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Analysis and stability study of retinoids in pharmaceuticals by LC with fluorescence detection☆

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Abstract

Liquid chromatographic (HPLC) methods with fluorescence detection at different wavelengths were developed for measurements of retinoic acids (13-*cis* and all-*trans*) in pharmaceutical dosage forms and components of 'retinoid solution' (all-*trans* retinoic acid, vitamin A palmitate and β-carotene), a galenical of 'Di Bella therapy', using reversed phase columns under isocratic conditions. The stability of all-*trans* retinoic acid in cream and all-*trans* retinoic acid and vitamin A palmitate in 'retinoid solution' was investigated. Solid-phase extraction (SPE), using C18 sorbent was applied to the analysis of retinoic acids (9-*cis*, 13-*cis* and all-*trans*) in the 'retinoid solution' to obtain a practical and reliable sample clean-up. The results showed that these preparations (cream and solution) can be conveniently stored in the dark (t.a. or 2–8°C); under these conditions about 86–87% of the all-*trans* retinoic acid initial concentration in both formulations and about 73–78% of vitamin A palmitate in the 'retinoid solution' remained after 90 days, while under sunlight exposure rapid degradation of the drugs was observed.

Keywords: Reversed-phase liquid chromatography; Retinoids; Fluorescence detection; Stability

1. Introduction

Retinoic acid is very potent in promoting growth and controlling differentiation and maintenance of epithelial tissue in vitamin A-deficient animals. Indeed, all-*trans*-retinoic acid (tretinoin) appears to be the active form of vitamin A in all tissues except the retina, and it is 10–100-fold more potent than retinol in various systems in vitro. Isomerization of this compound in the body yields 13-*cis*-retinoic acid (isotretinoin), which is nearly as potent as tretinoin in many of its actions on epithelial tissues [1]. Vitamin A and other retinoids have found wide application in the treatment of skin disorders and may find important roles in cancer chemoprevention and therapy [2]. Recently, all-*trans* retinoic acid was used as a component with vitamin A palmitate, β carotene and vitamin E acetate of 'retinoid solution', a galenical proposed from 'Di Bella therapy'. β-carotene is a

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carotenoid and is a precursor of vitamin A. β-carotene and vitamin E have antioxidant activity and have been studied for their possible protective benefit in a number of disorders.

Owing to the photolability and sensitivity to heat and oxidation of the retinoids their quantitative determination in pharmaceuticals is particularly important for quality control of finished products and for stability-indicating assays. Detailed studies on retinoid and carotenoid degradation products in commercial preparations and raw materials by HPLC [3–13], microcalorimetry [7], UV radiation monitor [14], TLC and PLC [15], capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MECC) [16] have been performed, but only preliminary informations have been reported on the stability of all-*trans* retinoic in the 'retinoid solution' [17]. Thus, it was considered necessary to gain further information on the chemical stability of 'retinoid solution' to suggest the most appropriate conditions for storage of these preparations.

In the present paper simple and selective procedures are proposed by high performance liquid chromatography (HPLC) with fluorescence detection for a reliable quality control of commercially available formulations. Fluorescence detection was chosen because, according previous reports [18–22], the method selectivity and sensitivity can be enhanced as required in stability studies. Particularly the purpose of this study was to develop HPLC methods suitable to determine the degradation products (13-*cis* and 9-*cis* retinoic acids) of tretinoin in a pharmaceutical dosage form (cream) and in the 'retinoid solution' formed after prolunged exposure of these samples to light, air and maintened at different temperatures. Moreover the stability of vitamin A palmitate contained in the galenical solution was investigated.

2. Experimental

2.1. Materials

All-*trans* retinoic acid (tretinoin), 13-*cis* retinoic acid (isotretinoin), 9-*cis* retinoic acid, vitamin A palmitate, vitamin E acetate, β-carotene were ob-

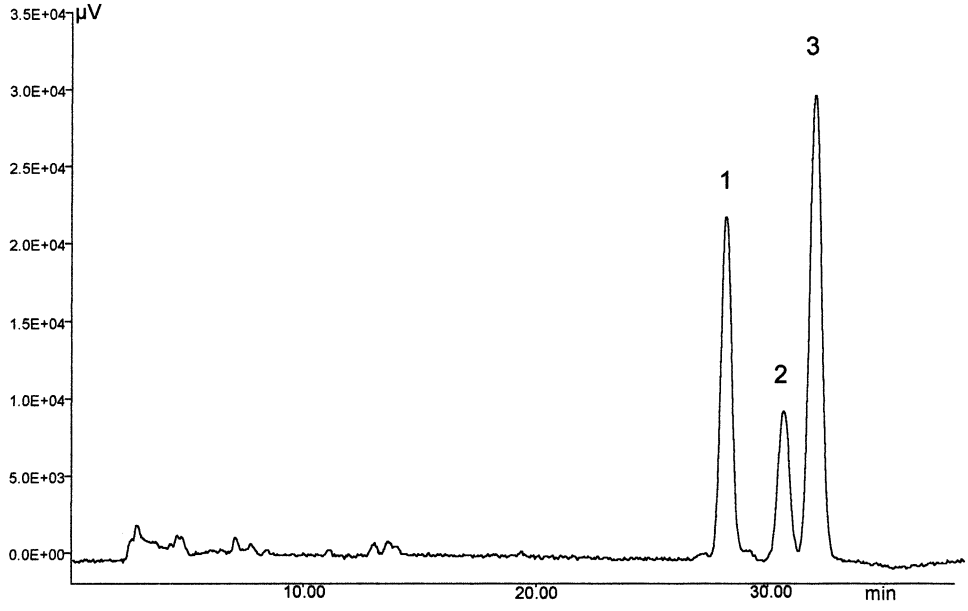

Fig. 1. Representative HPLC chromatogram obtained at 32 ± 2°C of retinoic acids. Peaks (1) 13-*cis* retinoic acid; (2) 9-*cis* retinoic acid; and (3) all-*trans* retinoic acid. Column, Phenomenex Prodigy 5ODS$_3$ (250 × 3.2 mm i.d.). Mobile phase: acetonitrile–ethanol–1% glacial acetic acid (68:8:24, v/v/v) at a flow rate of 0.4 ml min^{-1}. Fluorescence detection: $\lambda_{ex} = 350$ nm; $\lambda_{em} = 520$ nm.

