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Note

Measurement of vitamin E in premature infants by reversed-phase high-performance liquid chromatography

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Supplementation with vitamin E (α-tocopherol), a natural antioxidant, is currently the subject of controversy concerning its efficacy and toxicity in premature newborns. Numerous papers have assessed the effects of vitamin E administered with the aim of reducing the incidence of severe diseases, such as retrolental fibroplasia, intraventricular haemorrhage, bronchopulmonary dysplasia and haemolytic anaemia [1–4]. Although the benefit of vitamin E treatment has yet to be clearly proved, the drug is now widely prescribed, in large doses and by different schedules, in neonatal intensive care units [5]. Concern about its potential toxicity has been expressed for plasma levels exceeding 3.5 mg/dl [6,7], and monitoring of vitamin E levels was suggested as a guide for rational dosage adjustments [8]. However, as vitamin E is a natural component of biological material, other sources of supplementation, such as milk (human and standard formula) and transfusate specimens (blood, plasma, red

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blood cells) have to be taken into account for adequate dosage adjustment in newborns.

Previous methods for the determination of vitamin E by high-performance liquid chromatography (HPLC) coupled with ultraviolet (UV) detection required extensive sample preparation or temperature gradient during elution, or could only be used for plasma and/or serum [9–18].

As part of a wider project on preterm delivery [19,20], we set up an investigation to define vitamin E utilization in preterm babies, monitoring the levels in different specimens. The method described here is reliable, sensitive and rapid and only needs small samples.

EXPERIMENTAL

Chemicals and reagents

Vitamin E acetate (α-tocopherol acetate) and vitamin E (α-tocopherol) were purchased from Merck (Darmstadt, F.R.G.) and phytol and hydroquinone from Sigma (St. Louis, MO, U.S.A.). Methanol, ethanol, diethyl ether, *n*-hexane, formic acid, benzene and acetonitrile (LiChrosolv, Merck) were of UV grade. Sodium methoxide were obtained from Carlo Erba (Milan, Italy).

Standards

Stock solutions of compounds (100 μg/ml) in methanol were prepared weekly and stored under nitrogen in dark bottles at $-20°C$.

Tocol [3,4-dihydro-2-methyl-2-(4,8,12-trimethyltridecyl)-2*H*-1-benzopyran-6-ol; mol. wt. 388.61] is commonly employed as an internal standard for vitamin E assay but, as it is difficult to obtain commercially, it was synthesized in our laboratory according to Pendse and Karrer [21]. The product was purified by HPLC, collecting representative peaks and checking collected fractions by mass spectrometric analysis.

Two standard concentrations of vitamin E acetate (4 and 8 μg/ml) and vitamin E (5 and 10 μg/ml) were prepared by serial dilution from a pool of blood, whose basal concentration was first measured, to provide the material for quality control. Each pool was divided into 1-ml samples and frozen at $-20°C$ until analysis. The two sets of samples thus obtained were analysed over two months to obtain twenty replicates for each set.

Sample collection and storage

Blood samples from healthy volunteers, delivering women and premature newborns were collected in tubes containing disodium EDTA and potassium fluoride. Samples of blood, plasma, milk and transfusate were stored at $-20°C$ until analysis.

Extraction procedure

A 0.05–0.5 ml sample (commonly 0.1 ml for plasma and milk and 0.2 ml for the other matrices) was vortex-mixed in dark tubes for 1 min with 1.5 ml of 5% ascorbic acid in 0.1 *M* hydrochloric acid, 2 ml of ethanol, 4 ml of *n*-hexane and 0.1 ml of internal standard (1 μg). The tubes were shaken for 15 min and centrifuged for 15 min at 2000 *g* at 4°C. The *n*-hexane layer was transferred into another dark test-tube and evaporated to dryness under a gentle stream of nitrogen at 37°C. The tubes were rapidly removed from the bath and capped until analysis. The residue was dissolved in 0.2 ml of methanol and vortex-mixed, and a sample was injected into the chromatograph. A calibration graph (0.2–8.0 μg/ml vitamin E acetate and vitamin E) was plotted for each series of fifteen to twenty samples, prepared by adding increasing amounts of compounds to blank samples whose vitamin E basal content was known.

