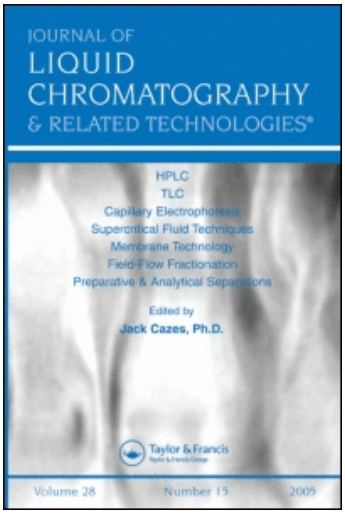

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DETERMINATION OF FAT-SOLUBLE VITAMINS BY LIQUID CHROMATOGRAPHY IN PEDIATRIC PARENTERAL NUTRITIONS

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ABSTRACT

A reversed phase high performance liquid chromatographic method using narrow-bore columns packed with 3 µm particles is described for the simultaneous determination of trans-retinol, ergocalciferol, DL-α-tocopherol and phytomenadione. The fat-soluble vitamins were separated in a C_{18} bonded phase column and eluted with methanol as the eluent pumped at a flow rate of 0.2 mL/min. A spectral detector was used and the wavelenghts were set at 325, 265, 284 and 250 nm for vitamin A, D_2, E and K_1 respectively. All vitamins were separated in less than 13 minutes. This method was applied to the determination of fat-soluble vitamins in the pediatric parenteral nutritions. The effect of the nutrition composition, daylight and the plastic infusion tubing on the stability of the vitamins was studied. Recovery studies showed good results for all solutes (91.0% - 110.5%) and the coefficients of variation were always less than 3%.

INTRODUCTION

The aim of parenteral nutrition is to improve or prevent malnutrition in those patients whose physio-pathological circumstances do not allow them to carry out certain necessities. Therefore, nutrient admixtures (aminoacids, glucose, vitamins, electrolites, oligoelements, water and in certain cases fats) are elaborated for intravenous administration, in accordance with different protocols.

During the neonatal period, the particular need to supply those nutritive elements which are necessary to achieve an adequate maturing of organs and systems is evident. This, together with metabolic and digestive immaturity, makes this subject group particularly sensitive to the administration of inadequate quantities of nutrients, especially the new-born who, due to incomplete fetal development or because of certain illnesses, can only receive an intravenous intake via parenteral nutrition during their first few days, weeks, or even months of life.

Clinical studies exist which relate fat-soluble vitamin deficiency states in newly-borns with certain pathologies (broncopulmonar dysplasia associated with a deficiency in retinol, haemolitic anaemia due to a lack of vitamin E or haemorraghing illness related to low plasma concentrations of clotting factors which depend of vitamin K), but, at the same time, clinical cases have been described which were triggered by high doses of fat-soluble vitamins (late growth owing to an excess in vitamin D or sepsis associated with a high vitamin E intake (1,2).

This all indicates that, for a correct supply of vitamins, the stability of said vitamins in pediatric parenteral nutritions must be known.

The mixing of the nutrients in a single container gives rise to problems of physico-chemical stability in some vitamins, yet in studies of said stability in parenteral nutrition, the data obtained to date with respect to degradation is disperse (3-5), due, in our opinion, not only to the distinct factors which affect the integrity of the vitamins (composition of the admixtures, the base products, the container and administration materials, exposure to the light, etc.) which depend on the protocols of elaboration and administration employed by each Hospital Nutrition Team, but also the methods and techniques of analysis employed.

The application of high performance liquid chromatography (HPLC) to the quantification of fat-soluble vitamins has been gaining acceptance as a sensitive and rapid analytical tool. In recent years many HPLC assays have been published, the principles of wich have been summarised (6,7). With respect to the evolution of the vitamin content in parenteral nutritions, most of the studies concentrate on the analysis of vitamin A, as it is one of the more labile vitamins and, what is more, recent papers (8,9), in which several vitamins are studied, excude vitamin D, probably since the vitamin is present in a very low concentration and the analytical methodologies used are pre-established, the latter are not sensitive enough,

especially when the concentration of vitamin D in the nutrition is expected to decrease with time.

This paper describes a practical and sensitive method for the simultaneous determination of fat-soluble vitamins in infant parenteral feeds by HPLC using narrow-bore columns (2.1 mm i.d.) packed with octadecylsilane. The proposed method was applied to the study of the evolution of the content in vitamins in infant parenteral feeds as a function of the influence of the feed composition, the infusion equipment employed and daylight.