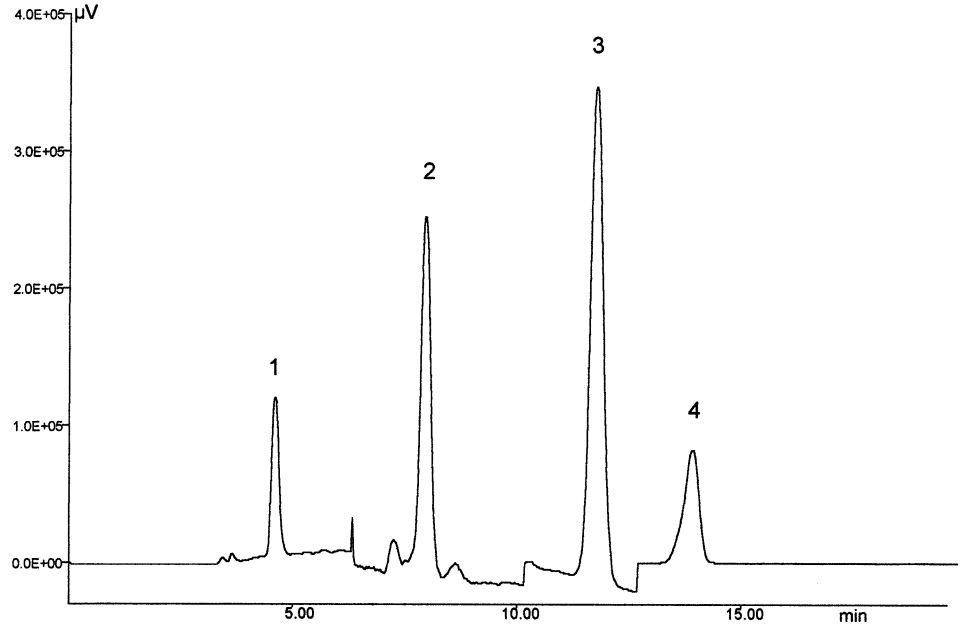

Fig. 2. Representative HPLC chromatogram obtained at 32 ± 2°C of (1) all-*trans* retinoic acid, (2) vitamin E acetate, (3) vitamin A palmitate and (4) β carotene. Column, Phenomenex Luna Phenyl-Hexyl (250 × 4.6 mm i.d.). Mobile phase, acetonitrile–methanol–1% glacial acetic acid mixture (86:10:4, v/v/v) at a flow rate of 0.8 ml min^{-1}. Fluorescence detection, programmable variable wavelength ($t = 0$ min $\lambda_{em} = 520$ nm with a $\lambda_{ex} = 350$ nm, gain 1000, for retinoic acid; $t = 6.2$ min $\lambda_{em} = 330$ nm with $\lambda_{ex} = 296$ nm, gain 1000, for vitamin E acetate; $t = 10$ min $\lambda_{em} = 520$ nm with $\lambda_{ex} = 350$ nm, gain 1000, for vitamin A palmitate; $t = 12.5$ min $\lambda_{em} = 520$ nm with $\lambda_{ex} = 450$ nm, gain 1000, for β-carotene).

tained from Sigma (St Louis, MO). Acetonitrile, methanol, ethanol for chromatography were HPLC grade, from Romil (Delchimica Scientific Glassware, Naples, Italy) and double distilled water was used. Other chemicals were from Carlo Erba Reagent (Italy). Solid phase exctractions were performed on Bond Elut cartridges (500 mg; 3 ml) from Analytichem (Varian, USA), using a Baker-10 SPE Vacuum manifold connected to a water aspirator.

2.2. Apparatus

The liquid chromatograph comprised a JASCO Model LG-980-02 ternary gradient unit, a Jasco PU-1580 pump, a Jasco FP-920 fluorescence detector with a programmable variable wavelengh, connected to a personal computer AcerView 34TL. The integration program Borwin was used. Manual injections were carried out using a Rheodyne model 7125 injector with 20 µl sample loop. The solvents were degassed on line with a degasser model Gastorr 153 S.A.S Corporation (Tokyo, Japan). Sonarex Super RK 102 (35 KMZ) Bandelin (Berlin, Germany) equipment with termostatically controlled heating (30–80°C) was used for ultrasonication.

2.3. Solutions

Stock solutions of all-*trans* retinoic acid, 13-*cis* retinoic acid, 9-*cis* retinoic acid, vitamin E acetate and vitamin A palmitate were prepared in acetonitrile, and β-carotene in dichlorometane; these solutions were then appropriately diluted to give standard solutions in the suitable mobile phase (concentration under calibration graphs) and were stable for at least 3 days at 4°C.

2.4. Chromatographic conditions

The separations of 9-*cis* retinoic acid, 13-*cis* retinoic acid and all-*trans* retinoic acid were carried out at 32 ± 2°C under isocratic reversed-phase conditions on a Phenomenex Prodigy 5ODS$_3$ (250×3.2 mm i.d.) using a mobile phase consisting of acetonitrile–ethanol–1% glacial acetic acid (68:8:24, v/v/v) mixture at flow rate of 0.4 ml min^{-1}. Fluorescence detection at $\lambda_{em} = 520$ nm with $\lambda_{ex} = 350$ nm was used.

