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Short Communication

Simultaneous determination of retinol, α-tocopherol and retinyl palmitate in plasma of premature newborns by reversed-phase high-performance liquid chromatography

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ABSTRACT

A reversed-phase high-performance liquid chromatographic method is described for the simultaneous determination of retinol, α-tocopherol and retinyl palmitate in plasma. Plasma containing an internal standard (tocol) was deproteinized with ethanol, then extracted with *n*-hexane. The organic layer was removed and evaporated under a nitrogen stream, and chromatographed on a reversed-phase RP-18 column using a water/acetonitrile–ethyl acetate/2-propanol gradient solvent system over 15 min at 305 nm. The recovery exceeded 93%. The detection limit was 0.1 μg/ml for retinol, 1.3 μg/ml for α-tocopherol and 0.95 μg/ml for retinyl palmitate. The reproducibility, precision (expressed as coefficients of variation) and accuracy were less than 8% for all analytes. The small sample requirement, the simplicity of extraction, the short run-time and the good reproducibility make this procedure particularly useful for monitoring retinol and α-tocopherol supplementation in premature newborns.

INTRODUCTION

Deficiencies of vitamin A (retinol) and vitamin E (α-tocopherol) may be associated with severe pathological conditions in premature newborns. A supplementation from early postnatal life with vitamin A was apparently effective in promoting regenerative healing of lung injury [1], and supplementation with vitamin E was proposed as prophylaxis against syndromes caused by oxygen toxicity or membrane instability [2–7]. Measuring the plasma concentrations of the fat-soluble vitamins, retinol, α-tocopherol and retinyl palmitate (palmitic ester of retinol), is therefore particularly important in neonatal intensive care units as a guide for dosage adjustment during vitamin supplementation.

Several high-performance liquid chromatographic (HPLC) procedures for quantitating retinol and α-tocopherol simultaneously [8–16] and retinyl palmitate [17–20] have been published. Because vitamin A supplements are given as esters of retinol (retinyl acetate or retinyl palmitate), and retinyl palmitate appears in the blood both in the absorptive state and in cases of hypervitaminosis A [21–23], retinol, α-tocopherol and retinyl palmitate must be measured simultaneously to assess supplementation therapy.

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Considering the small size and the clinical severity of these patients, a reliable, sensitive and rapid method (retinyl palmitate eluted in less than 15 min) using small samples is mandatory.

EXPERIMENTAL

Chemicals and reagents

All-*trans*-retinol and retinyl palmitate were purchased from Sigma (St. Louis, MO, USA) and α-tocopherol from Merck (Darmstadt, Germany). Tocol (internal standard) was synthesized in our laboratory according to Pendse and Karrer [24] and currently used for α-tocopherol monitoring [25].

Acetonitrile, ethyl acetate, ethanol, 2-propanol and *n*-hexane (LiChrosolv, Merck) and methanol (J. T. Baker, Phillipsburg, NJ, USA) were UV grade.

Standards

Individual stock standards (0.1 mg/ml) of retinol and α-tocopherol were prepared in methanol, and retinyl palmitate (first diluted in a small amount of water) was dissolved in ethanol. All standards were stored at −20°C in brown glass vials under nitrogen.

A pool of plasma obtained from healthy adult subjects was used for quality control. The pool was divided into 1-ml samples and stored at −20°C under a nitrogen atmosphere until analysis. The average endogenous concentrations were 0.9 μg/ml for retinol and 8 μg/ml for α-tocopherol.

Sample collection and storage

Blood samples from healthy volunters and excess blood submitted for routine analysis from newborns were collected in tubes (protected from light with aluminium foil to minimize light-induced degradation of vitamins) containing disodium EDTA and potassium fluoride, and centrifuged. Plasma was transferred to a clean test-tube (protected from light) and stored at −20°C until analysis.

Extraction procedure

A 0.2-ml plasma sample was mixed with 0.02 ml of internal standard (0.1 mg/ml) and 0.2 ml of ethanol as precipitating agent, and vortex-mixed. The mixture was then extracted with 0.6 ml of *n*-hexane and vortex-mixed for 3 min. After centrifugation for 10 min at 3000 *g* to remove precipitated proteins, the *n*-hexane layer was transferred to dark test-tubes and evaporated to dryness under a gentle stream of nitrogen. The tubes were rapidly capped until analysis. The residue was dissolved in 0.15 ml of a mixture (1:1, v/v) of chromatographic eluents and vortex-mixed, and 0.10 ml were injected into the chromatograph. A calibration curve (for each series of ten to fifteen samples) for retinol, α-tocopherol and retinyl palmitate was prepared by adding increasing amounts of a combined working standard solution to blank plasma samples whose retinol and α-tocopherol basal contents were known.

