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Simultaneous high-performance liquid chromatographic determination of retinol by fluorometry and of tocopherol by ultraviolet absorbance in the serum of newborns.

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Abstract

The simultaneous determination of retinol and tocopherol by isocratic HPLC in 100 μl serum from preterm newborns is described. Retinol (t_R 2.02±0.04 min) and retinyl acetate were detected fluorometrically, and were baseline-resolved in 4 min. Tocopherol (t_R 8.4±0.16 min) and tocopheryl acetate were detected by UV absorbance. Intra- and inter-assay RSD were: retinol, 5.6 and 8.1, and tocopherol, 3.6 and 6.7, respectively. This method is fast, selective and highly sensitive for retinol. It permits the measurement of serum concentrations of retinol and tocopherol with good accuracy and precision.

Keywords: Retinol; Tocopherol

1. Introduction

The lung of preterm newborns is particularly sensitive to oxygen toxicity [1]. Clinical interest in the simultaneous determination of retinol and tocopherol in the serum of preterm newborns stems from their scavenger effect on free radicals [2] and from the possible involvement of retinol in the pathogenesis of bronchopulmonary dysplasia [3,4]. Since only small serum volumes can be obtained from preterm newborns, sensitivity is a major requirement of procedures for the determination of retinol and tocopherol.

Fluorometric detection usually confers more sensitivity and selectivity than ultraviolet absorbance detection to a HPLC procedure. Although serum tocopherol concentrations in newborns are of the order of mg/dl [5], HPLC procedures with fluorometric detection for tocopherol determination are available [6,7]. Several chromatographic systems have been described for the simultaneous separation of retinol and tocopherol [8–10]. But, in contrast to

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tocopherol, serum retinol concentrations are lower [11], and a convenient method for serum retinol determination by HPLC with fluorometric detection has not yet been reported. To address this issue, we have developed an isocratic HPLC procedure for the simultaneous determination of serum retinol and tocopherol, with fluorometric detection of retinol and UV absorbance detection of tocopherol.

2. Experimental

2.1. Equipment

The liquid chromatograph used here (Waters Associates, Milford, MA, USA) consisted of: a model 6000A pump, a model 420-AC fluorescence detector fitted with a 8 μl flow cell, a F4T5 BL lamp, and 360 and 455 nm wavelength excitation (band-pass, 310 to 390 nm) and emission (long-pass) filters, respectively, and a model 441 absorbance detector fitted with a 10 μl flow cell, and a 280 nm filter. The detectors were placed in series, with the fluorometer nearer to the column. Samples were injected (Rheodyne model 9725i injector fitted with a 200 μl loop) using a 100 μl Hamilton syringe. Chromatograms were recorded using a RB201 recorder (ECB, São Paulo, Brazil), modified to permit switching between output from fluorometer and UV detector. For the sake of convenience, the Waters detectors described above were referred to as detector set 1. Unless otherwise stated, all experiments described here were carried out using the above equipment.

In a second series of experiments to compare the sensitivity of retinol determination by fluorometry and by UV absorption, we have used a Shimadzu model RF-535 fluorometer (Kioto, Japan), with a 12 μl flow cell, set at 325 and 465 nm wavelength for excitation and emission, respectively. A LDC Analytical (Riviera Beach, FL, USA) programmable wavelength absorbance detector, model SM4000, fitted with a 14 μl, 10 mm pathlength flow cell, was set at 325 nm. Fluorescence and absorbance data collection was carried out using the LCTalk software, version 2.03.02 (LDC Analytical), with 1 V full scale for both detectors. These detectors are referred to as detector set 2. All other HPLC modules were as described above.

2.2. Reagents

All-trans-retinol (99% pure), all-trans-retinyl acetate, D-α-tocopherol (99% pure), D-α-tocopheryl acetate were from Acros Organics (Geel, Belgium). All solvents used were HPLC grade (Lichrosolv), and were from Merck (Darmstadt, Germany). Distilled water was purified using a Milli-Q apparatus (Millipore, Bedford, MA, USA).