The separation of retinoic acid, vitamin E acetate, vitamin A palmitate and β-carotene was carried out at 32 ± 2°C under isocratic reversed-phase conditions Phenomenex Luna Phenyl-Hexyl (250×4.6 mm i.d.) using a mobile phase consisting of acetonitrile–methanol–1% glacial acetic acid mixture (86:10:4, v/v/v) at flow rate of 0.8 ml

Table 1
Data for calibration graphs ($n = 6$) of retinoids, β carotene and vitamins

Drug	Slope	y-Intercept	Correlation coefficient	Concentration range (μg ml^{-1})	$\lambda_{ex}/\lambda_{em}$ (nm)
Tretinoin[a]	391040 ± 1727.7	-1741.6 ± 8010.1	0.9999	0.13–11.45	350/520
Tretinoin[b]	241566 ± 1704.0	-17899 ± 17379	0.9999	1.24–19.76	350/520
Isotretinoin[a]	203858 ± 772.07	2326.4 ± 1583.6	1.0000	0.06–9.40	350/520
	214570 ± 2450.5	423510 ± 185518	0.9999	12.77–102.20	350/520
9-*cis* Retinoic acid[a]	61405 ± 1010.8	3996.1 ± 914.74	0.9997	0.11–1.74	350/520
β-Carotene[b]	231686 ± 1653.3	-333341 ± 63488	0.9999	4.65–74.40	450/520
Vitamin A palmitate[b]	2051670 ± 41720	-385691 ± 242802	0.9996	1.26–10.10	350/520
Vitamin E acetate[b]	180906 ± 2217.7	303869 ± 58181	0.9998	2.57–51.40	296/330

[a] HPLC analysis with Phenomenex Prodigy 5 ODS$_3$ column (250×3.2 mm i.d.).
[b] HPLC analysis with Phenomenex Luna Phenyl-Hexyl column (250×4.6 mm i.d.).

Table 2
Precision of the HPLC analysis of retinoids, β carotene and vitamins

Compound	Concentration (μg ml^{-1})	Intraday (% RSD)	Interday (% RSD)
Tretinoin[a]	2.22	0.108	0.136
Tretinoin[b]	7.72	0.158	0.356
Isotretinoin[a]	2.35	1.98	3.16
9-*cis* Retinoic acid[a]	4.35	0.215	0.347
β-Carotene[b]	39.70	1.368	3.045
Vitamin A palmitate[b]	4.64	0.499	0.501
Vitamin E acetate[b]	21.60	0.199	0.220

[a] HPLC analysis with Phenomenex Prodigy 5 ODS$_3$ column (250×3.2 mm i.d.).
[b] HPLC analysis with Phenomenex Luna Phenyl-Hexyl column (250×4.6 mm i.d.).

Table 3
Detection limit of retinoids, β carotene and vitamins (S/N = 3)

Drug	pmol	$\lambda_{ex}/\lambda_{em}$ (nm)
Tretinoin[a]	7.12	350/520
Tretinoin[b]	7.64	350/520
Isotretinoin[a]	10.33	350/520
9-*cis* Retinoic acid[a]	11.09	350/520
β-Carotene[b]	4.87	450/520
Vitamin A palmitate[b]	0.81	350/520
Vitamin E acetate[b]	11.89	296/330

[a] HPLC analysis with Phenomenex Prodigy 5 ODS_3 column (250 × 3.2 mm i.d.).
[b] HPLC analysis with Phenomenex Luna Phenyl-Hexyl column (250 × 4.6 mm i.d.).

min^{-1}, selecting a programmable variable wavelength ($t = 0$ min $\lambda_{em} = 520$ nm with a $\lambda_{ex} = 350$ nm, gain 1000, for retinoic acid; $t = 6.2$ min $\lambda_{em} = 330$ nm with $\lambda_{ex} = 296$ nm, gain 10 or 1000, for vitamin E acetate; $t = 10$ min $\lambda_{em} = 520$ nm with $\lambda_{ex} = 350$ nm, gain 1000, for vitamin A palmitate; $t = 12.5$ min $\lambda_{em} = 520$ nm with $\lambda_{ex} = 450$ nm, gain 1000, for β-carotene).

2.5. Calibration graphs

Standard solutions of 13-*cis* retinoic acid (0.06–9.40 and 12.77–102.20 μg ml^{-1}), 9-*cis* retinoic acid (0.11–1.74 μg ml^{-1}), all-*trans* retinoic acid (0.13–11.45 μg ml^{-1}) and vitamin E acetate (2.57–51.40 μg ml^{-1}) were prepared in the mobile phase.

Stock solutions of all-*trans* retinoic acid (1.24–19.76 μg ml^{-1}), vitamin A palmitate (1.26–10.10 μg ml^{-1}) and β-carotene (4.65–74.40 μg ml^{-1}) were prepared in a mixture of acetonitrile–ethanol–dichloromethane (60:30:10, v/v/v). Triplicate injections for each standard solution were made and the peak-area was plotted against the corresponding compound concentration to obtain the calibration graphs.

2.6. Solid phase extraction (SPE)

Before use, the SPE column, C18 sorbent, was properly conditioned by rinsing with 2 ml of acetonitrile and 3 ml of a mixture of acetonitrile–

Table 4
Results by HPLC analysis of retinoids, β carotene and vitamins in pharmaceuticals

Formulation[a]	Drug	Found[g]	Found[h]	RSD%	Recovery[i]	RSD%
Capsules[b]	Isotretinoin	100.68		2.03	100.28	1.23
	Tretinoin		1.84	3.08	97.89	3.57
Gel[c]	Isotretinoin	101.93		0.76	100.04	2.70
	Tretinoin		2.72	2.54	98.65	2.88
Cream[d]	Tretinoin	107.13		1.88	101.46	0.08
	Isotretinoin		1.90	3.12	98.03	3.54
	9-*cis* retinoic acid		2.11	2.78	98.66	2.96
Capsules[e]	Vitamin E acetate	91.13		4.98	100.57	2.89
	Vitamin A palmitate	135.13		4.04	100.40	1.24
'Retinoid solution'[f]	Tretinoin	110.10		1.17	100.32	1.54
	Vitamin A palmitate	89.53		0.52	99.52	0.99
	β carotene	83.49		3.98	99.05	3.08

