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Short communication

Determination of retinoids in galenicals by column liquid chromatography with fluorescence and diode-array detection

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Abstract

Simple and rapid reversed-phase gradient column liquid chromatography (LC) with fluorescence detection at different wavelengths was developed for the simultaneous analysis of all-*trans*, 13-*cis*, 9-*cis* retinoic acids, vitamin A palmitate and β-carotene in galenicals. The assay results agreed with those obtained by an LC method with diode-array UV detection. A post-column on-line photochemical reactor (irradiation at 254 and 366 nm) was inserted between the LC column and the fluorescence detector to enhance the performance of the method. Two fluorescence spectra (photoreactor on and off) were obtained for each analyte which proved useful for the unambiguous identification of the various analytes.

Keywords: Derivatization, LC; Retinoids; Vitamins

1. Introduction

Retinoids include the natural compounds and synthetic derivatives of retinol that exhibit vitamin A activity. The essential role of vitamin A in vision is well documented. Retinoids also have prominent effects on epithelia and have revolutionised dermatological therapy in the last two decades [1]. They influence a wide variety of biological activities, including cellular proliferation and differentiation, immune function, inflammation, and sebum production. The association of vitamin A deficiency with squamous metaplasia, increased cell proliferation, hyperkeratosis, and carcinoma suggested that retinoids might be valuable in the treatment and prevention of cutaneous premalignant and malignant disorders. Recently, the so-called ''Multiterapia Di Bella'' has been proposed as an anti-cancer therapy. Besides other drugs, this therapy proposes the administration of the ''retinoid solution''; the formulation is the sole commercially available containing all-*trans* retinoic acid (0.5 g), vitamin A palmitate (0.5 g), β-carotene (2 g) and vitamin E acetate (1000 g), which is present in the galenical as diluent in large quantities.

Recently, the HPLC analysis and stability study of retinoids in pharmaceutical preparations have been carried out [2], owing to the fact that retinoids are unstable compounds, being readily oxidized and/or isomerized, especially in the presence of oxidants and air, light, and excessive heat [3]. Therefore, the isomers of all-*trans* retinoic acid can be present in the formulations as degradation products or impurities. Moreover, since the structure and polarity of the ''retinoid solution'' components and retinoic acid

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isomers are different, the separation and quantification of all analytes in a single run in short time is difficult. In order to analyse the retinoic acid isomers, solid-phase extraction (SPE) was found to be necessary to eliminate the components with long retention times such as vitamin E acetate, vitamin A palmitate and β-carotene. Two LC runs proved to be necessary for reliable quality control of these galenicals. In recent years, several isocratic and gradient LC procedures [4–15] for the analysis of very polar, polar and non-polar retinoids, carotenoids and vitamin E in pharmaceutical and biological samples were reported. However, none of these included the separation of ''retinoid solution'' components. To the best of our knowledge, one report only was published on an LC method for the stability evaluation of all-*trans* retinoic acid present in the ''retinoid solution'' [16].

In the present work, gradient LC with fluorescence and diode-array UV detection were developed for the simultaneous analysis of the ''retinoid solution'' components and the potential impurities, 13-*cis* and 9-*cis* retinoic acids, in galenical samples from different sources.

Chromatographic separation techniques in combination with selective and sensitive detection methods have gained a notable popularity in all fields of analytical chemistry [17]. Among the derivatization techniques, photoreactions appear to be rapid and the light sources do not exhibit the stability problems of the chemical derivatization reactions in solution [18,19]. In previous studies, we have successfully used a combination of fluorescence detection with post-column on-line photochemical derivatization to enhance the selectivity in the LC analysis of oestrogens [20,21]. In the present work, this approach was extended to the analysis of ''retinoid solution'' components. The LC effluent was subjected to UV irradiation (λ=254 and 366 nm) and photo-induced alterations resulted in modified spectral properties of the analytes.