Chromatographic conditions

A Beckman Model 342 liquid chromatograph (Fullerton, CA, USA) equipped with a Model LC-95 detector (Perkin-Elmer, Norwalk, CT, USA), and a reversed-phase column (Superspher RP-18, 4 μm, 25 mm × 4 mm I.D., LiChroCART HPLC cartridge, Merck) were used. A guard column (Merck), 4 mm × 4 mm I.D., packed with 5 μm particles, was attached on-line before the main column. A linear gradient of 90:10 (v/v) acetonitrile–water (solvent A) and 90:10 (v/v) ethyl acetate–2-propanol (solvent B) was used. The column was equilibrated using 100% solvent A, then a 9 min linear gradient was run to 60% of solvent B and maintained for an additional 6 min, followed by a 10 min re-equilibration with solvent A. The flow-rate was 1.5 ml/min and the detection wavelength was 305 nm.

Calculations

Quantitation was performed using peak-height measurements and the ratio of analyte to internal standard. The endogenous concentrations of retinol and α-tocopherol in the unspiked samples were subtracted from the total concentration in the spiked samples. Results are expressed as the

mean, standard deviation (S.D.) and coefficient of variation (C.V., %). Regression lines were obtained by the least-squares method [26].

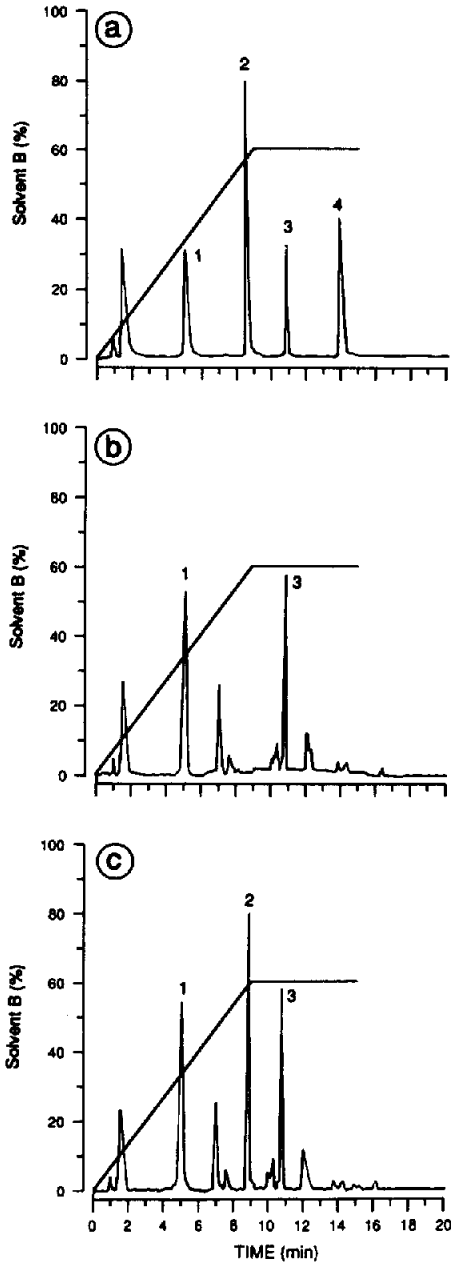

Fig. 1. Chromatograms of extracted standard solution (a), adult plasma (b) and adult plasma with internal standard added (c). Chromatographic conditions are given in the text. Peaks: 1 = retinol, 0.5 μg/ml in (a); 2 = tocol, 10 μg/ml (internal standard); 3 = α-tocopherol, 5 μg/ml in (a); 4 = retinyl palmitate, 5 μg/ml in (a).