[a] Other ingredients; [b]Capsules: partially hydrogenated soybean seed oil, yellow wax, hydrogenated soybean seed oil, soybean oil. Gelatin sheat elements: gelatin, glycerin, partiallly hydrolysate starch hydrogenation product, titanium dioxide, natural dye E161g (canthaxanthin); [c]Gel: butyhydroxytoluene (BHT), hydroxypropylcellulose, ethanol; [d]Cream: cetilic alcohol, stearic acid, isopropyl myristate, polysorbate 60, sorbitan monostearate, sorbitol, sorbic acid, edetate sodium, butylhydroxytoluene, purified water; [e]Capsules: piridoxine hydrochloride, peanut oil, hydrogenated soybean oil, hydrogenated vegetable oils, white wax, soybean lecithin, buthylhydroxyanisole (BHA), buthylhydroxytoluene (BHT); sheath: gelatin, glycerin, sodium ethyl *p*-hydroxybenzoate; sodium propyl *p*-hydroxybenzoate, ethyl vanillin, titanium dioxide, E127; [f]'Retinoid solution': vitamin E acetate.
[g] Mean of five determinations and expressed as a percentage of the claimed content.
[h] Mean of five determinations of impurity and expressed as a percentage of the drug.
[i] Mean of five determinations and expressed as a percentage recovery of the added standard (20% of the claimed content).

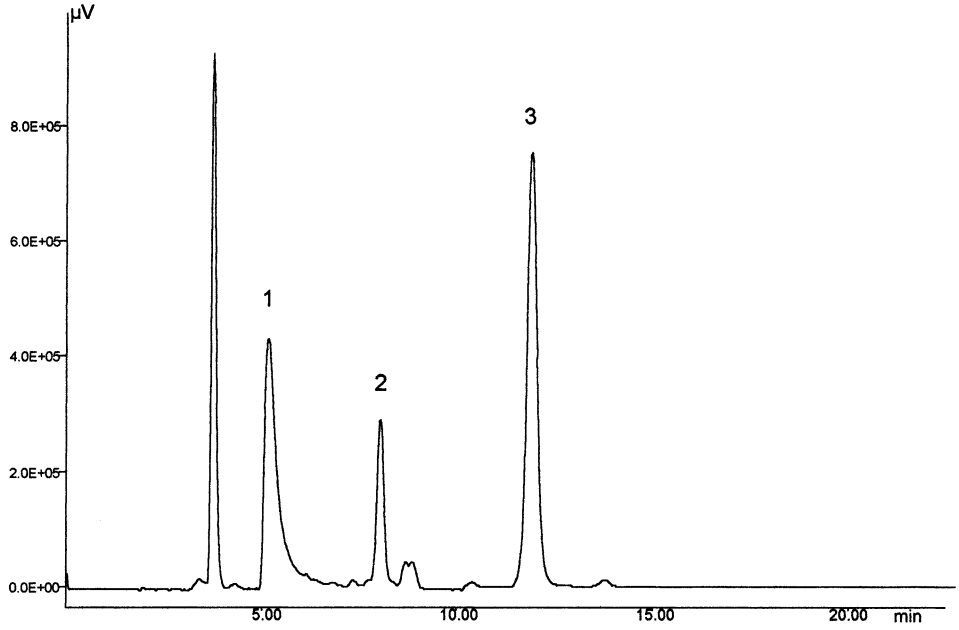

Fig. 3. HPLC chromatogram of vitamin E acetate and vitamin A palmitate (capsules). Peaks, (1) piridoxine hydrochloride; (2) vitamin E acetate; and (3) vitamin A palmitate. Chromatographic conditions as in Fig. 2. Fluorescence detection: programmable variable wavelength ($t = 0$ min $\lambda_{em} = 330$ nm with $\lambda_{ex} = 296$, gain 1000, for piridoxine hydrochloride and vitamin E acetate; $t = 10$ min $\lambda_{em} = 520$ nm with $\lambda_{ex} = 350$, gain 1000, for vitamin A palmitate).

ethanol (80:20, v/v), then a 2 ml aliquot of the sample solution was applied to the SPE column.

2.7. Analysis of pharmaceutical formulations

2.7.1. Sample preparation

The sample handling was carried out according to the nature and composition of the commercial dosage forms avoiding exposure to light and using amber-coloured glassware.

2.7.1.1. Isotretinoin (capsules). Ten capsules, equivalent about to 100 mg of 13-*cis* retinoic acid, were opened carefully using a sharp blade, without loss of shell material into a centrifuge tube. The capsules were extracted (3×40 ml) with a mixture consisting of acetonitrile–ethanol–1% acetic acid (70:20:10, v/v/v) by ultrasonication at 30°C for 5 min. After centrifugation for 6 min at 3500 rpm, the supernatants were filtered and combined quantitatively into a 250 ml volumetric flask. Then a 1 ml aliquot of the resulting clear solution was diluted to 10 ml volume with the mobile phase to obtain the final solution. At last the resulting solution was filtered with 0.45 μm nylon 25 mm filter and analysed by HPLC in comparison with an appropriate standard solution (40 μg ml^{-1}).

2.7.1.2. Isotretinoin (gel). An amount of the gel equivalent to about 0.25 mg of 13-*cis* retinoic acid was accurately weighed into a centrifuge tube and treated with 8 ml of mobile phase by ultrasonication for 1 min. After centrifugation for 10 min at 3500 rpm, the supernatant was filtered and quantitatively transferred to a 10 ml volumetric flask. Then a 1 ml aliquot of this solution was diluted to 5 ml volume with the mobile phase to obtain the final concentration. At last the solution was filtered with 0.45 μm nylon 25 mm filter. HPLC analysis was performed using a standard solution (5 μg ml^{-1}).

