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Analysis of all-in-one parenteral nutrition admixtures by liquid chromatography and laser diffraction: study of stability

A. Sforzini [a,*], G. Bersani [a], A. Stancari [a], G. Grossi [b], A. Bonoli [c], G.C. Ceschel [d]

[a] *Pharmacy Service, S. Orsola-Malpighi University Hospital, 40126 Bologna, Italy*
[b] *Centralized Analysis Laboratory, S. Orsola-Malpighi University Hospital, 40126 Bologna, Italy*
[c] *Chemical, Mining and Environmental Engineering Department, University of Bologna, Bologna, Italy*
[d] *Pharmaceutical Sciences Department, University of Bologna, Bologna, Italy*

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Abstract

All-in-one parenteral nutrition admixtures are complex lipid emulsions (oil/water) which require absolute sterility, stability and no precipitates. Particle diameter must be in the range 0.4–1 μm in order to mime the size of chylomicra. Added vitamins must not degrade during infusion time (24 h). In this study, the physicochemical stability of parenteral nutrition admixtures was tested in the course of time at different storage temperatures. Two liquid chromatographic methods, based on solid phase extraction (SPE), were developed for fat-soluble vitamin determination. Stability studies were carried out on three industrial lipid emulsions and on six compounded all-in-one admixtures. They were stored at three different temperatures: 4°C (storage), 25°C (compounding) and 37°C (infusion); then they were analyzed at starting time and at 24, 48 and 72 h after compounding. Particle diameter was determined by means of *Laser Particle Sizer Analysette* 22, which uses laser diffraction technique (light scattering — reverse Fourier optics). Fat-soluble vitamins (retinol palmitate and α-, δ-, γ-tocopherol) were determined in admixtures with a branded vitamin compound called Idroplurivit® Liofilizzato. Samples were extracted by SPE on C_{18} cartridges, then they were separated by reversed-phase liquid chromatography (LC) and detected by ultraviolet detection (retinol palmitate) and electrochemical detection (tocopherols). *Laser diffraction analysis* pointed out that particle size did not change in the course of time at the tested temperatures. *LC analysis* showed that vitamins interact each other and degrade after compounding at different times and storage temperatures; only retinol palmitate is stable at 37°C. Retinol palmitate recovery was 98%, coefficient of variation (CV) 5.4%, detection limit 25 μg/l, limit of quantitation 75 μg/l and there were not interfering substances. Tocopherols average recovery was 99%, CV 3.5%, detection limit 15 ng/l and limit of quantitation 50 ng/l. In conclusion, all-in-one parenteral admixtures were proved to be physically

* Corresponding author. Tel.: +39-51-6364861; fax: +39-51-6364897.
E-mail address: co14651@iperbole.bologna.it (A. Sforzini).

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stable under analysis conditions, but degradation of retinol palmitate and tocopherols requires admixtures with vitamins to be infused within 24 h after compounding.

Keywords: All-in-one admixtures; Stability; Laser diffraction; Sample clean-up; Retinol palmitate determination; Tocopherols determination; Reversed-phase liquid chromatography analysis

1. Introduction

Parenteral nutrition is a fundamental technique of artificial nutrition which allows to provide the right nutritional intake to the patients who cannot feed themselves and the patients who reject food or do not eat enough. Parenteral nutrition admixtures are infused by intravenous injection, so their stability is an essential requirement in clinical practice. All-in-one admixtures are complex lipid emulsions, which require absolute sterility, stability and no precipitates. The various substrata which are used in admixture compounding (amino acids, carbohydrates, lipids, water for injection, electrolytes, trace elements and vitamins) interact according to different reactions which are only partly known; especially the biphasic characteristic of lipid emulsion (oil/water) can cause physicochemical instability, thus it is necessary a careful control during compounding phase and in clinical practice. The most critical parameter of admixtures is particle diameter which must be in the range 0.4–1 μm in order to mime the size of chylomicra [1]; no particles larger than 6 μm are allowed (diameter of pulmonary capillary vessels) [2]. Alteration of physical characteristics of lipid emulsion can be related to the effects of aggregation, creaming, coalescences and oiling out. Several methods [3–7] are available to value particle size changes: visual inspection, optical microscopy, electron microscopy, Coulter counter, photon correlation spectroscopy and laser diffraction (light scattering — reverse Fourier optics).

The last technique measures number and size of particles and evaluates the angular distribution of a beam of incident laser light that goes through the diluted emulsion sample. This method has the advantage of requiring a small dilution of sample avoiding the redispersion of possible aggregates, that is why, nowadays, laser diffraction technique is the most used to evaluate particle size of emulsions.

Vitamins added to parenteral admixtures must not degrade during infusion time (24 h) to assure the recommended daily vitamin amount to patients. Determination of vitamins is carried out by liquid chromatography (LC) [8,9]. Several LC methods are available in chemical literature to determine fat-soluble vitamins, but most of them refer to biological matrixes, such as blood, serum, mother's milk, etc. [10–16] and not to parenteral admixtures. For this reason, in this study, two specific LC methods, based on solid phase extraction (SPE), had to be developed.