EXPERIMENTAL

Reagents and Samples

Methanol was HPLC grade and was employed as supplied by the manufacturers. Ultrapure water was obtained though a Millipore Milli-Q system (Mildford, M.A., U.S.A.). Analytical grade trans-retinol, ergocalciferol, DL-α-tocopherol acetate and phytomenadione standard supplies by Merck (Darmstadt, Germany) were used. Butylated hydroxytoluene (BHT) was purchased from Sigma (St Louis, M.O., U.S.A.). Absolute ethanol and hexane were purchased from Romil (Loughborough, Leics, U.K.).

Individual stock solutions of each vitamin were prepared in ethanol with 0.025% of BHT to provide a concentration of 5 mg/mL for trans-retinol and phytomenadione and 2.5 mg/mL for DL-α-tocopherol acetate

and ergocalciferol. These solutions were degassed with helium and stored in dark glass flasks under -18°C refrigeration.

Individual or mixed standard solutions were prepared by appropiate dilution of the stock solution and filtered through a 0.45 µm membrane (Millex-HV 13, Millipore) before being injected into the chromatographic system.

The parenteral nutritions formulas investigated are shown in the Tables I and II. All components were mixed in a glass container and transferred to polyvinyl chloride (PVC) (Viaflex 500 mL, Baxter) plastic bags. Both the composition of the parenteral feed as the well as the plastic bags employed were similar to those used in the neonatal intensive care unit at Central Hospital of Asturias (Spain).

Apparatus and Conditions

The experiments were carried out using an HPLC system equipped with two Kontron 422 pumps; a 8125 Rheodyne injector with a 5 µL injection loop; a Kontron model 430 UV-VIS detector with a 3 µL flow cell and a data station with Data System 450 software (Kontron Instruments, Milan, Italy). The wavelenghts of the detector were switched at 325, 265, 284 and 250 nm for vitamins A, D_2, E and K_1 repectively.

The column employed was a Spherisorb ODS-2 (100 x 2.1 mm i.d., 3 µm). An ODS guard column was used to protect the analytical column. The mobile phase was pumped at a flow rate of 0.2 mL/min and used in

TABLE 1

Parenteral Nutritions Content

	PN-1 mL	PN-2 mL	PN-3 mL	PN-4 mL	PN-5 ml
Trophamine (Aminoacids Solution, 6%)	12	12	16	24	32
L-Cisteine Hydrochloride 8.8%	0.6	0.6	0.8	1.2	1.6
Glucose 50%	10	15	17	20	20
Sodium Chloride 0.9%	20	16	16	13	13
Potassium Chloride 18.5%	0.2	0.2	--	--	--
Calcium Gluconate 9.2%	2.9	2.9	3.5	4.0	4.1
Potassium Monohydrogenphosphate 1M	0.5	0.5	0.6	0.7	0.8
Magnesium Sulphate 15%	0.2	0.2	0.4	0.4	0.4
Oligoelements Pediatric Solution	0.7	0.7	0.7	0.7	0.7
Sodium Heparine 1%	0.2	0.2	0.2	0.2	0.2
Pediatric Multivitamins	5	5	5	5	5
Nonpyrogenic Sterile Water	47.7	46.7	39.8	30.8	22.2

isocratic mode (100% methanol). Before use, the mobile phase was vacuum-filtered 0.45 µm nylon filter and degassed with helium.

The chromatographic experiments were carried out at room temperature (20 ± 2°C).

TABLE 2.Pediatric Multivitamin Composition

FAT-SOLUBLE VITAMINS	
Trans-Retinol	0.7 mg
Ergocalciferol	10 μg
DL-α-Tocopherol Acetate	7 mg
Phytomenadione	0.2 mg
WATER-SOLUBLE VITAMINS	
Riboflavine	1.4 mg
Pyridoxine Hydrochloride	1.0 mg
Niacinamide	17.0 mg
Pantothenic Acid	5.0 mg
Thiamine	1.2 mg
Biotin	20 μg
Folic Acid	0.14 mg
Cyanocobalamin	1 μg
Ascorbic Acid	80 mg
EXCIPIENTS	
Mannitol	375 mg
Polysorbate 80	50 mg
Polysorbate 20	0.8 mg
Butylated Hydroxytoluene	58 μg
Butylated Hydroxyanisole	14 μg

RESULTS AND DISCUSSION

Using previous studies as a basis, we chose high performance reversed-phase liquid chromatography for the separation and determination of the fat-soluble vitamins in pediatric parenteral nutritions (PPN). We studied the effect of the elution conditions, particularly mobile phase composition and flow, on the resolution of fat-soluble vitamins. This was achieved with narrow-bore columns because, in comparising with ordinary columns, it is clear that less mobile phase solvent is consumed and the analytical cost is therefore less. Furthermore, the detection limits obtained with the narrov-bore columns are lower than those provided by the ordinary column. This is quite significant for the analysis of samples such as the PPN in wich the vitamin concentration, because of its marked lability, decrease with the time.