2.7.1.3. Tretinoin (cream). An amount of the cream equivalent to about 0.25 mg of all-*trans* retinoic acid, accurately weighed into a centrifuge

tube, was extracted (2 × 4 ml) with a mixture consisting of acetonitrile–ethanol–1% acetic acid (70:20:10, v/v/v) by ultrasonication for 5 min. After centrifugation for 6 min at 3500 rpm, the supernatants were filtered and combined quantitatively to a volumetric flask and diluted to 10 ml with the mixture used for the extraction. Then a 2 ml aliquot of this solution was diluted to 5 ml volume with mobile phase to obtain the final concentration. At last the solution was filtered with 0.45 μm nylon 25 mm filter. The standard solution contained 10 μg ml^{-1} of the drug.

2.7.1.4. Vitamin E acetate and vitamin A palmitate (capsules). Ten capsules, equivalent to about 0.50 g of vitamin A palmitate and 1.80 g of vitamin E acetate, were open carefully using a sharp blade, without loss of shell material into a centrifuge tube. The capsules were extracted (3 × 55 ml) with ethanol by ultrasonication at ambient temperature for 5 min. After centrifugation for 5 min at 3500 rpm, the supernatants were filtered and combined quantitatively to a 250 ml volumetric flask and diluted to volume with ethanol. Then a 0.05 ml aliquot of the resulting clear solution was diluted to 20 ml volume with mobile phase to obtain the final concentration. At last the solution was filtered with 0.45 μm nylon 25 mm filter and analysed by comparison with vitamin A palmitate (5.00 μg ml^{-1}) and vitamin E acetate (18.00 μg ml^{-1}) standard solutions.

2.7.1.5. 'Retinoid solution'. Each sample of the 'retinoid solution' was examined by two procedures: method A for the analysis of all-*trans* retinoic acid and its degradation products (13-*cis* and 9-*cis* retinoic acids) and method B for the determination of the components (retinoic acid, vitamin A palmitate, β carotene) of the galenical, using the chromatographic conditions described above.

Method A. A 100 mg amount of 'retinoid solution' was accurately weighed and diluted to 10 ml volume with acetonitrile. A 2 ml aliquot of this

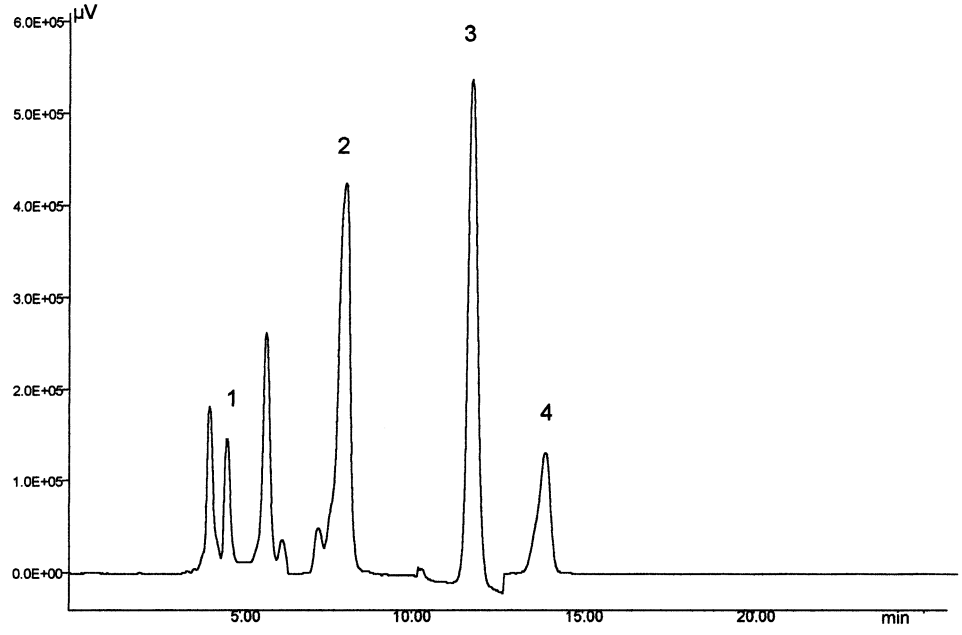

Fig. 4. HPLC chromatogram of 'retinoid solution'. Peaks (1) retinoic acid; (2) vitamin E acetate; (3) vitamin A palmitate; and (4) β carotene. Chromatographic conditions as in Fig. 2. Fluorescence detection, programmable variable wavelength ($t = 0$ min $\lambda_{em} = 520$ nm with a $\lambda_{ex} = 350$ nm, gain 1000, for retinoic acid; $t = 6.2$ min $\lambda_{em} = 330$ nm with $\lambda_{ex} = 296$ nm, gain 10, for vitamin E acetate; $t = 10$ min $\lambda_{em} = 520$ nm with $\lambda_{ex} = 350$ nm, gain 1000, for vitamin A palmitate; $t = 12.5$ min $\lambda_{em} = 520$ nm with $\lambda_{ex} = 450$ nm, gain 1000, for β-carotene).