Vitamins are particularly unstable and may degrade during storage and infusion; moreover parenteral emulsion stability can be influenced by storage conditions. As nutritional admixtures are infused at different times after compounding, the aim of this study was to test their physicochemical stability in the course of time and at different storage temperatures in order to state the expiry date.

2. Experimental

2.1. Materials

Stability studies were carried out on three industrial lipid emulsions: Intralipid® 20% (the most widely used in Europe), Liposyn® 20% (commonly used in Italy) and ClinOleic® 20% (recently put on the market in Italy) (Table 1) and on six compounded all-in-one admixtures (Table 2). The admixtures were prepared into ethyl-vinyl acetate (EVA) plastic bags by Parenteral Nutrition Centralized Pharmacy Service, S. Orsola-Malpighi University Hospital, Bologna, Italy by means of filling up automatic system, in a sterile room characterized by positive pressure and vertical air flow filtered by HEPA absolute filters.

Table 1
Composition of industrial lipid emulsions

	Intralipid® 20% 500 ml Pharmacia-Upjohn (Stockolm, Sw)	Liposyn® 20% 500 ml Abbott (Latina, I)	ClinOleic® 20% 500 ml Clintec-Baxter (Roma, I)
Soybean fractionated lipids	200.0 g/l	100.0 g/l	40.0 g/l
Safflower oil	0.0 g/l	100.0 g/l	0.0 g/l
Purified olive oil	0.0 g/l	0.0 g/l	160.0 g/l
Egg yolk micronized			
Phospholipids	12.0 g/l	12.0 g/l	12.0 g/l
Phosphate	27 mEq/l	27 mEq/l	27 mEq/l
Glycerol	22.5 g/l	25.0 g/l	22.5 g/l
Fatty acids composition (% on total lipids)			
Linoleic acid	52.0	65.8	18.5
Linolenic acid	8.0	4.2	2.0
Oleic acid	22.0	17.7	59.5
Palmitic acid	13.0	8.8	13.5
Stearic acid	4.0	3.4	2.9
Amount of vitamin E	200.0 mg/l	99.1 mg/l	70.0 mg/l

Table 2
Composition of the six tested compounded all-in-one admixtures (A, B, C, D, E and F formula)

Formula	A	B	C	D	E	F
Complete amino acids (g)	100.00	100.00	100.00	100.00	100.00	100.00
Glucose (g)	250.00	250.00	250.00	250.00	250.00	250.00
Lipids (g)						
Liposyn® 20%	75.00	75.00				
Intralipid® 20%			75.00	75.00		
ClinOleic® 20%					75.00	75.00
Sodium (mEq)	60.00	60.00	60.00	60.00	60.00	60.00
Potassium (mEq)	60.00	60.00	60.00	60.00	60.00	60.00
Chloride (mEq)	60.00	60.00	60.00	60.00	60.00	60.00
Phosphate (mEq)	40.00	40.00	40.00	40.00	40.00	40.00
Magnesium (mEq)	16.00	16.00	16.00	16.00	16.00	16.00
Calcium (mEq)	9.00	9.00	9.00	9.00	9.00	9.00
Trace elements (ml)	3.00	3.00	3.00	3.00	3.00	3.00
Idroplurivit® Liofil. (ml)		2.00		2.00		2.00
Vitamina C® (ml)		5.00		5.00		5.00
Volume (ml)	2500.00	2507.00	2500.00	2507.00	2500.00	2507.00
Total (kcal)	2160.00	2160.00	2160.00	2160.00	2160.00	2160.00
Non-protein (kcal)	1750.00	1750.00	1750.00	1750.00	1750.00	1750.00
Glucose %kcal	57.00	57.00	57.00	57.00	57.00	57.00
Nitrogen (g)	15.23	15.23	15.23	15.23	15.23	15.23
Non-protein (kcal/N_2 kcal)	115.00	115.00	115.00	115.00	115.00	115.00
kcal/ml	0.83	0.83	0.83	0.83	0.83	0.83
Osmolarity (mM/l)	1051.00	1051.00	1051.00	1051.00	1051.00	1051.00
pH	6.32	6.32	6.32	6.32	6.32	6.32

Table 3
Composition of Idroplurivit® Liofilizzato (A. Menarini, Fi, I)

1. Lyophilized powder composition	
Active principles	
Retinol palmitate	16 600 units = 9.13 mg/vial
D-L-α-tocopherol acetate	8.00 mg/vial
Colecalcipherol	3300 units = 82 500.00 ng/vial
Thiamine hydrochloride	3.20 mg/vial
Riboflavin-5′-monophosfate	3.20 mg/vial
Pyridoxine chloride	1.60 mg/vial
Nicotinamide	32.00 mg/vial
Ascorbic acid	100.00 mg/vial
Sodium pantothenate	3.20 mg/vial
Excipients	
Polysorbate 80, mannite, polyvinylpyrrolidone, metyl *p*-hydroxybenzoate, sodium edetate, butylhydroxyanisole	
2. Solvent solution composition	
Sodium phosphate, sodium citrate, *p*-hydroxybenzoic acid esters, water for injection	

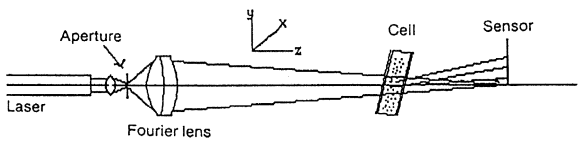

Fig. 1. Reverse Fourier optics.