Chromatographic conditions

A Perkin-Elmer (Norwalk, CT, U.S.A.) Series 2/2 liquid chromatograph equipped with a Model LC15 detector and a reversed-phase column (Superspher RP-18, 4-μm, 25 mm × 4 mm I.D., LiChroCART HPLC cartridge, Merck) was used. The column was eluted isocratically at room temperature with acetonitrile–water–methanol (49:3:48, v/v). The flow-rate was 2.0 ml/min and the detection wavelength was 280 nm.

Mass spectrometry

For the identification of tocol and vitamin E, a TS-250 mass spectrometer (VG, Manchester, U.K.) operating in the electron-impact mode was used. The accelerating voltage was 6 kV and the electron energy 70 eV. The samples, purified by HPLC, were introduced using the direct inlet system.

Calculations

The concentrations of vitamin E and vitamin E acetate were calculated from the ratios of the peak areas or peak heights of the compounds to those of tocol by a Shimadzu C-R3A integrator. The endogenous concentration of vitamin E in the unspiked samples was 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 [22].

RESULTS AND DISCUSSION

Fig. 1 illustrates chromatograms from blank blood and milk samples and the same spiked with 30 μl of vitamin E acetate and vitamin E (10 μg/ml standard

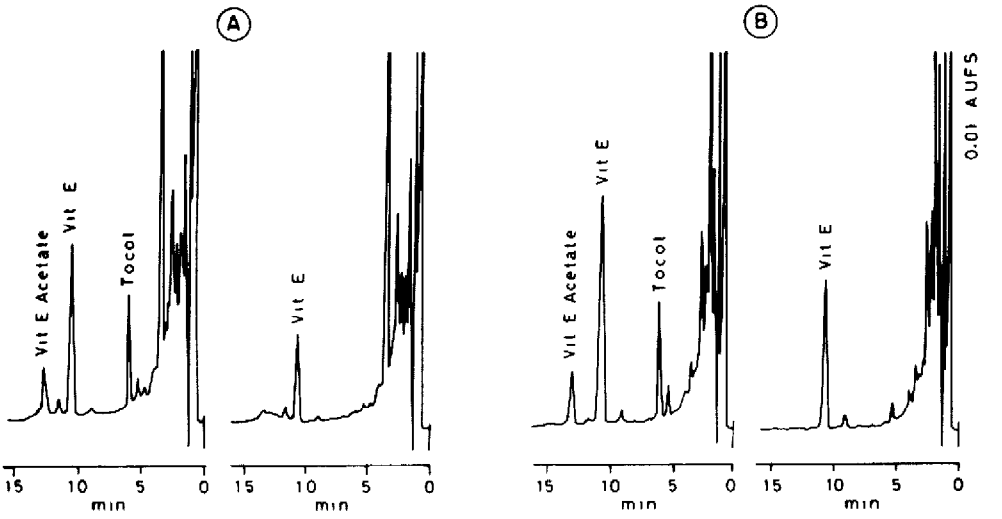

Fig. 1. Chromatograms showing resolution of vitamin E acetate, vitamin E and tocol (internal standard) in 0.2 ml of human blood (A) and breast milk (B), blank and spiked (30 μl of 10 μg/ml standard solutions) samples

solutions). No interfering peaks were found at retention times close to those of the two compounds measured (capacity ratio $k' = 8.33$ and 6.66, respectively) and of tocol ($k' = 3.33$) under the chromatographic conditions used.

In order to identify the peaks potentially corresponding to the compounds in different matrices, after injection into the HPLC system eluted fractions were collected, using the UV absorption trace on the recorder as a reference, and analysed by mass spectrometry. The substance collected at the retention time corresponding to vitamin E gave an M^+ ion at m/z 430 (base peak). Important peaks appeared at m/z 205 and 165, due to the progressive loss of the aliphatic moiety of the molecule. The compound present in the fraction corresponding to tocol gave an M^+ ion at m/z 388 (base peak). The other major peaks were at m/z 163 and 123, reproducing the fragmentations observed for vitamin E. These spectra were identical with those obtained from pure compounds.

