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Simultaneous determination of low plasma concentrations of retinol and tocopherols in preterm infants by a high-performance liquid chromatographic micromethod

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Abstract

A method for the simultaneous determination of low concentrations of retinol and tocopherols from 100 μl plasma using isocratic reversed-phase HPLC is described. Retinol is quantified with a programmable UV–Vis detector, whereas tocopherols are quantified by fluorescence detection using tocol as the internal standard. Intra- and inter-assay precision are 3.7 and 4.3% for retinol and 2.3 and 6.1% for α-tocopherol, respectively. The accuracy as determined with standard material from the US National Institute of Standards and Technology with low, medium and high concentrations is in the range of 0.2–6.0% bias for retinol and of −3.0 to 5.5% for α-tocopherol, respectively. This method is highly sensitive and selective and has a good precision and accuracy for measuring low concentrations of vitamins in small plasma volumes.

Keywords: Vitamins; Retinol; Tocopherol

1. Introduction

The supply and metabolism of lipid soluble antioxidants in preterm infants is receiving increasing attention. The formation of oxygen radicals appears to play an important role in several disorders which frequently occur in immature babies, including bronchopulmonary dysplasia, retinopathy of prematurity, persistent ductus arteriosus, necrotizing enterocolitis and intracranial haemorrhage [1,2]. Moreover, preterm neonates often require parenteral infusion of lipid emulsions, which are highly susceptible to peroxidation particularly in combination with phototherapy [3], which indicates the need to optimize antioxidant status in these patients. Plasma levels of retinol and tocopherol are usually low in preterm infants. In the past, research on lipid soluble vitamin status has been somewhat limited by the need to obtain relatively large volumes of plasma in these small babies to detect such low plasma concentrations.

Several high-performance liquid chromatography methods have been described for the simultaneous determination of retinol and tocopherols, which require 200 μl or more of plasma or serum [4–10]. Few methods for vitamin assessment in 100 μl of plasma have been developed and validated for vitamin concentrations found in adults, but no validation data were published for the relatively low vitamin concentrations found in preterm infants [11–

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15]. Preterm neonates have plasma concentrations of α-tocopherol which are approximately one third of the concentration in maternal serum (0.29±0.09 mg/dl vs. 0.97±0.16 mg/dl) [16]. Thus, one needs a sensitive, precise and accurate method to assess low concentrations of retinol and tocopherols in small plasma volumes available from preterms. We developed a method that meets these requirements.

2. Experimental

2.1. Chemicals

We used the following chemicals: retinol, DL-α-, DL-γ- and DL-δ-tocopherol (Merck, Darmstadt, Germany), BHT (butylhydroxytoluol), ammonium acetate (all from Fluka, Buchs, Switzerland); acetonitrile LiChrosolv, *n*-hexane LiChrosolv, methanol LiChrosolv, gradient grade (all from Merck); ethanol absolute p.A. (Baker, Deventer, Netherlands); tetrahydrofurane (Fluka) and Standard Reference Material 968a (National Institute of Standards and Technology, Gaithersburg, MD, USA). The internal standard tocol was a gift from Hoffmann-La Roche (Basel, Switzerland).

2.2. Equipment

The HPLC system consisted of the following instruments: autosampler AS-2000; chromato-integrator D-2500; UV–Vis detector L4250; fluorescence spectrophotometer F-1050; intelligent-pump L6200 (all from Merck) and a column oven (Gynkothek, Germering, Germany). The UV–Vis spectrophotometer program was run as follows: the detector was programmed to monitor 325 nm from 0 to 4.05 min for retinol and 292 nm from 4.05 to 8.5 min for the internal standard. The fluorescence detector was set to an excitation wavelengh of 298 nm and to an emission wavelengh of 330 nm to detect tocopherols. The injection volume was 20 μl (cut-volume technique). The column temperature was held constant at 30°C.

2.3. Calibration solutions

The stock solutions and dilutions of retinol and tocopherols were prepared in ethanol. The working standards were made with *n*-hexane–BHT. The stock solution of the internal standard tocol was made with ethanol–BHT (0.0625%). For the preparation of the tocol working solution an aliquot of the stock solution was evaporated under a stream of nitrogen and resolved in mobile phase–BHT (0.02%). The stock solutions can be stored at −80°C for at least two months [4,17]. The dilutions were made immediately prior to use, their absorbances were determined photometrically and the concentrations calculated by using the standard absorbances E (1 cm/1%): retinol 1780 at 325 nm, α-tocopherol 75.8 at 292 nm, γ-tocopherol 92.8 at 298 nm and δ-tocopherol 91.2 at 298 nm. A mixture of the working standards based on the expected concentrations was made. For the calibration curve, various amounts of the standard mixture were evaporated after addition of BHT (final concentration 0.05%) and finally resolved in tocol working solution.