All-in-one emulsions were stored at three different temperatures: 4°C (storage), 25°C (compounding) and 37°C (infusion); then they were analyzed at starting time and at 24, 48, 72 h after compounding. Sample total number was equal to 90. The branded vitamin compounds used were Idroplurivit® Liofilizzato, made up of a vial of lyophilized powder and a vial of solvent solution (Table 3) and Vitamina C® 500 mg vial, made up of ascorbic acid (active principle) and sodium hydrate, sodium metabisulphite, sodium edetate and water for injection (excipients).

LC determination of vitamins was carried out making use of the following materials: *cis*-retinoic acid, all *trans*-retinoic acid, all *trans*-retinol, all *trans*-retinol palmitate, β-carotene, (±)-α-tocopherol acetate, (+)-δ-tocopherol, (±)-α-tocopherol, (+)-γ-tocopherol and butylated hydroxytoluene (BHT) were obtained from Sigma (St. Louis, MO); methanol, chloroform, ethanol, acetonitrile, 2-propanol and *n*-hexane (all of LC grade), sodium dihydrogen phosphate monohydrate and sodium acetate (both of analytical-reagent grade) were obtained from E. Merck (Darmstadt, D); sodium dodecyl sulphate (SDS) (specially purified for biochemical work) was purchased from BDH (Poole, Dorset, UK); glacial acetic acid (of INSTRA grade) was supplied by J.T. Baker (Deventer, NL); 85% ortophosphoric acid was obtained from C. Erba (Mi, I). SPE extraction cartridges containing octadecyl-bonded silica (100 mg, 40 μm) were obtained from J.T. Baker.

The reversed-phase analytical column, Genesis C_{18}, 4 μm (50 × 4.6 mm^2 ID) was supplied from Jones Chromatography (Mid Glamorgan, GB).

2.2. Apparatus

Particle diameter determination was carried out by means of Laser Particle Sizer Analysette 22 (Fritsch GmbH, Idar-Oberstein, D), which uses laser diffraction technique (light scattering – reverse Fourier optics). This technique allows us to evaluate the particle size of lipid emulsion according to the capability of scattering a beam of incident light: the smallest is the size of particles, the largest is the diffraction angle of light.

The granulometer consists of three units and an additional printer: a measuring unit containing a helium–neon laser (wavelength 632 nm), the related optical system (Fourier lens) which produces a convergent laser beam, the measuring cell for emulsions and the multielement detector which allows particle size distribution into 31 classes (Fig. 1); a dispersion unit which contains an ultrasonic bath, an agitator and a centrifugal pump for dispersing and transporting the sample into measuring unit; an interfaced screen computer which controls the measuring sequence and calculates the measurement results.

Measurement field can be covered by two optical configuration, conventional and reverse Fourier optics. In the reverse configuration, the order of measuring cell and Fourier lens is inverted in comparison with conventional optic; this reversal of position and the employment of a

convergent laser beam allow to measure particles smaller than 0.1 μm which scatter light at much wider angles than it is possible by the conventional method.

The chromatographic system for the determination of retinol palmitate consisted of a model 9010 gradient LC pump (Varian, Walnut Creek, CA), a model 460 autosampler (Kontron, Mi), a model LC-95 ultraviolet (UV) detector (Perkin–Elmer, Norwalk, CT) and a model HP 3395 integrator (Hewlett Packard, Palo Alto, CA).

The chromatographic system for the determination of α-, δ-, γ-tocopherol consisted of a model 2510 isocratic LC pump (Varian), a model 9125 injection valve fitted with a 20 μl injection loop (Rheodyne, Cotati, CA), a Coulochem 5100A electrochemical detector (ED) fitted with a model 5021 conditioning cell and a model 5011 analytical cell, all from Environmental Sciences Association (ESA) (Bedford, MA) and a model Servogor 200 recorder (BBC Goerz Electro, Graz, Au). The SPE clean-up was performed using a model SPE-21 vacuum manifold (J.T. Baker)

2.3. Methods

2.3.1. Particle diameter determination

After setting the granulometer at zero, sample (5 ml) was introduced into the dispersion unit containing distilled water (15 ml) in order to reach sample/water ratio (1:3 v/v). The measuring range used was between 0.16 and 1160 μm. Sample turnover was on the average 15–20 min, because measuring cell had to be carefully washed after each test.

Results were calculated by the interfaced computer and presented both numerically and graphically. For every tested admixture (Intralipid® 20%, Liposyn® 20%, ClinOleic® 20% , A, B, C, D, E and F formula) the following dimensional parameters were read: d10 (maximum diameter of 10% of particles), d50 (maximum diameter of 50% of particles) and d90 (maximum diameter of 90% of particles).