2.3. Preparation of standard solutions

Stock solutions of retinols and tocopherols were prepared dissolving each vitamin in ethanol (HPLC grade) to 349 μmol/l and 11 600 μmol/l, respectively. Aliquots of stock solutions were stored at −20°C protected from light, and used for up to 2 months. The working solutions were prepared weekly by diluting stock solutions in ethanol to obtain concentrations of retinol and tocopherol in the range 0.17 to 4.2 μmol/l and 11.6 to 232 μmol/l, respectively. An ethanol solution containing both retinyl acetate (0.91 μmol/l) and tocopheryl acetate (106 μmol/l) was used as the internal standard solution.

2.4. Sample preparation

Serum from newborns was stored at −85°C protected from light for up to 1 month prior to analysis. Retinol and tocopherol were extracted from serum according to a minor modification of the procedure of Catignani and Bieri [8]. Briefly, 100 μl serum was added to 100 μl of the internal standard solution in 4 mm I.D.×65 mm pyrolyzed glass tubes, and vigorously vortex-agitated for 2 min. Then, 100 μl *n*-hexane was added, and intermittently and vigorously vortex-agitated for an additional 2 min. The tubes were centrifuged at 3800 *g* for 10 min, and 75 μl of the upper layer were transferred to a glass vial and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 100 μl of mobile phase, and 90 μl were injected into the HPLC. All operations were carried out under reduced light, at room temperature.

2.5. Mobile phase preparation

Solvent was prepared by mixing methanol and water, 95:5, v/v, and was degassed by sonication under reduced pressure for 3 min before use.

2.6. Chromatographic conditions

All-trans-retinol, all-trans-retinyl acetate, D-α-tocopherol, and D-α-tocopheryl acetate were separated by isocratic elution chromatography, at a flow-rate of 1.5 ml/min and at 25°C, using a stainless steel 4 μm NovaPak C_{18} column (3.9 mm I.D.×150 mm). A 5 cm Bondapak precolumn (37–50 μm C_{18}/Corasil packing, Waters) was placed between the injector and column. The effluent was monitored fluorometrically (retinol and retinyl acetate) and by UV absorbance (tocopherol and tocopheryl acetate). The overnight shut down procedure consisted of washing the column with about 20 ml methanol, followed by 20 ml mobile phase, at 1 ml/min.

2.7. Assay precision

A pool of sera from newborn infants was analysed seven times in one day, and the average, standard deviation and intra-assay relative standard deviation (RSD) were calculated. The inter-assay RSD was determined analysing the same pool of sera on fourteen different days.

2.8. Assay accuracy

The accuracy was evaluated by the method of analyte addition recovery [9]. Five different serum samples were spiked with all-trans-retinol and D-α-tocopherol, such that their concentrations were increased by 0.52 and 23.2 μmol/l, respectively. The vitamins were added in ethanol. The samples were analysed as described here, using the internal standard method for quantification. Recovery was calculated subtracting the serum concentration before serum spiking from the serum concentration determined after the addition of the vitamins, and dividing the resulting value by the concentration of vitamin added. Recovery is reported as percent.

3. Results and discussion

3.1. HPLC separation of retinol and tocopherol

Several combinations of mobile phases with C_{18} columns have been proposed for the simultaneous HPLC determination of retinol and tocopherol with UV detection [6,8–10,12,13], among them methanol–water at 95:5, v/v [8]. Retinol and retinyl acetate exhibit broad peaks of excitation and emission, with maxima at wavelengths 325 and 470 nm (retinol) and at 325/340 and 465 nm (retinyl acetate), respectively (data not shown). This is in agreement with the values reported [14] for ethanol solutions of retinol. Tocopherol isomers α, β, γ and δ, and the internal standard tocol, have been separated by HPLC with fluorometric detection [6,13]. However, the fluorescence of tocopherol and tocopheryl acetate was not detected with the fluorescence filters available for the detector used here. Therefore, we have detected retinol and retinyl acetate fluorometrically in the present method, whereas tocopherol and tocopheryl acetate were detected by absorbance at 280 nm.