Taking into account the important structural differences among the vitamins to be separated, as well as the high retention capacity of the octadecylsilane packing chosen as the stationary phase, mobile phases with a high elution capacity, such as methanol or mixtures of methanol with extremely low water contents (99:1), were employed. With respect to the mobile phase flow employed, this was modified over a range of values between 0.15 and 0.25 mL/min, the resolution obtained in all cases always being higher than 1.0. The optimum working conditions were found to be the following: mobile phase 100% methanol, flow rate 0.2 mL/min, isocratic mode, room temperature.

A chromatogram of a mixture of standards demonstrating the separation of vitamins A, D_2, E and K_1 is presented in Figure 1. As can be seen, the analysis time is less 13 minutes under isocratic conditions, thus avoiding re-equilibration delay between runs. Retention times of analysis performed on the same day were reproducible within ±1% (Table III).

Figure 2 shows the chromatogram obtained, under optimum experimental conditions, for an ethanolic solution of the vitamin complex wich is added to the nutrient units. As can be seen, a considerable front of scarcely retained or unretained compounds appears which interferes wich the chromatogram peaks corresponding to

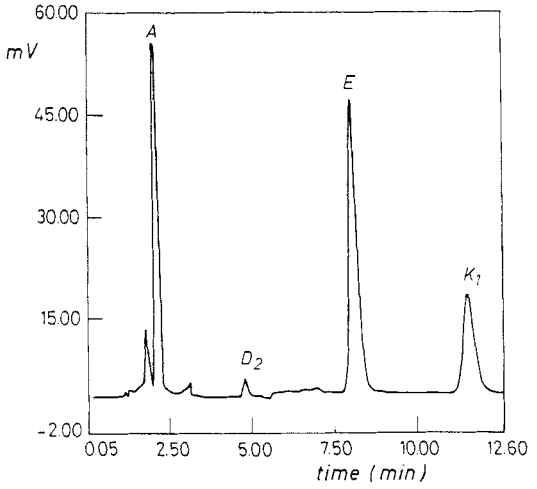

FIGURE 1. Chromatogram obtained from standard solutions of fat-soluble vitamins by using a Spherisorb ODS-2 (100x2.1 mm I.D. 3 µm). Mobile phase, 100% methanol. Flow-rate, 0.2mL/min.

TABLE 3

Retention Times And Their Repeatability In The Investigated Vitamins

Vitamin	Retention Time (min)	C.V. (%)
A	2.18	0.69
D_2	5.03	0.82
E	8.77	0.83
K_1	12.45	0.93

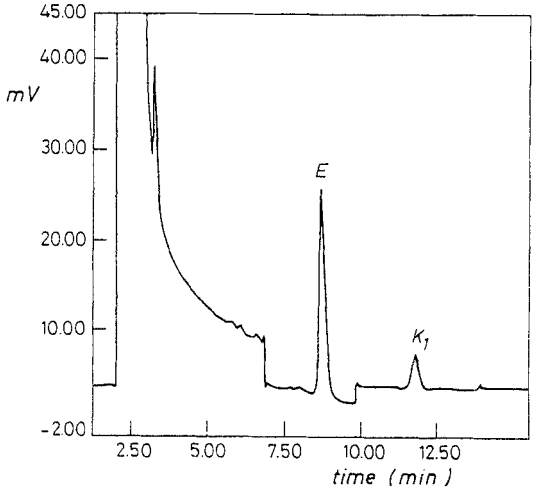

FIGURE 2. Typical chromatogram of fat-soluble vitamins contained in an ethanolic solution of the multi-vitamin added to the nutritional formulas. Column and chromatographic conditions as in Figure 1.

vitamins A and D_2. Since the number of species present in the nutritions is even higher, a clean-up of the sample was carried out using hexane as extractant according to the following procedure: PPN (1mL) was transferred into a 10 mL glass centrifuge tube and 3 mL of hexane were added and the admixture was vortexed for 5 min. The solution was then centrifuged at 2000 g for 10 min. The organic layer was transferred and the extraction process was repeated with 3mL of hexane. The organic layers were jointed and washed with 2 mL of methanol-water (9:1). The organic upper layer was separated and passed through a 0.45 µm filter. After this, it was evaporated under nitrogen until dry and reconstituted in 1 mL ethanol with the help of an ultrasonic bath. A 5 µL aliquot of this solution was injected into the HPLC system.