2.3.2. Retinol palmitate determination

2.3.2.1. Sample preparation. Before analysis, sample was mixed by a reversal mixer for an hour; then buffer solution (1 ml) and internal standard solution (retinol 500 mg/l, 10 μl) were added to sample (200 μl) and vortex mixed. One milliliter volume of the sample was passed through a 100 mg octadecyl-bonded silica SPE cartridge, previously conditioned by 1 ml of methanol and 2×1 ml of buffer solution; the cartridge was then washed with 2×1 ml of buffer solution and dried under a 500-mbar vacuum for 1 min. Retinol palmitate was eluted with 2×0.5 ml of 2-propanol. Eluted fraction was put into a dark glass vial and sealed under argon flow. Each sample was treated in duplicate.

2.3.2.2. Dilution of Idroplurivit® Liofilizzato vial. Buffer solution (1 ml) was added to Idroplurivit® Liofilizzato vial (200 μl) and vortex mixed.

2.3.2.3. Preparation of standard solutions, buffer- and mobile-phases. A *stock solution* containing (±)-α-tocopherol acetate-β-carotene-all *trans*-retinoic acid-all *trans*-retinol palmitate-chloroform (1.9437:0.1906:0.0476:0.0507:100 w/w/w/w/v) was prepared; it was stored at −20°C.

Internal standard solution (retinol, 500 mg/l) was prepared by dissolving 3.6 mg of retinol into ethanol (5 ml) and adding BHT in 2-propanol (22.2 g/l, 2.3 ml). To obtain *standard solutions*, the internal standard was diluted with BHT in 2-propanol and stock solution was added in order to obtain six solutions containing the same amount of internal standard (4.2 mg/l) and 0, 0.2, 0.4, 0.8, 1 and 11.9 mg/l of retinol palmitate, respectively. These standards were fresh and daily prepared.

The composition of buffer solution was methanol–acetonitrile–sodium dihydrogen phosphate monohydrate (pH 2.8; 50 mM) (15:8:77 v/v/v) with 200 mg/l of SDS added. It was stored at room temperature. A and B LC mobile-phases were prepared as follows:

- A: sodium acetate (10 mM) in glacial acetic acid–water–methanol (4.8:387:1545 v/v/v).
- B: sodium acetate (10 mM) in glacial acetic acid–2-propanol–methanol (4.8:225:1706 v/v/v).

2.3.2.4. Chromatographic conditions. Separation was carried out at room temperature on a C_{18} Genesis analytical column (50×4.6 mm^2 ID), 4

μm. Injection volume was 20 μl. It was used a binary gradient between *A* and *B* mobile-phases with the following programme: 10%, *B* mobile-phase for 5 min; from 10 to 100%, *B* in 0.1 min; and 100%, *B* for 8.9 min. The flow rate was 2 ml min^{-1}; back pressure was 60 atm. The eluted peaks were monitored by an UV set at 325 nm wavelength.

2.3.2.5. Calculation. Retinol palmitate concentrations were calculated by means of internal standard method; chromatographic peak height was evaluated. Standard solutions were directly injected and the ratio between retinol palmitate peak height and internal standard peak height versus retinol palmitate concentration was utilized for the calibration graph. The extracted sample concentration was calculated from the graph.

2.3.2.6. Assay validation. LC method was validated in terms of accuracy, precision, linearity, parallelism, detection limit, limit of quantitation, specificity and selectivity. In order to know retinol palmitate amount added to admixtures, the vial of Idroplurivit® Liofilizzato was tested. Accuracy was expressed as percent recovery of retinol palmitate and retinol standard.

Precision was expressed as percent coefficient of variation (CV%) of the method. Linearity and parallelism of response were tested by extraction of diluted samples at four different sample–internal standard–water–buffer ratios (v/v/v/v): (400:10:0:800); (200:10:0:1000); (100:10:100:1000); (50:10:150:1000).

Specificity and selectivity were tested by injection of possible interfering substances.

2.3.3. α-, γ-, δ-tocopherol determination

2.3.3.1. Sample preparation. Before analysis, sample was mixed by a reversal mixer for an hour; then buffer solution (1 ml) was added to sample (200 μl) and vortex mixed. One milliliter volume of the sample was passed through a 100 mg octadecyl-bonded silica SPE cartridge, previously conditioned by 1 ml of methanol and 2×1 ml of buffer solution; then the cartridge was washed with 2×1 ml of buffer solution and dried under a 500-mbar vacuum for 1 min. Tocopherols were eluted with 0.5 ml of 2-propanol. Eluted fraction was put into a dark glass vial and sealed under argon flow. Each sample was treated in duplicate.