2.4. Sample preparation

Plasma was stored at −80°C before analysis. For analysis, 100 μl of plasma and 100 μl of ethanol–BHT (0.0625%) [18] were pipetted into 1.5-ml brown glass test tubes to minimize light-induced degradation of vitamins, and vortexed for 15 s. A 1-ml volume of *n*-hexane–BHT (0.005%) was added, the tubes were shaken mechanically for 10 min and centrifuged (3 min, 2000 *g*). After centrifugation, the tubes were placed on ice to improve phase separation. An aliquot of 900 μl of the supernatant was pipetted into a second test tube and evaporated to dryness under a stream of nitrogen. This extraction procedure was repeated twice with pure *n*-hexane removing 1000 μl of the hexane layer and combining it with the extracts of the former steps. Extracts were reconstituted in 100 μl of the working tocol solution, shaken mechanically for 10 min and transferred into microvials before injecting into the HPLC system.

2.5. Column

A LiChrospher 100, RP-18 (5 μm) column (LiChroCART 250-3) in combination with a precolumn, LiChrospher 100, RP-18 (5 μm) (LiChroCART 4-4) (both from Merck) were used.

2.6. Mobile phase

An isocratic mobile phase consisting of acetonitrile–tetrahydrofurane–methanol–1% ammonium acetate (684:220:68:28, v/v) was used [19]. To avoid the formation of explosive peroxides from tetrahydrofurane, the mobile phase was prepared prior to use and degassed ultrasonically. The flow-rate of 0.65 ml/min was held constant for a run time of 9 min. After a run time of 20 min, the flow-rate was reduced to the initial rate.

2.7. Standard curves and calculation

Vitamins were quantified from calibration curves of peak-area ratios (vitamin/tocol) vs. vitamin concentrations. Since fluorescence detection of tocopherols is much more sensitive than UV–Vis detection, tocopherol concentrations were calculated exclusively by means of fluorescence detection. β- and γ-Tocopherol elute as a single peak, but usually the concentration of β-tocopherol is negligible.

3. Results

3.1. Linearity of calibration curves

The following numbers reflect the used working range of the different vitamins, calculated from expected plasma concentrations plus and minus 50% [16,20]. The calculated coefficients of determination obtained with our method in these ranges using six vitamin concentration levels are noted in parenthesis: retinol 0.19–0.86 μmol/l (r^2=0.9988), δ-tocopherol 0.62–2.78 μmol/l (r^2=0.9990), γ-tocopherol 0.88–3.96 μmol/l (r^2=0.9993), α-tocopherol 2.37–10.68 μmol/l (r^2=0.9897).

3.2. Quantification limit

The quantification limits, defined as the lowest quantitatively measurable concentration of the different compounds (μmol/l) were calculated with an accuracy of 95% according to Funk et al. [21]: retinol 0.192, δ-tocopherol 0.617, γ-tocopherol 0.878, α-tocopherol 2.370.

3.3. Intra-assay variation with cord blood

Since it is not ethically justifiable to perform validation experiments with the blood of preterm infants, we used cord blood which can be obtained without any adverse effects for the infant. A cord blood plasma pool made from 30 term infants was analysed 20 times and mean values, standard deviations and R.S.D. were calculated. In biological assays, R.S.D. below 5% are considered desirable, while 10% are tolerated with micromethods. All R.S.D. were below 5% (Table 1).

3.4. Inter-assay variation with cord blood

A deep frozen cord blood plasma pool made from 30 term infants was analysed during a period of seven weeks at 20 different days to test the day-to-day reproducibility. The following R.S.D. were obtained: retinol 4.3%, γ-tocopherol 11.1%, α-tocopherol 6.1%. No δ-tocopherol was measurable in this plasma pool.

3.5. Recovery from cord blood

Recovery was assessed by adding six progressively increasing standard concentrations, equidistantly distributed in the working range, to cord blood plasma according to Funk et al. [21], Carey et al.

Table 1
Intra-assay with cord blood

Vitamin	Mean (μmol/l)	S.D. (μmol/l)	R.S.D. (%)
Retinol	0.681	0.025	3.7
δ-Tocopherol	0.668	0.033	4.9
γ-Tocopherol	1.400	0.024	1.7
α-Tocopherol	7.607	0.174	2.3

Mean vitamin concentrations resulting from analysis of 20 extracts of a cord plasma pool.