Fig. 1 shows baseline resolution, within 14 min, of retinol and tocopherol in the serum of a newborn, and of the internal standards retinyl and tocopheryl acetate. Retention times (min±SEM, for 12 runs) were: 2.02±0.04, 3.0±0, 8.4±0.16, 12.7±0.48, for retinol, retinyl acetate, tocopherol and tocopheryl acetate, respectively. Retinol and tocopherol extracted from serum exhibited the same retention times as the authentic compounds (data not shown). When the guard column was changed after about every 120 analysis, retinol, tocopherol, and the internal standards used were well separated for at least 2000 analysis per column.

One of the problems commonly reported with the simultaneous HPLC determination of retinol and tocopherol is an incomplete resolution of these vitamins from serum contaminants. This led to the development of several chromatographic systems [9,10]. We have overcome this problem by detecting retinol and retinyl acetate fluorometrically (Fig. 1), since fluorescence detection is generally more selective than absorbance detection. A second problem described in the literature is that the separation of

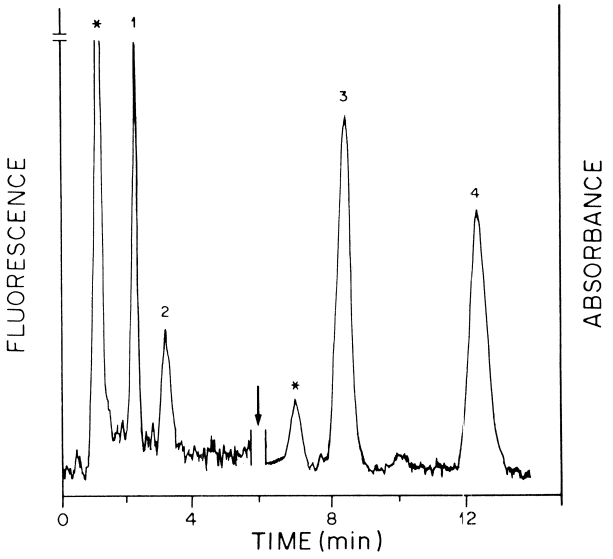

Fig. 1. Reverse-phase HPLC of retinol and tocopherol from 100 μl serum from a newborn, using the method described here. Retinol (peak 1) and retinyl acetate (peak 2; 61.4 pmoles) were detected by fluorometry, at gain 64. Tocopherol (peak 3) and tocopheryl acetate (peak 4; 7.15 nmoles) were detected by absorbance at 280 nm, at 0.01 AUFS. Unidentified serum peaks are indicated by *. The concentrations of retinol and tocopherol found in serum were 1.59 and 62.7 μmol/l, respectively. The arrow indicates the change in the input to the recorder, from the fluorescence to the UV detector.

retinol from retinyl acetate is sometimes incomplete in the context of the simultaneous determination of retinol and tocopherol [6]. The solvent system used here permits the tailoring of the mobile phase so that small changes (1% or less) in the proportion of methanol and water can separate retinol from retinyl acetate, without impairing the resolution of tocopherol from tocopheryl acetate. The chromatographic system described here is simple and reproducible. The peaks are symmetrical and well resolved from each other and from serum contaminants. Furthermore, when only the determination of retinol is required, this method permits the injection of a sample every 4 min, without the problem of interfering peaks (data not shown).

3.2. Linearity of standard curves

The ratios of the peak heights of all-trans-retinol/retinyl acetate and D-α-tocopherol/tocopheryl acetate were linearly related to their concentration ratios in the following ranges: for all-trans-retinol, 0.17 to 4.2 μmol/l (6 points, each determined in triplicate) [height ratio=(1.239±0.0163)×concentration ratio+(−0.0279±0.0460); r^2=0.9972]; for D-α-tocopherol, 11.6 to 232 μmol/l (6 points, each determined in triplicate) [height ratio=(1.597±0.0185)×concentration ratio+(−0.0235±0.0228); r^2=0.9979].

The ratios of peak height/noise for the lower limits of quantification for retinol (0.17 μmol/l) and tocopherol (11.6 μmol/l) were 3.8 and 10.3, respectively.

3.3. Analytical characteristics of the assay

The relative standard deviations for retinol and tocopherol were: intra-assay 5.6 and 3.6, inter-assay, 8.1 and 6.7, respectively.