RESULTS AND DISCUSSION

Fig. 1a shows a chromatogram of extracted standard solutions. Fig. 1b depicts a typical chromatogram of two fat-soluble vitamins extracted from plasma, and Fig. 1c the elution profile of the same plasma sample with internal standard added. The chromatograms in Fig. 1b and c compare well with a chromatogram of pure standard (Fig. 1a). The retention times were 5.0 min for retinol, 8.9 min for tocol, 10.9 min for α-tocopherol and 14 min for retinyl palmitate. Retinol, tocol (internal standard) and α-tocopherol have similar polarity and eluted close to one another under isocratic conditions, whereas retinyl palmitate took much longer. The initial (9 min) gradient from 100% of solvent A to 60% of solvent B allowed us to maintain good resolution of the first three peaks and also to elute retinyl palmitate in a reasonable time. Besides, compared with a previously reported method for the simultaneous determination of retinol, α-tocopherol and retinyl palmitate [17], the use of acetonitrile instead of methanol in the linear gradient allowed us to work at a low pressure (*ca.* 6.9–8.3 MPa). Under the chromatographic conditions used, the chromatograms demonstrate baseline separation and peak symmetry. Most of methods reported in literature used retinyl acetate [8,10–12,14–17] as internal standard. However, in many of our plasma chromatograms, there was an intrinsic peak that nearly co-eluted with retinyl acetate. This peak was also seen by other authors [12]. Retinyl acetate was stable for only a short time, and a slight shoulder appeared on the peak after about two weeks of standard storage. Thus the use of tocol represents an improvement to the assay, because it is much more stable and no interfering peaks were found close to its retention time. Calibration graphs after subtraction of the basal concentration of retinol and α-tocopherol passed through the origin and were linear up to at least 8 μg/ml for all analytes.

Detection limits, recovery, intra- and inter-day precision and accuracy are shown in Table I. The detection limit was estimated to be 0.1 μg/ml for retinol, 1.3 μg/ml for α-tocopherol and 0.95 μg/

TABLE I

DETECTION LIMITS, RECOVERY AND INTRA- AND INTER-DAY PRECISION AND ACCURACY

Analyte	Limit of detection (μg/ml)	Recovery (mean ± S.D., n = 6) (%)	Coefficient of variation (%)		Accuracy (mean ± S.D., n = 18) (%)
			Intra-day (n = 16)	Inter-day (n = 10)	
Retinol	0.1	96 ± 4	5	3	6 ± 1
α-Tocopherol	1.3	93 ± 4	4	3	8 ± 2
Retinyl palmitate	0.95	98 ± 5	6	4	7 ± 2

ml for retinyl palmitate at a signal-to-noise ratio of 3. This assay is *ca.* 13 times more sensitive for retinol than α-tocopherol on account of the detection wavelength used. Nevertheless, because plasma ordinarily contains *ca.* 10–15 times more α-tocopherol than retinol, the two peak heights are usually comparable.

The analytical recovery of the three analytes added to plasma at concentrations of 2.5 and 5 μg/ml averaged 96 ± 4% for retinol, 93 ± 4% for α-tocopherol and 98 ± 5% for retinyl palmitate. Recovery of retinyl palmitate depends largely on its solubility in the solvent used to dissolve the concentrated plasma extract. A 1:1 (v/v) mixture of the two eluents allowed the best recovery of this analyte, without changes in the analytical response of other compounds.

Inter- and intra-day precisions were assessed using plasma samples containing 1 μg/ml retinol and retinyl palmitate and 8 μg/ml α-tocopherol. Inter-day precision was calculated by measuring the levels in ten aliquots of the same plasma sample during one day. The C.V. for retinol, α-tocopherol and retinyl palmitate were 3%, 3% and 4%, respectively. When a plasma sample was analysed in duplicate each day for sixteen times over two months, the intra-day precisions for retinol, α-tocopherol and retinyl palmitate were 5%, 4% and 6%, respectively.

Accuracy was calculated as the percentage deviation from the true value on processing samples containing 2.5 and 5 μg/ml of each analyte; the average values were 6% for retinol, 8% for α-tocopherol and 7% for retinyl palmitate. With the procedures described, the lifespan of the analytical column was very long; over 800 runs were made on the same column with no serious loss of resolution. The guard column was changed once during this time.

To assess the clinical usefulness of this method we determined the plasma concentrations of retinol, α-tocopherol and retinyl palmitate in premature newborns and adults (Table II). The concentrations of retinol and α-tocopherol are in good agreement with literature concentrations [27]. Retinyl palmitate, a minor component of total plasma vitamin A, was not detected in our samples in view of the low concentrations reported in plasma in the post-absorptive state.

The method described is an improvement on previously described assays. It is efficient and sensitive, and needs only routine instrumentation. Small volumes of sample are needed, and only limited time and costs are involved in sample analysis. The procedure is routinely used in our laboratory and appears ideal for clinical and research use.

TABLE II

RETINOL AND α-TOCOPHEROL IN PLASMA

Data are expressed as mean ± S.D., with range in parentheses.

Subject	n	Retinol (μg/ml)	α-Tocopherol (μg/ml)
Newborns	9	0.33 ± 0.12 (0.18–0.52)	2.5 ± 0.3 (2.0–2.8)
Adult volunteers	7	0.68 ± 0.11 (0.60–1.00)	10.0 ± 2.0 (7.1–12.3)

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