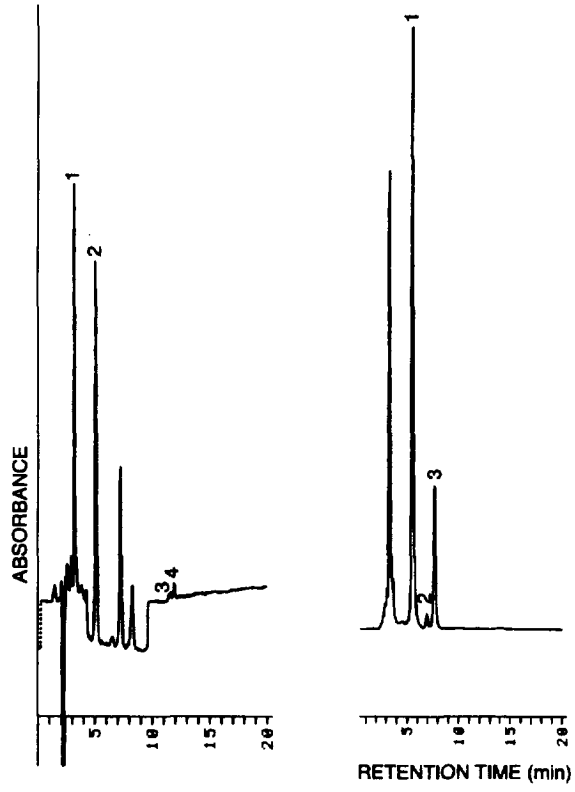

Fig. 1. Chromatograms of cord blood plasma, vitamin concentrations in (μmol/l) in parenthesis. UV–Vis detection (left); peaks: 1=retinol (0.626); 2=tocol (internal standard). Fluorescence detection (right); peaks: 1=tocol; 2=γ-tocopherol (0.383); 3=α-tocopherol (7.843).

[22] and Koch et al. [23]. The standards in *n*-hexane were pipetted into the tubes, evaporated to dryness, plasma was added and analysed as described. Each level was analysed in quadruplicate. Recovery was calculated by subtracting from the concentration measured the baseline concentration obtained by intra-assay analysis (see Table 1, Fig. 1), and dividing the resulting value by the concentration of substance added. This yielded a recovery figure expressed in percent. The mean recoveries of 96 to 101% for tocopherols and retinol were very satisfactory (Table 2).

3.6. Accuracy

The accuracy of this method was verified with lyophilized standard reference material 986a obtained from the US National Institute of Standards and Technology (NIST), which contains the vitamins in low, medium and high concentrations. Nine samples of each concentration level were analysed. Our results were in good agreement with the approved values. A bias between 0 and 5% is regarded as excellent, while a bias between 5 and 10% is considered acceptable by the NIST. The results we achieved under the conditions employed were all in the acceptable to excellent range (Table 3).

4. Discussion

The development of a sensitive and reliable method for determination of low lipid soluble vitamin concentrations from small plasma volumes poses a considerable challenge. In an attempt to determine the most suitable stationary phase for chromatography, we tested 6 columns listed below with different lengths, inner diameters and characteristics of stationary phases: (a) EcoCART 125-3, LiChrospher 100 RP-18 (5 μm), Merck; (b) LiChroCART 125-4,

Table 2
Recoveries from cord blood

Vitamin	Ranges of added vitamins (μmol/l)	Ranges of measured plasma concentrations (μmol/l)	Recovery (%)
Retinol	0.174–0.836	0.840–1.454	89±4
δ-Tocopherol	0.527–2.659	1.262–3.244	101±6
γ-Tocopherol	0.827–3.851	2.276–5.083	98±4
α-Tocopherol	2.426–10.138	9.989–17.605	96±7

Six concentration levels of a standard mixture were added to cord blood pool plasma and analysed four times. The added concentration found was calculated by substracting the pool plasma concentration measured in the intra-assay analysis (see Table 1) from the measured plasma concentration after standard addition. Recovery (mean±S.D.) was calculated for each concentration level by dividing the added concentration found by the actually added vitamin concentration.

Table 3
Accuracy determined with standard material from the US National Institute of Standards and Technology (NIST)

Vitamin Level	Mean (μmol/l)	R.S.D. (%)	Cert. val.[b] (%)	Bias (%)
Retinol				
Low	0.627	1.9	0.667	6.0
Medium	1.759	1.4	1.763	0.2
High	2.194	1.2	2.318	5.3
γ-Tocopherol				
Low	1.966	2.5	2.1[a]	6.4
Medium	6.653	1.0	6.9[a]	3.6
High	9.079	0.8	8.8[a]	−3.2
α-Tocopherol				
Low	10.031	1.5	10.61	5.5
Medium	25.089	1.2	24.36	−3.0
High	36.164	1.4	36.99	2.2

Mean vitamin concentrations and R.S.D. at three concentration levels, each analysed nine-fold. The bias from the assigned values was calculated as follows: bias (%)=[(reference value−mean)/reference value]×100%.

[a] Reference values determined by a limited number of analyses, provided by NIST for information only.

[b] Cert. Val.: certified value.