2.3.3.2. Preparation of standard solutions, buffer- and mobile-phases. Three different *stock solution*s containing (±)-α-tocopherol (1 mM/l), (+)-γ-tocopherol (1 mM/l) and (+)-δ-tocopherol (1 mM/l) were prepared in ethanol and stored at +4°C in the dark. To obtain standard solutions, the stock solutions were mixed and diluted in ethanol in order to obtain six solutions containing 0, 1, 1.5, 2, 2.5 and 3 mg/l, (α-tocopherol), 0, 2, 4, 1.5, 6 and 8 mg/l (γ-tocopherol) and 0, 0.5, 1, 1.5, 2 and 2.5 mg/l (δ-tocopherol) respectively. They were stored at +4°C in the dark.

The composition of buffer solution was 200 mg/l of SDS in sodium dihydrogen phosphate monohydrate (50 mM) aqueous solution; the pH was adjusted to pH 2.8 with ortophosphoric acid 85%. It was stored at +4°C.

2.3.3.3. Chromatographic conditions. Separation was carried out at room temperature on a C_{18} Genesis analytical column (50×4.6 mm^2 ID), 4 μm. Injection volume was 20 μl. The LC mobile-phase was *B*; an isocratic LC pump was used. The flow rate was 2 ml/min; back pressure was 130 atm. The eluted peaks were monitored by the ED with the conditioning cell set at −600 mV and the analytical cell set as follows: detector 1 at −150 mV, detector 2 at +450 mV; response 4; gain 10×1. Monitored signal was Detector 2.

2.3.3.4. Calculation. Tocopherols concentrations were calculated by means of external standard method; chromatographic peak height was evaluated. Standard solutions were directly injected and the peak heights versus concentrations were utilized for the calibration graphs. The extracted sample concentrations were calculated from the graphs.

2.3.3.5. Assay validation. LC method was validated in terms of accuracy, precision, linearity, parallelism, detection limit, limit of quantitation, specificity and selectivity. Accuracy was expressed

Table 4
Dimensional parameter of B, D and F formula (given in μm)

Time (h)	B formula			D formula			F formula		
	$T = 4$°C	$T = 25$°C	$T = 37$°C	$T = 4$°C	$T = 25$°C	$T = 37$°C	$T = 4$°C	$T = 25$°C	$T = 37$°C
0	d10 = 0.04 d50 = 0.18 d90 = 0.33	d10 = 0.04 d50 = 0.18 d90 = 0.33	d10 = 0.04 d50 = 0.18 d90 = 0.33	d10 = 0.06 d50 = 0.23 d90 = 0.53	d10 = 0.06 d50 = 0.23 d90 = 0.53	d10 = 0.06 d50 = 0.23 d90 = 0.53	d10 = 0.04 d50 = 0.19 d90 = 0.37	d10 = 0.04 d50 = 0.19 d90 = 0.37	d10 = 0.04 d50 = 0.19 d90 = 0.37
24	d10 = 0.04 d50 = 0.18 d90 = 0.33	d10 = 0.04 d50 = 0.18 d90 = 0.33	d10 = 0.04 d50 = 0.18 d90 = 0.33	d10 = 0.06 d50 = 0.23 d90 = 0.53	d10 = 0.06 d50 = 0.23 d90 = 0.53	d10 = 0.06 d50 = 0.23 d90 = 0.53	d10 = 0.04 d50 = 0.19 d90 = 0.40	d10 = 0.04 d50 = 0.19 d90 = 0.40	d10 = 0.04 d50 = 0.19 d90 = 0.41
48	d10 = 0.04 d50 = 0.18 d90 = 0.33	d10 = 0.04 d50 = 0.18 d90 = 0.33	d10 = 0.04 d50 = 0.18 d90 = 0.33	d10 = 0.06 d50 = 0.24 d90 = 0.54	d10 = 0.06 d50 = 0.24 d90 = 0.54	d10 = 0.06 d50 = 0.24 d90 = 0.54	d10 = 0.04 d50 = 0.19 d90 = 0.41	d10 = 0.04 d50 = 0.19 d90 = 0.41	d10 = 0.04 d50 = 0.19 d90 = 0.41
72	d10 = 0.04 d50 = 0.18 d90 = 0.33	d10 = 0.04 d50 = 0.18 d90 = 0.33	d10 = 0.04 d50 = 0.18 d90 = 0.33	d10 = 0.06 d50 = 0.24 d90 = 0.54	d10 = 0.06 d50 = 0.24 d90 = 0.54	d10 = 0.06 d50 = 0.24 d90 = 0.54	d10 = 0.04 d50 = 0.19 d90 = 0.42	d10 = 0.04 d50 = 0.19 d90 = 0.43	d10 = 0.04 d50 = 0.19 d90 = 0.43

as percent recovery of pure standards; the addition method was used to test sample recovery, because initials tocopherols amount was not known. In fact, both industrial lipid emulsions and Idroplurivit® Liofilizzato contained tocopherols, but the ratio between α-, γ- and δ-tocopherol of branded lipid emulsions was not provided by industries.

Precision was expressed as CV% of the method. Linearity and parallelism were tested by extraction of diluted samples at four different sample–water–buffer ratios (v/v/v): (400:0:800); (200:0:1000); (100:100:1000); (50:150:1000).