Accuracy was good, as indicated by the recoveries of known amounts of authentic retinol and tocopherol added to serum. Recoveries were 98.6±5.9% (range 85 to 115) and 102.8±3.9% (range 94 to 116).

3.4. Further studies of retinol quantification limit

The small amount of blood that can be collected in some clinical (for example, of very very low birth weight preterm newborn) and experimental situations (small laboratory animals) requires a method for the determination of retinol with high sensitivity. A comparison of relative sensitivities of retinol HPLC determination by fluorometry and absorbance was carried out using detector set 2. We have used 325 nm excitation and 465 nm emission wavelengths for fluorescence determination, and 325 nm wavelength for absorbance detection. Fig. 2 shows that the sensitivity of retinol determination by fluorometry was higher than that obtained measuring absorbance at 325 nm wavelength. The ratio of peak height/noise for the lower limit of quantification of retinol (0.017 μmol/l) by fluorometry was 3.6. In addition, although it has been reported [9] that unidentified compounds interfere with retinol determination using absorbance at 325 nm wavelength, no interfering serum peaks were detected by fluorometry nor by absorbance in 20 sera from preterm newborns analysed using the detection conditions above (data not shown).

Furthermore, using detector set 2, there was a linear relationship between peak height ratio of all-trans-retinol/retinyl acetate and their concentration ratios in the range: 0.35 to 0.017 μmol/l: (5 points, each determined in triplicate) [height ratio = $(0.9112 \pm 0.0329) \times$ concentration ratio + (-0.0425 ± 0.0972); $r^2 = 0.9833$]. The sensitivity for the quantification of retinol reported here (0.017 μmol/l) is about 10-fold higher than that reported recently [6] using a programmable absorbance detector at 325 nm wavelength. This increase in the sensitivity of retinol quantification permits the use of less than 100 μl serum for retinol determination.

4. Conclusions

There is a need for a high sensitivity analytical method for the determination of retinol and

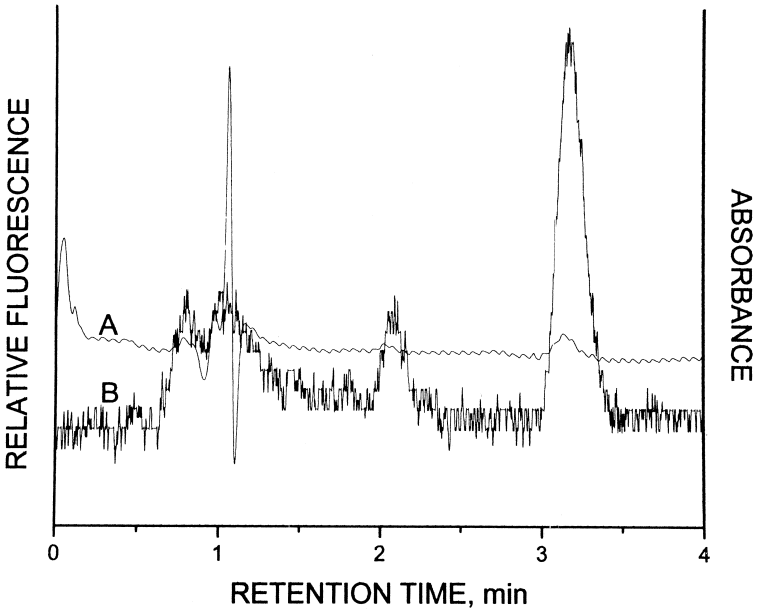

Fig. 2. HPLC of retinol: comparison between the sensitivity of absorbance (A) and fluorescence (B) detection. Ninety μl of a sample containing retinol (0.017 μmol/l) and retinyl acetate (0.061 μmol/l) were injected. Fluorescence detection was carried out at 325 nm excitation and 465 nm emission wavelengths. Absorbance detection was at 325 nm. Detector set 2 was used. The 1 V output of both detectors was used with the LCTalk interface and software program.

tocopherol. We describe here a rapid (4 min), selective, sensitive and convenient procedure for the determination of retinol by fluorometric detection. The same chromatographic procedure can be used for the simultaneous determination (in 14 min) of retinol by fluorometry and tocopherol by absorbance in 100 μl serum, with good accuracy and precision.

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