Since vitamins oxidise easily, as well as being photo-sensitive and thermo-labile, the preparation of the samples has to be carried out under diffuse light, in the presence of an anti-oxidant, and it must then be preserved at low temperatures. In fact, the nutrition samples tested contain butylated hydroxytoluene (BHT) as anti-oxidant. Although this compound is extractable in hexane, considering that the solubility of oxygen in hexane is greater than in ethanol, it was decided to add an equivalent quantity of BHT (0.58ppm) to the hexane employed in the extraction so as to preserve the vitamins extracted whilst these remained in hexane.

Figure 3 shows the chromatograms obtained on extracting two similar aliquots of the nutrition in hexane, with and without adding BHT in the organic

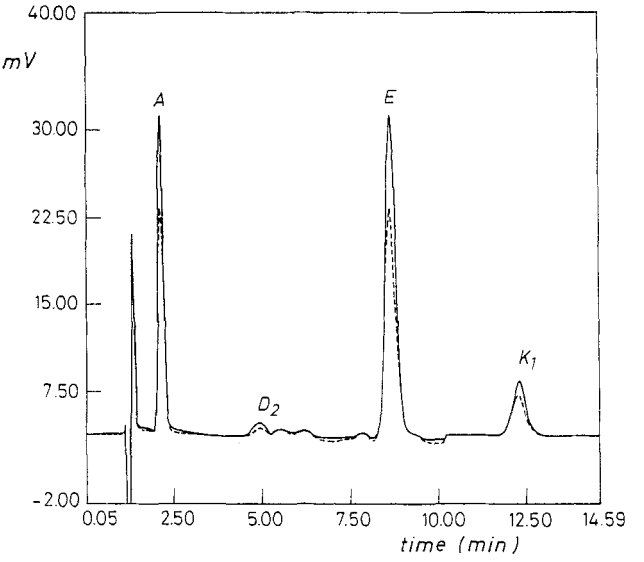

FIGURE 3. Chromatograms of hexane extract of a pediatric parenteral nutrition admixture:
— with BHT as antioxidant in the organic layer.
-- without BHT in the organic layer.
Chromatographic conditions as in Figure 1.

layer, where it can be seen that their behaviour is practically the same, although an important increase in the peaks corresponding to vitamins A and E is appreciated with BHT, for which reason said anti-oxidant was systematically incorporated in the preparation procedure of the sample.

Table IV lists the minimum quantities detectable for the four vitamins based on a signal-to-noise ratio of 3:1. In order to check the accuracy of the proposed method, we determined recoveries by analysing PPN as such and spiked with known amounts of the vitamins. All analyses were carried out in triplicate at three

TABLE 4

Detection Limits Of Fat-Soluble Vitamins Determined By Using Narrow-Bore Column

Vitamin	D.L. (ng)
A	0.75
D_2	0.85
E	4.85
K_1	0.95

concentration levels. For determination of vitamin D_2 in the samples, a preconcentration step was neccesary. The results obtained are given in Table V. The average recoveries obtained, wich ranged between 91.0±0.6 and 110.5±0.9 testify to the accuracy of the proposed method.

The precision of the method was checked using five different PPN and by analysing each sample in triplicate. The coefficients of variation were always less than 3%.

Under the above described working conditions the vitamins in the PPN were quantified by the external standard method. Standards were injected and the resulting integrator response factors were computed and then processed by the integrator to deliver the unknown concentrations. The injected volume used was always 5 µL and the amount of each vitamin present was directly obtained from the data module. Regular recalibrations were carried out.

TABLE 5

Recovery Studies Of Fat-Soluble Vitamins Added To The Nutritional Formulation.

Vitamin	Amount in sample µg/mL	Amount added µg/mL	Amount found µg/mL	% Recovery
A	6.63	1.04	7.60	99.1±4.2
		2.08	7.93	91.0±0.6
		3.12	10.02	102.8±2.6
D_2	0.08	0.03	0.103	94.0±1.8
		0.06	0.154	110.1±0.3
		0.09	0.188	110.5±0.9
E	67.7	5.00	73.35	100.9±5.9
		10.0	79.64	102.5±2.7
		20.0	78.40	94.8±0.6
K_1	1.85	0.3	2.35	109.4±1.0
		0.6	2.44	99.4±2.5
		0.9	2.79	101.5±2.4

To check the influence of nutritional parenteral composition on vitamin stability, five formulas were prepared (see Table I) the vitamin content being determined over time (recently prepared, at 10 hours and after 24 hours). The bags were maintained at room temperature and unprotected from the light. From the results obtained, it can be seen that the composition of the nutritions has not notable influence on the evolution of the vitamin content, or at least does not

have the same degree of influence as the other external factors. As an example, Figure 4 represents the decrease that the concentration of vitamin K_1 suffers with respect to the composition of the sample over time.