Specificity and selectivity were tested by injection of possible interfering substances.

3. Results and discussion

3.1. Particle diameter

With regard to particle diameter and distribution, analysis results were homogenous and comparable in the course of time (0, 24, 48 and 72 h after compounding) among the different industrial lipid emulsions and the compounded formulae stored at 4, 25 and 37°C. In particular, the graphs obtained by the analysis of samples did not point out particle diameter larger than 1 μm; only admixtures compounded by Intralipid® 20% pointed out some values >1 μm, which were also found in Intralipid® 20% single bottle.

To make an example, the dimensional parameters (d10, d50, d90) of the most critical formulae are shown in Table 4; in addition, particle size distribution of admixtures with vitamins stored for 72 h at 37°C, is reported in Figs. 2–4.

3.2. Fat-soluble vitamins

Two specific LC methods based on SPE were developed, because tested admixtures were complex lipid emulsions, which made it difficult to

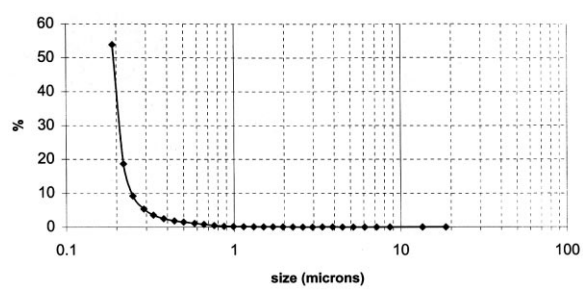

Fig. 2. Particle size distribution of B formula ($t = 72$ h, $T =$ 37°C).

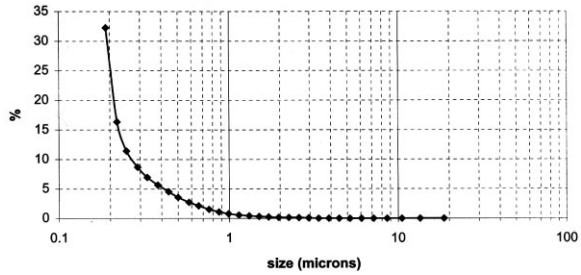

Fig. 3. Particle size distribution of D formula ($t = 72$ h, $T = 37$°C).

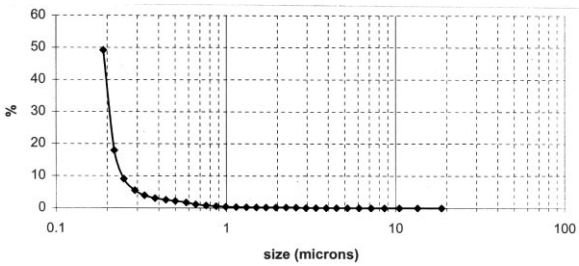

Fig. 4. Particle size distribution of F formula ($t = 72$ h, $T = 37$°C).

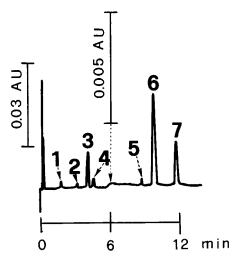

Fig. 5. Analytical profile obtained by injection of a standard solution. Peaks: 1, BHT; 2, *cis*-retinoic acid; 3, retinol (I.S.) (4.20 mg/l); 4, *trans*-retinoic acid; 5, α-tocopherol acetate; 6, retinol palmitate (0.80 mg/l); 7, β-carotene. Chromatographic conditions: column, Genesis C_{18}, 4 μm (50×4.6 mm^2 ID) (Jones Chromatography); mobile-phases, *A*: sodium acetate (10 mM) in glacial acetic acid–water–methanol (4.8:387:1545 v/v/v); *B*: sodium acetate (10 mM) in glacial acetic acid–2-propanol–methanol (4.8:225:1706 v/v/v); binary gradient between *A* and *B*: 10% *B* mobile-phase for 5 min; from 10 to 100% *B* in 0.1 min; 100% *B* for 8.9 min; flow rate, 2 ml/min; detection, UV, 325 nm.

extract fat-soluble vitamins. A previous liquid/liquid extraction method [17] was tested; the extraction mixture was made up of *n*-hexane and ethanol 95% (2:1 v/v), the recovery was good, but suffered of lack of repeatability. By applying the SPE, the recoveries were comparable, but SPE was preferred for its higher precision. Moreover, it did not require solvent evaporation, which caused fat-soluble vitamin degradation.

3.2.1. Retinol palmitate

The typical chromatographic profiles obtained by injection of the standard solution and of an extracted sample are shown in Figs. 5 and 6, respectively. Solutions of pure retinol and retinol palmitate are highly unstable, thus a specific and complex solution had to be compounded by mixing them with other stabilizing molecules; particularly α-tocopherol acetate was added as an antioxidant agent, β-carotene (the most insoluble antioxidant agent) was added to exclude all possible insolubility phenomena, all *trans*-retinoic acid was added for monitoring mixture stability (time and temperature influence *trans–cis* isomerism).