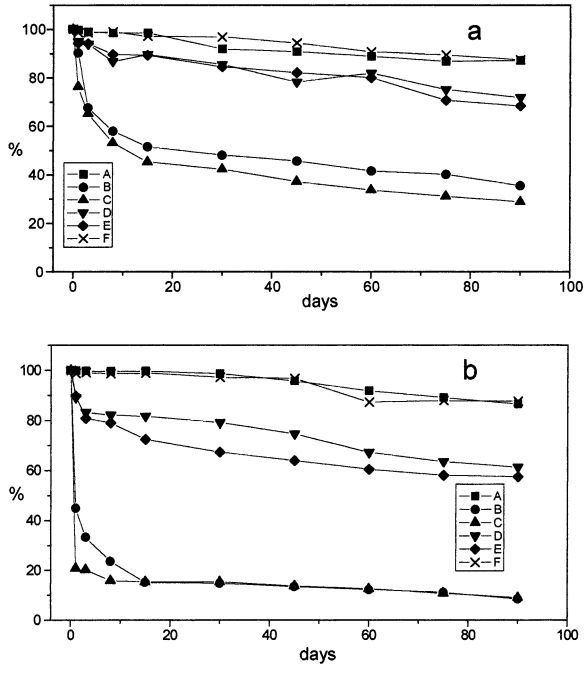

Fig. 5. Stability of tretinoin in 'retinoid solution' and cream. Mean percentage of initial tretinoin concentration in 'retinoid solution' (a) and cream (b) found over 3 month period. Storage conditions (A) a.t. in the dark; (B) a.t. in sunlight without air; (C) a.t. in sunlight with air; (D) 37°C in the dark without air; (E) 50°C in the dark without air; and (F) 2–8°C in the dark without air.

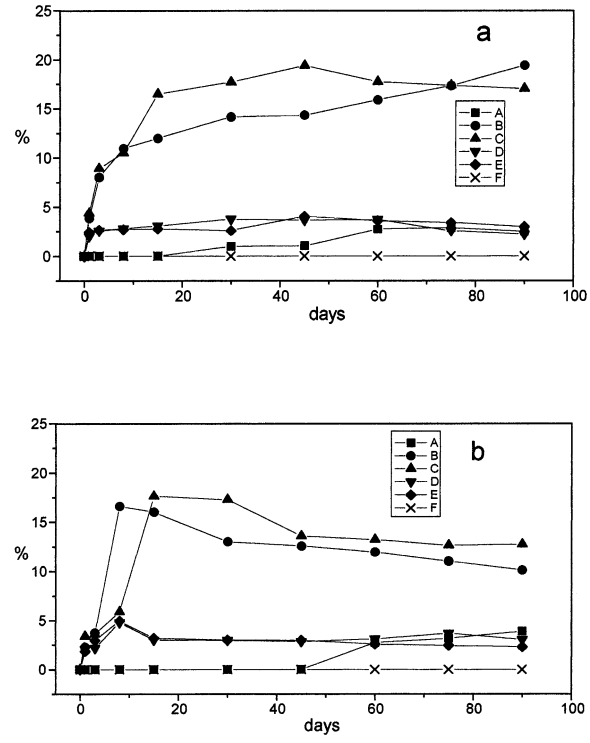

Fig. 6. Degradation products of tretinoin in 'retinoid solution'. Mean percentage of isotretinoin (a) and 9-*cis* retinoic acid (b) respect to initial tretinoin concentration found over 3 month period. Storage conditions as in Fig. 5.

solution was applied to a conditioned SPE C18 column, and the retinoic acids were eluted with 2 ml of a mixture of acetonitrile-methanol (50:50, v/v). The eluate was filtered with 0.45 μm nylon 25 mm filter and analysed by HPLC in comparison with an appropriate standard solution (5.00 μg ml^{-1} for all-*trans* retinoic acid and about 0.10 μg ml^{-1} for 9-*cis* retinoic acid and 13-*cis* retinoic acid)

Method B. A 100 mg amount of 'retinoid solution' was accurately weighed and diluted to 10 ml volume with a mixture consisting of acetonitrile–ethanol–dichlorometane (60:30:10, v/v/v). The resulting solution was filtered with 0.45 μm nylon 25 mm filter. The standard solutions contained 5.00 μg ml^{-1} of all-*trans* retinoic acid, 20 μg ml^{-1} of β-carotene and 5.00 μg ml^{-1} of vitamin A palmitate.

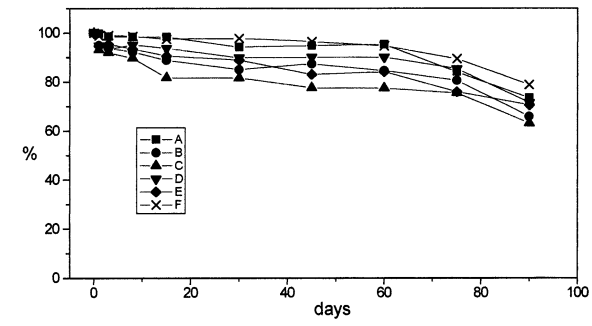

Fig. 7. Stability of vitamin A palmitate in 'retinoid solution'. Mean percentage of initial vitamin concentration in 'retinoid solution' found over 3 month period. Storage conditions as in Fig. 5.

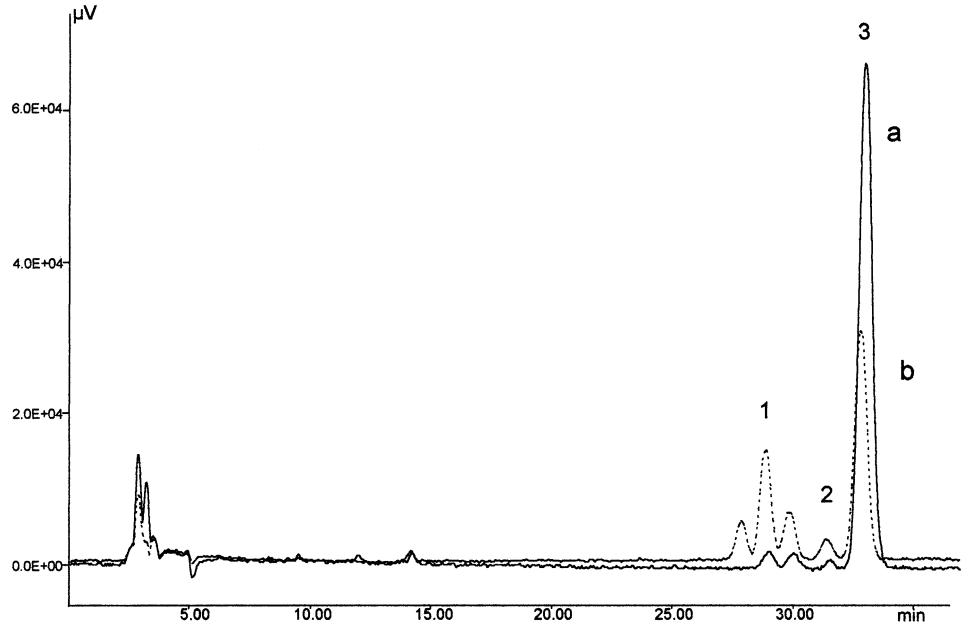

Fig. 8. HPLC chromatogram of tretinoin (cream) before (a) and after (b) exposure to the sunlight and air for 4 h. Peaks (1) 13-*cis* retinoic acid; (2) 9-*cis* retinoic acid; and (3) all-*trans* retinoic acid. Chromatographic conditions and detection as in Fig. 1.