The photo-sensitivity of the different vitamins in the bags of nutrition during administration was studied in one of the samples (PN-2). In order to do this, two bags were prepared, one of which was protected from the light. Its vitamin content being determined when recently prepared and after 24 hours. As can be seen in Figure 5, the degradation of the vitamins in the bag exposed to the light is greater than in the protected bag, vitamin E being the least affected by this factor. The small variation in the concentration of vitamin E may be attributed to other factors such as oxidation, due, amongst other causes, to the permeation of atmospheric oxygen through the bag.

Vitamin A is affected to an important degree by the light, in fact, protecting it enables around 30% more to be recuperated after 24 hours. Vitamin D_2, as opposed to the other three, seems to degrade fundamentally because of this factor. The concentrations of vitamins A, E, D_2 and K_1 decrease over time, independently of whether they are exposed to the light or not, as a consequence of the other causes mentionet, vitamin A being the most affected.

Another possible cause of loss of vitamin content in the parenteral nutritions may be attributed to proccesses of adsorption onto the plastic material with

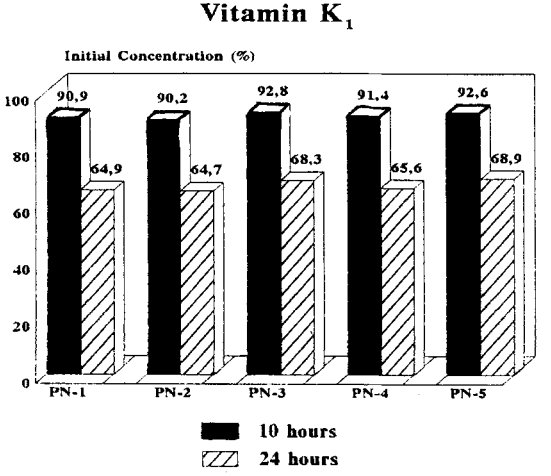

FIGURE 4. Evolution of vitamin K_1 concentration according to the pediatric parenteral nutrition admixture composition over time.

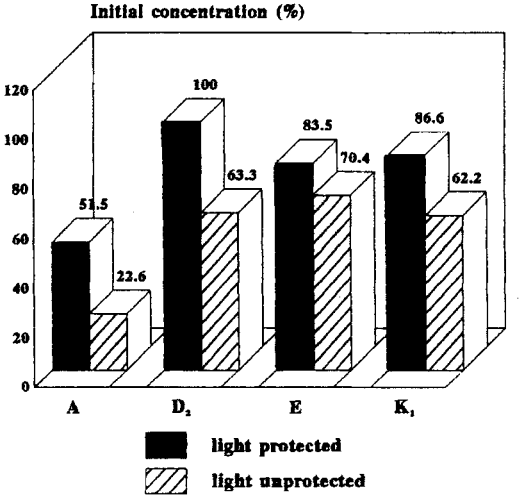

FIGURE 5. Stability of fat-soluble vitamins in a pediatric parenteral nutrition admixture, in a polyvinyl chloride plastic bag, hanging with and without to protection from daylight.

which the intravenous perfusion equipment is elaborated. In order to study this possibility, the same nutrient mix (PN-2) was passed through the infusion equipment (Abbot Venisystems, Lifecare 5000 IV). After purging the system, a flow of 3 mL/hour was established and aliquots were taken from the bag and from the distal end of the equipment (connection point with the patient's intravenous administration tube) which was subject to room tempetarure and protected from the light. The chromatographic analysis carried out shows that vitamin A is highly absorbed, there existing an average difference in concentration between the distal end of the tube and the bag of 41.9%, while the rest of the vitamins practically do not experiment interaction with the perfusion equipment -which may be relevant with respect to therapy- the slight differences obtained were attributed to error in the method.

CONCLUSIONS

Reversed-phase chromatography using narrow-bore columns packed with 3 µm particles provides a rapid and simple alternative to the determination of fat-soluble vitamins. The method described in this report is suitable for determining the vitamins A, D_2, E and K_1 in pediatric parenteral nutritions (PPN).

The results of the experiments in this study demonstrate that under the conditions of preparation and administration employed, vitamins losses, particularly vitamin A, appear in PPN infused to neonatal patients. Given the clinical importance of intravenous delivery of vitamin A for premature neonates the use of more inert

bags and perfusion equipment protected from the light is considered highly convenient.

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