(1) Assay validation

Table 5 shows the recoveries of retinol and retinol palmitate and the CV of LC-UV method. Retinol palmitate recovery was calculated by the ratio between retinol palmitate found amount and the retinol palmitate nominal amount.

The calibration graph fits a simple straight-line equation ($r = 0.996$) with a typical calibration equation of $y = 0.354x + 0.003$ (y = retinol palmitate, mg/l; x = retinol palmitate peak height/retinol peak height) ($n = 6$, slope RSD = 4.9% , intercept RSD = 5.2%). The internal standard added to the sample before extraction compensated for possible variation in SPE recovery and

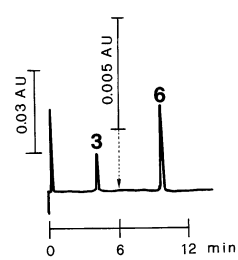

Fig. 6. Analytical profile obtained by injection of an extracted sample. Peaks: 3, retinol (I.S.) (4.20 mg/l); 6, retinol palmitate (0.78 mg/l). Chromatographic conditions, same as in Fig. 5.

Table 5
Recovery of retinol and retinol palmitate and CV [a]

	Recovery %	Intra-assay CV % ($n = 6$)	Inter-assay CV % ($n = 4$)
Retinol (I.S.)	99	5.1	6.2
Retinol palmitate	98	5.4	6.5

[a] n – number of sample or session repetitions.

in eluted volume, so it was not necessary to extract the standard solutions. Vitamins were soluble in the sample because they were dispersed in the lipophilic phase of the emulsion. SDS in the buffer solution was necessary to keep vitamins in solution also in absence of organic solvents. Organic solvents in buffer solution must be kept low in order to optimize SPE recovery. Diluted sample was homogeneous and clear, with no precipitates; the use of larger amounts of organic solvents in the buffer solution caused sample precipitation.

The linearity test gave a linear graph, parallel to calibration graph. The capacity factor of extraction cartridge was good and higher than the sample volumes utilized. The detection limit was 10 μg/l (retinol) and 25 μg/l (retinol palmitate) (concentration that gives rise to a signal which is $3x$ for the noise value). The limit of quantitation was 30 μg/l (retinol) and 75 μg/l (retinol palmitate) (concentration at which RSD = 10%, with $n = 6$). No interfering peaks were pointed out, because of the high selectivity of chromatographic system, wavelength used and solid-phase extraction method.

(2) Idroplurivit® Liofilizzato. In tested vial retinol palmitate was 12.4 mg/2 ml.

(3) B, D and F compounded formulae. Chromatographic profile were comparable among the different tested formulae. Changes in retinol palmitate amount were pointed out in the course of time (0, 24, 48 and 72 h after compounding) at tested storage temperatures.

To make an example, Table 6 shows the results obtained for B formula.

Analysis of the storage temperature of admixtures pointed out that retinol palmitate was highly unstable at room temperature (50% degradation 72 h after compounding), while it was more stable when stored at 4°C (28.5% degradation 72 h after compounding) and it was completely stable at 37°C. The last experimental datum was very interesting; it could be explained as a synergistic effect between retinol palmitate and another admixture component (tocopherols, ascorbic acid, lipids), which acts as an antioxidant agent at that temperature. Similar vitamin interaction phenomena have been quoted also by other authors [18] while studying complex vitamin admixtures.

3.2.2. Tocopherols

The typical chromatographic profile of an extracted sample is shown in Fig. 7. An interference was pointed out between γ-tocopherol and α-tocopherol acetate. Both eluted at the same retention time, but the former showed a response coefficient 2300 times higher, thus the last molecule was not detectable under analytical conditions. No other interfering peaks were pointed out, because of the high selectivity of chromatographic system, detector and solid-phase extraction method.

(1) Assay validation results. Table 7 shows the recoveries of δ-, γ-, α-tocopherol and α-tocopherol acetate and the CV of LC-ED method. For

Table 6
Retinol palmitate amount/bag of B formula at $T = 4$, 25 and 37°C

Time (h)	Retinol palmitate (mg [a])		
	$T = 4$°C	$T = 25$°C	$T = 37$°C
0	11.8	11.8	11.8
24	11.4	7.9	12.7
48	10.5	7.3	12.6
72	9.3	5.8	12.7

[a] mg — arithmetic mean of two determinations.

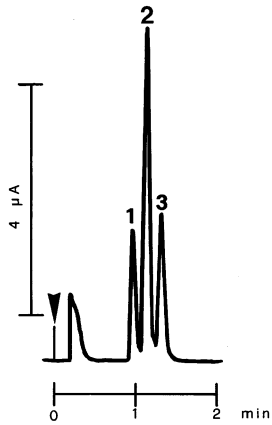

Fig. 7. Analytical profile obtained by injection of an extracted sample. Peaks: 1, δ-tocopherol (1.80 mg/l); 2, γ-tocopherol (4.62 mg/l); 3, α-tocopherol (2.08 mg/l). Chromatographic conditions: column, C_{18}, 4 μm (50×4.6 mm^2 ID) (Jones Chromatography); mobile-phase: sodium acetate (10 mM) in glacial acetic acid-2-propanol-methanol (4.8:225:1706 v/v/v); flow rate, 2 ml/min; electrochemical detector (ESA): conditioning cell, -600 mV; detector 1, -150 mV; detector 2, $+450$ mV (signal monitored).

recovery study pure standards were extracted and the recovery was calculated by the ratio between the found amount and the nominal amount. Sample recovery obtained by the addition method gave the same results as pure standards.