LiChrospher 100 RP-18 (5 μm), Merck; (c) LiChroCART 250-4, LiChrospher 100 RP-18 (5 μm), Merck; (d) LiChroCART 250-3, LiChrospher 100 RP-18 (5 μm), Merck; (e) Ultraspher ODS (C_{18}), 5 μm, 250×4.6 mm, Beckman; (f) Ultraspher ODS (C_{18}), 5 μm, 150×4.6 mm, Beckman. We aimed at optimizing sensitivity and selectivity of the chromatography and achieving good peak-shapes and separation of peaks. All these columns revealed good separation of the tocopherols.

We also compared several published mobile phase systems and different flow-rate programs in order to optimize chromatographic separation and peak-shape. The solvent systems used were acetonitrile–dichlormethane–methanol (7:1:1; 7:1:2; 7:2:1 [4,6]; 8:1:1; 6:2:2); acetonitrile–tetrahydrofurane–methanol (7:1:2; 7:2:1; 6:2:2); acetonitrile–methanol with 0.05 *M* ammonium acetate–ethyl acetate, and 0.05% triethylamine (solvent gradient) [5]; acetonitrile–tetrahydrofurane–methanol–ammonium acetate 1% [19]. Comparing the first two solvent systems, the use of tetrahydrofurane resulted in symmetric peak-shapes, while dichlormethane has a greater tendency towards asymmetric peak-shapes especially among the tocopherol peaks. Since Epler et al. [5] found improved carotenoid recoveries by adding buffers such as ammonium acetate, we also tested the more complex systems used by these authors, as well as by Hess et al. [19], and found comparable results with both these solvent systems. However, since the mobile phase used by Hess et al. [19] does not require a solvent gradient, it is easier to handle and less solvent- and time-consuming. We therefore chose to use this system.

Commonly used internal standard substances for analysis of fat soluble vitamins are retinyl acetate [9,11], tocopheryl acetate [4,8,11] and tocopheryl nicotinate [24]. The retention time of retinyl acetate was so close to that of retinol that the peaks could not be sufficiently separated with the mobile phase used. A further disadvantage of the esters is that they show no fluorescence at concentrations comparable to that of the vitamins, which does not permit their fluorescence detection. In contrast, the synthetic vitamin E analogue tocol can be used as an internal standard both for UV–Vis and for fluorescence detection, and its peak can be well separated from the tocopherols.

There is no internal standard available whose extraction behavior is comparable to that of the vitamins determined in the complex plasma matrix. Efficacy of extraction may be rather different for tocopherols incorporated in the complex plasma lipoprotein matrix and an added internal standard. Moreover, changes in the relative efficacy of extraction are conceivable, particularly in preterm infants whose plasma lipoprotein pattern is subject to marked changes induced by the introduction of enteral and parenteral feeding. Hence, we decided to add the standard at the end of the sample preparation, which only compensates for the chromatography deviations. Our intra- and inter-assay experiments document very satisfactory results of this approach. We injected the standard mixture for calibration purposes into the HPLC without further treatment, because the vitamins in the mixture of the standard substances in organic solvents behave differently during extraction than the lipoprotein bound vitamins in plasma. Both these procedures chosen require an almost complete extraction of the vitamins. Therefore, we tried to optimize the extraction procedure and tested various agents as to their protein precipitation abilities, as well as the numbers of extraction steps used. Since the protein precipitate after addition of ethanol to plasma is coarsely

grained, it appeared possible that the vitamins were partly enclosed in the precipitate. Isopropanol acidified by hydrochloric acid led to more finely grained precipitates. We investigated four precipitation reagents (ethanol, acid ethanol, isopropanol and acid isopropanol) as to their suitability for increasing vitamin yields in a pool-plasma. While retinol showed no appreciable differences, highest yields of tocopherol were achieved with ethanol used as the precipitation medium, whereas their concentrations were lower with the use of acid isopropanol. Seven pool plasma samples were extracted two and three times respectively. The mean values of vitamin concentrations did not differ, but the R.S.D. were two to three times lower when three extraction steps were employed (Table 4). The greater precision of the results obtained with three extractions justifies their use.

5. Conclusions

This isocratic HPLC method for the simultaneous analysis of retinol and tocopherols is sensitive, selective, precise and accurate while allowing determination of low vitamin contents in only 100 μl of plasma. It is therefore well suitable for measurements of the low plasma vitamin concentrations found in preterm infants, in whom only small sample sizes can be obtained.

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Table 4
Comparision two and three extraction steps

Vitamin	Two extractions		Three extractions	
	Mean (μmol/l)	R.S.D. (%)	Mean (μmol/l)	R.S.D. (%)
Retinol	1.460	5.9	1.448	1.8
γ-Tocopherol	2.091	7.5	2.190	3.2
α-Tocopherol	30.076	7.9	32.111	3.3

Results from the analysis of seven extracts of pool plasma vitamin concentrations.

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