Calibration graphs after subtraction of the basal concentration of vitamin E passed through the origin and were linear up to at least 20 μg/ml for both compounds. The analytical recovery averaged 96 and 91% for vitamin E acetate and vitamin E, respectively, over the range 0.5–20 μg/ml for all specimens. The detection limit was 0.01 μg/ml for both compounds at a signal-to-noise ratio of 3:1. The reproducibility, expressed as the C.V. of the slopes of ten blood calibration graphs (80 determinations) used in routine analysis of compounds was 8.6 and 12.4% for vitamin E acetate and vitamin E, respectively. The day-to-day ($n = 20$) precision of the assay was assessed over a two-month period

by determining the C.V. at two concentrations: vitamin E acetate 4 μg/ml (5.5%) and 8 μg/ml (5.7%); vitamin E 5 μg/ml (2.3%) and 10 μg/ml (4.5%). Accuracy was calculated as the percentage error from the true value on processing twenty samples containing different amounts of the two compounds; the average value was 8.5%. According to the procedures described, the lifespan of the analytical column averaged 1000 h.

Although the detailed data here concern blood specimens, similar performances were obtained on processing other sample matrices such as plasma, serum, milk and red blood cells. The concentrations of vitamin E in different human specimens are reported in Table I.

To assess the potential clinical usefulness of the method, we followed the plasma concentrations of vitamin E acetate and vitamin E in ten premature newborns given intramuscular injections of 20 mg/kg vitamin E acetate (Ephynal®, Hoffman-LaRoche, Basle, Switzerland) for prophylaxis on three consecutive days starting within 8 h of birth. Vitamin E plasma levels were also assayed in a group of non-supplemented infants. Serial blood samples were drawn over a five-day period from administration (Fig. 2). The highest average plasma concentrations of vitamin E acetate [1.01 ± 0.57 mg/dl (mean ± S.D.)] and vitamin E (3.48 ± 1.06 mg/dl) were observed on the third day. The calculated concentrations and observed disposition profile are in good agreement with previous findings [2].

As there is general agreement that the prophylactic use of vitamin E in premature infants must continue to be considered experimental [23] and monitoring of plasma levels of vitamin E, like many other pharmacological agents used in premature infants, has been suggested as a basis for rational utilization of the drug [24], an easy and reliable analytical method is essential for clinical use.

The method described, routinely used in our laboratory, offers simplicity and

TABLE I

CONTENT OF VITAMIN E IN DIFFERENT HUMAN SPECIMENS

Sample	Concentration (mean ± S.D., $n = 10$) (mg/dl)
Maternal blood (at delivery)	0.33 ± 0.11
Premature newborn blood (at delivery)	0.15 ± 0.07
Adult volunteer.	
Blood	0.44 ± 0.13
Red blood cells	0.33 ± 0.13
Plasma	0.61 ± 0.49
Breast milk	1.11 ± 0.53
Infant formula	0.23 ± 0.08

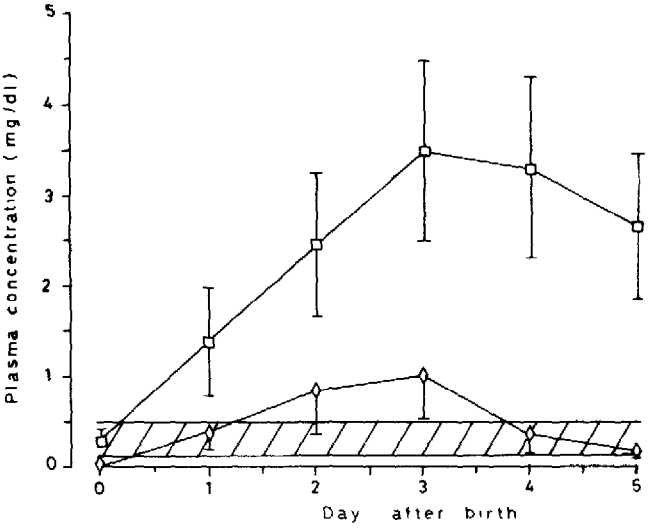

Fig. 2. Mean (with S.D.) plasma (◇) vitamin E acetate and (□) vitamin E concentrations in babies given 20 mg/kg vitamin E acetate intramuscularly on three consecutive days. The shaded area depicts range of concentrations in non-supplemented infants.

rapidity and requires common instrumentation; the method is efficient and only limited time and costs are involved in sample analysis. Further, small samples are needed and different matrices can be processed with the same performance to determine vitamin E acetate and vitamin E. All the findings reported here are well within the performance limits of an analytical technique required for medical management [25], particularly of paediatric patients.

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