The calibration graphs fit a simple straight-line equation ($r = 0.998$). Equations ($y =$ tocopherols, mg/l; $x =$ tocopherols peak height) were $y = 0.0339x + 0.003$ ($n = 6$, slope RSD = 4.8% , intercept RSD = 5.2%) for δ-tocopherol, $y = 0.0342x + 0.003$ ($n = 6$, slope RSD = 4.9% , intercept RSD = 5.1%) for γ-tocopherol and $y = 0.0347x - 0.002$ ($n = 6$, slope RSD = 4.8%, intercept RSD = 5.1%) for α-tocopherol. Also for tocopherols, it was necessary to use SDS in the buffer solution.

The linearity test gave a linear graph, parallel to calibration graph. The capacity factor of extraction cartridge was good and higher than the sample volumes utilized. The detection limit was 15 ng/l (δ-, γ-, α-tocopherol) and 30 μg/l (α-tocopherol acetate). The limit of quantitation was 50 ng/l (δ-, γ-, α-tocopherol) and 100 μg/l (α-tocopherol acetate).

(2) B, D and F compounded formulae. Chromatographic profile were comparable among the different tested formulae. Changes in tocopherols amount were pointed out in the course of time (0, 24, 48 and 72 h after compounding) at tested storage temperatures.

To make an example, Table 8 shows the results obtained for B formula. Analysis of the storage temperature of admixtures pointed out that tocopherols were enough stable at room temperature (25% degradation, 72 h after compounding), while both at 4 and 37°C they showed an irregular trend. In particular, at 4°C it was pointed out a quick drop in concentration during the first 24 h (about 34% of initial amount); then tocopherols amount rose again until it reached almost initial value. At 37°C concentration dropped quickly during the first 48 h (about 42% of initial amount); afterwards, 84% of initial value was reached.

Those experimental data could be explained as a free conversion between the oxidized and the reduced form of tocopherols according to admixture different balances at various storage temperatures. In particular, ascorbic acid could influence that conversion. On the other hand, regeneration of tocopherols reduced form by ascorbic acid is confirmed by other authors [19].

Table 7
Recovery of δ-, γ-, α-tocopherol and α-tocopherol acetate and CV [a]

	Recovery %	Intra-assay CV % ($n = 6$)	Inter-assay CV % ($n = 4$)
δ-tocopherol	99	3.5	4.7
γ-tocopherol	98	3.7	4.8
α-tocopherol acetate	99	3.6	4.8
α-tocopherol	99	3.5	4.7

[a] n — number of sample or session repetitions.

Table 8
δ-, γ-, α-tocopherol amount/bag at $T = 4$, 25 and 37°C

Time (h)	δ-tocopherol (mg [a])			γ-tocopherol (mg [a])			α-tocopherol (mg [a])		
	$T = 4$°C	$T = 25$°C	$T = 37$°C	$T = 4$°C	$T = 25$°C	$T = 37$°C	$T = 4$°C	$T = 25$°C	$T = 37$°C
0	9.6	9.6	9.6	25.7	25.7	25.7	11.0	11.0	11.0
24	6.4	9.0	6.6	16.4	23.2	16.7	7.7	10.4	7.6
48	7.6	7.2	5.4	19.9	18.8	13.9	9.7	8.5	7.3
72	9.3	7.1	8.1	24.0	18.1	20.5	11.5	7.6	9.6

[a] mg — arithmetic mean of two determinations.

4. Conclusions

In this study three industrial lipid emulsions (Intralipid® 20%, Liposyn® 20% and ClinOleic® 20%) and six different compounded formulae have been compared by laser diffraction, in order to value the stability of all of them and their technical interchangeability in clinical practice. Stability studies have confirmed they have a superimposable behavior and they are physically stable at different storage temperature (4, 25 and 37°C) for at least 72 h after compounding. It allows to set a four days expiry date in clinical practice.

The LC described methods are effective for quantifying retinol palmitate and δ-, γ-, α-tocopherol in parenteral admixtures; they allow to obtain clearer and more replicable chromatographic profiles and they simplify the extraction and analytical procedures. These methods have a good accuracy, precision, selectivity and a very good sensitivity for the quantitation of analyzed vitamins. The use of SPE allows to shorten sample preparation time and to decrease analytical errors.

The results of LC analysis have pointed out that vitamins interact each other and degrade in different way according to various storage temperatures and times; in particular, vitamin amount begins decreasing 24 h after compounding, that is why all-in-one admixtures with vitamins must be fresh and daily prepared.

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