2.8. Stability studies

Qualitative and quantitative analysis were made using 'retinoid solution' and tretinoin (cream). Each formulation was divided in six parts and stored in the following conditions: (a) in the dark without air (ambient temperature, 2–8, 37 and 50°C); (b) exposed to sunlight at the laboratory window (ambient temperature with and without air). Samples of 100 mg of 'retinoid solution' (equivalent to about 50 μg of all-*trans* retinoic acid and 50 μg of vitamin A palmitate) and 250 mg of tretinoin cream (equivalent to about 0.125 mg of all-*trans* retinoic acid) were accurately weighed and were periodically analysed over a 3 month period following the methods described above.

In the second step additional stability-indicating studies were performed to quantify tretinoin in cream and 'retinoid solution' and vitamin A palmitate in 'retinoid solution' under different conditions for 90 days. Because of the presence of conjugated double bonds, retinoids in general are unstable compounds. They are readily oxidized and/or isomerized to altered products, especially in the presence of oxidants (including air), light and excessive heat. They are also labile toward strong acids and solvents that have dissolved oxygen or peroxides [14]. Anhydrous solvents containing even traces of acid cause structural changes of retinoids. Thus, use of strong acid should be avoided. Alkali usually is not harmful to retinoids. Indeed, samples containing esters of retinol are routinely saponified in the presence of alkali to hydrolyze the esters to free retinol [23].

3. Results and discussion

The first objective of this work was the development of practical HPLC methods for the retinoid and vitamin determination in pharmaceuticals.

3.1. Chromatography

The HPLC separations were carried out under isocratic conditions at 32 ± 2°C on reversed phase columns: $5ODS_3$ and Phenyl–Hexyl. In the devel-

opment of HPLC methods the type of stationary phase, the effects of composition and pH of the mobile phase on the resolution and fluorescence intensity of retinoids and vitamins were investigated. Different chromatographic separation conditions (C8, C18, Phenyl–Hexyl columns and diverse mobile phases) were evaluated because of their different separation characteristics with regard to the retinoic acids and vitamins. In order to obtain the satisfactory chromatographic separation of retinoic acid isomers (13-*cis*, 9-*cis*, and all-*trans* retinoic acids) the ODS column was selected. Various mobile phase with different composition were carefully tried to achieve maximum separation in a minimum time. A solvent mixture consisting of acetonitrile–ethanol–1% glacial acetic acid (68:8:24, v/v/v) allowed to obtain acceptable resolution. High percentage of organic solvent was needed for eluting of the isomers in reasonable time. Acetic acid was used to reduce the tailing of the retinoic acids. Using lower percentage of acetic acid solution ($\leq 20\%$) only partial separation of 9-*cis* and all-*trans* retinoic acid was achieved. Both columns, ODS and Phenyl–Hexyl, with different and appropriate mobile phase, were suitable to obtain a good separation of all-*trans* retinoic acid, vitamin E acetate, vitamin A palmitate and β-carotene. The Phenyl–Hexyl column was chosen because allowed to reduce the run time. The typical chromatograms obtained using the optimized methods described are reported in Figs. 1 and 2. Attempts to analyze simultaneously all of these analytes (isomers, β-carotene and vitamins) in a single run using different columns and solvent mixture were unsuccessful.

3.2. Analysis of pharmaceutical formulations

Under the described chromatographic conditions (Figs. 1 and 2) a linear relationship between the peak-area (y) and analyte concentration (x) were obtained (Table 1). The precision of the methods (repeatability intraday and interday) were indicated by the relative standard deviation of the peak-area from the replicated ($n = 8$) analyses of a single standard solution (Table 2). The robustness study was performed in order to investigate the reproducibility of the method when controlled and limited variations are applied around normal operating conditions. The obtained results demonstrated that within the studied variations (organic modified percentage, acetic acid concentration, temperature and batch-to-batch variability of column) the chromatographic parameters of the method do not affect the column efficiency and provide acceptable resolution. The detection limit (signal to noise ratio = 3) is reported in Table 3.

The method with $5ODS_3$ column was then applied to the HPLC analyses of commercial formulations of isotretinoin (capsules and gel), tretinoin (cream), while the method with Phenyl–Hexyl column to the determination of vitamin E acetate and vitamin A palmitate (capsules). A relative simple and fast sample preparation procedure can be followed for the analyses of pharmaceuticals. The appropriate solvent (or solvent mixture) suitable for the complete drug solubilization was studied and added to sample. The dosage forms (cream, gel, capsules) was extracted by ultrasonication and the other unsoluble ingredients were removed by centrifugation. Then, after described dilution, an aliquot of resulting clear solution was injected into the HPLC system.

Both methods A and B were applied to the HPLC analyses of the 'retinoid solution' to obtain a reliable quality control of the preparation. The solid-phase extraction (SPE) (method A) was found to be effective for the sample clean-up. This procedure allowed elimination of the components (vitamin E acetate, vitamin A palmitate and β-carotene) with longer retention times. These compounds are retained by a sorbent, while the retinoic acid isomers are then recovered in the eluate.

