol and retinyl acetate (I.S.), 300 nm for α-tocopherol, and 450 nm for α,β-carotene, and lycopene.

Stability studies on standard solutions at concentration of 10 μg/mL and 1 μg/mL indicated that, in the first three days, a loss of initial concentration ranging between 20 and 30% was observed in the case of retinol and α-tocopherol, between 50 and 55% for α,β-carotene, and lycopene, and more than 90% in case of lycopene. These results proved that once opened and equilibrated at room temperature, solutions of fat-soluble nutrients rapidly degraded. For this reason, it was decided to discard the remaining portion of the vial used to prepare daily working solutions.

Analytical recovery of retinol and α-tocopherol ranged from 80–90%, while those of α and β-carotene and lycopene never exceed 50%. For this reason, and since the course of recovery was supposed to be highly dependent from external variables (e.g. room temperature, humidity), a calibration curve was checked every day, examining three repetitions of five different concentrations. Spiked samples were used, instead of standard solutions, as elsewhere reported (7).

The intraday and interday variabilities, evaluated at three different concentrations on six days, were always below 6% for retinol and α-tocopherol and below 10% for α and β-carotene and lycopene.

Table 1. Concentration Ranges of Fat-Soluble Nutrients from 980 Women Subjects, Ages 23–39 Years

Analyte	Study Ranges (ng/mL)	Reference Ranges (ng/mL)
Retinol	300–900	370–700
α-Tocopherol	6000–20 000	9300–20 000
α-Carotene	10–130	50–500
β-Carotene	35–550	160–950
Lycopene	50–500	70–880

Limits of quantitation were 5 ng/mL for retinol, 200 ng/mL for α-tocopherol, and 5 ng/mL for α,β-carotene, and lycopene, respectively.

More than 980 samples from women participating in a National Nutrition Examination Survey were analyzed by this method, without ever needing dilution of samples or being under the limit of quantitation. Typical ranges obtained for fat-soluble nutrients are shown in Table 1.

In conclusion, the above results demonstrate that a precise and accurate determination of fat-soluble nutrients in serum by reversed-phase liquid chromatography and multiwavelength detection, although easy to perform, requires special care in the pre-analytical phase due to the chemical nature of the compounds under examination.

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