The results obtained for each formulation (Table 4) were found to be in good agreement with the claimed content of the drugs. The USP XXIII monograph for oil-soluble vitamins capsules provide that the capsules must contain not less than 90.0% and not more than 165.0% of the labeled amounts of vitamin A palmitate and vitamin E acetate and for tretinoin cream not less than 90.0% and not more than 130.0% of the labeled amount of *trans* retinoic acid [13]. The

accuracy of the methods was verified by analysing commercial samples spiked with known amounts of drugs (20% of the claimed content); essentially quantitative recoveries were obtained. This suggests that the analytes were stable during the steps of the sample preparation. Method precision/ruggedness for five assays, with two different analysts and systems was 99.4% (2.5% RSD) for isotretinoin and 1.80% (3.6% RSD) for the impurity tretinoin, respectively (capsules, Table 4). The other ingredients of the formulations did not interfere with the analysis. Chromatograms obtained from commercial dosage forms and 'retinoid solution' are illustrated in Figs. 3 and 4, respectively. These data support the suitability of the methods for the quality control of retinoid commercial formulations.

3.3. Stability studies

Stability studies were performed on commercial formulations and therefore the effects of temperature and light exposure on their storage were examined. The effect of pH on the retinoids is well known [23] and was not object of this investigation.

The proposed methods were applied to the evaluation of the stability of the components of 'retinoid solution' and tretinoin (cream). In particular retinoic acids (all-*trans* retinoic, 9-*cis* and 13-*cis* retinoic acids) and vitamin A palmitate were studied as these retinoids are known to undergo Z–E isomerization (due to the presence of four unsaturated double bounds) and oxidation when exposed to light and air. Because of its chemical structure, retinol and its esters are highly reactive molecules and can be readily oxidized by chemical and physical agents [23–25]. The samples ($n = 2$) were analysed after 0, 1, 3, 8, 15, 30, 45, 60, 75, 90 days and triplicate analyses were performed for each sample. The results of 'retinoid solution' expressed as percentage of the initial concentration remaining for each condition are illustrated in Fig. 5a. As shown, only all-*trans* retinoic acid stored in sunlight with (C) or without (B) air decomposed rapidly after 1 day. This transformation is more rapid in the cream (Fig. 5b). The degradation products, 13-*cis* and 9-*cis* retinoic acids, increased till about 15–20% in these conditions, with respect to initial tretinoin concentration (Fig. 6). The samples of 'retinoid solution' and cream maintened in the dark at a.t. and 2–8°C displayed notable stability after 3 months. Their tretinoin content being reduced to about 86% at a.t. and 87% at 2–8°C of the initial concentration, while at 37 and 50°C was about in the range of 57–72% of the initial levels. The quantitative data of tretinoin in 'retinoid solution' are reported in Table 5. Vitamin A palmitate shows in the described conditions good stability in the time, although its degradation is not at all negligible (about 73% at a.t. and 78% at 2–8°C in the dark initial concentration remained in 90 days) (Fig. 7). These results suggest that the relative stability of vitamin A palmitate in the 'retinoid solution' might be due to the protective effect of vitamin E present at remarkable quantity and β carotene [1].

A representative chromatogram of retinoic acids obtained from cream sample maintained in sunlight for 4 h is reported in Fig. 8.

4. Conclusions

The proposed HPLC methods with fluorescence detection showed good linearity, precision and accuracy for sensitive and selective determination of tretinoin, vitamin A palmitate, β carotene and vitamin E acetate in pharmaceutical formulations. Moreover these procedures are useful to verify the suitable conditions for the storage of these formulations. The stability-indicating HPLC method for tretinoin in cream and 'retinoid solution' samples is able to quantify both tretinoin and degradation products formed in degradation studies.

The data achieved in the applications on pharmaceutical formulations are concordant and suggest that both preparations should be stored in the dark. Under these conditions the examined retinoid level was found to remain considerably high for 3 months, while in sunlight, the drugs were subjected to severe degradation.

Table 5
Stability of all-trans retinoic acid in 'retinoid solution'[a]

Storage conditions[b]	% Initial concentration at day								
	1	3	8	15	30	45	60	75	90
A	99.76 (1.32)	98.98 (1.57)	98.45 (1.22)	98.54 (1.98)	91.89 (2.01)	90.73 (1.76)	88.81 (1.99)	86.74 (1.58)	87.15 (1.25)
B	90.30 (2.05)	67.70 (1.65)	57.93 (1.08)	51.58 (2.13)	48.06 (2.50)	45.69 (2.38)	41.57 (2.08)	40.16 (2.66)	35.50 (2.91)
C	76.35 (2.36)	65.20 (2.72)	53.12 (2.53)	45.28 (2.81)	42.28 (1.98)	37.15 (2.31)	33.66 (2.05)	31.07 (2.71)	28.86 (2.85)
D	94.98 (1.54)	93.90 (1.36)	86.70 (1.23)	89.57 (1.55)	85.44 (1.81)	78.23 (2.07)	81.95 (2.14)	75.08 (2.02)	71.89 (1.58)
E	94.27 (1.68)	94.17 (1.51)	89.66 (1.12)	89.28 (1.64)	84.50 (1.24)	82.10 (1.51)	80.00 (1.30)	70.66 (2.15)	68.40 (2.88)
F	98.64 (1.03)	98.50 (1.22)	98.97 (0.96)	97.12 (0.78)	96.83 (1.65)	94.35 (1.84)	90.72 (1.90)	89.39 (1.69)	87.42 (2.05)

[a] The data are expressed as percentage (mean and RSD%) of the initial concentration ($n = 3$). Time zero concentration 5.5 μg ml^{-1}.

[b] (A) a.t. in the dark; (B) a.t. in sunlight without air; (C) a.t. in sunlight with air; (D) 37°C in the dark without air; (E) 50°C in the dark without air; and (F) 2–8°C in the dark without air.

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