2. Experimental

2.1. Chemicals

All-*trans* retinoic acid (tretinoin), 13-*cis* retinoic acid (*iso*-tretinoin), 9-*cis* retinoic acid, vitamin A palmitate, vitamin E acetate and β-carotene were obtained from Sigma (St. Louis, MO, USA). Acetonitrile, methanol, ethanol and tetrahydrofuran for chromatography (HPLC grade) were from Romil (Delchimica Scientific Glassware, Naples, Italy) and double distilled water was used. Other chemicals were from Carlo Erba Reagent (Italy).

2.2. Equipment

The liquid chromatograph comprised a Jasco Model LG-980-02 ternary gradient unit, a Jasco PU-1580 pump and a Jasco FP-920 fluorescence detector with a programmable variable wavelength, connected to a personal computer AcerView 34TL. The integration program Borwin was used. A second liquid chromatograph consisted of Varian Model 5020 chromatograph and a photometric diode-array detector (HP 1040A) connected to an HP 79994A workstation. 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). A Beam Boost Model C6808 photoreactor (ICT, Frankfurt, Germany) was inserted on-line between the analytical column and the fluorescence detector. The eluate was irradiated on-line in capillary PTFE tubing (20 m×0.3 mm I.D.) in a crocheted geometry by an 8 W low-pressure mercury lamp with the main spectral emission at 254 and 366 nm.

2.3. Solutions

Stock solutions of all-*trans*, 13-*cis* and 9-*cis* retinoic acids were prepared in methanol, vitamin A palmitate in methanol–tetrahydrofuran (70:30, v/v) and β-carotene in tetrahydrofuran. These solutions were appropriately diluted to give standard solutions in methanol–tetrahydrofuran (84:16, v/v) (concentration under calibration graphs) and were stable for at least 2–3 days at 4°C. Ammonium acetate (10 m*M*), used in the mobile phase, was prepared dissolving about 770 mg of ammonium acetate to 1000 ml of water.

2.4. Chromatographic conditions

Routine chromatographic separations were performed at ambient temperature on a Phenomenex Luna 3μ C_{18} column (150×4.6 mm I.D.) under gradient elution conditions using a mobile phase consisting of a mixture of A:B, where A is methanol–10 m*M* ammonium acetate (75:25, v/v) and B is methanol–tetrahydrofuran (84:16, v/v) at a flow-rate of 0.8 ml/min. The gradient profile adopted was: $t=0$ min, 0% B; $t=25$ min, 0% B; $t=35$ min, 100% B; $t=45$ min, 100% B; $t=50$ min, 0% B. The LC analysis of the ''retinoid solution'' components, 13-*cis* and 9-*cis* retinoic acids with diode-array detector (DAD) was carried out at $\lambda=350$ nm, while using fluorescence detection was selected a programmable variable wavelength ($t=0$ min $\lambda_{em}=520$ nm with $\lambda_{ex}=350$ nm for retinoic acid isomers and vitamin A palmitate; $t=48.5$ min $\lambda_{em}=520$ nm with $\lambda_{ex}=450$ nm for β-carotene).

The LC separations of ''retinoid solution'' components under isocratic reversed-phase conditions were carried out at 32±2°C on a Phenomenex Prodigy 5ODS$_3$ column (250×3.2 mm I.D.) with a mobile phase consisting of acetonitrile–ethanol–tetrahydrofuran (73:25:2, v/v/v) at a flow-rate of 0.5 ml/min selecting a programmable variable wavelength ($t=0$ min, $\lambda_{em}=520$ nm with $\lambda_{ex}=350$ nm for retinoic acid; $t=15$ min, $\lambda_{em}=330$ nm with $\lambda_{ex}=296$ nm for vitamin E acetate; $t=30$ min, $\lambda_{em}=520$ nm with $\lambda_{ex}=450$ nm for β-carotene; $t=38$ min, $\lambda_{em}=520$ nm with $\lambda_{ex}=350$ nm for vitamin A palmitate) with gain 1000.

2.5. Calibration graphs

Standard solutions of β-carotene, vitamin A palmitate, all-*trans*, 13-*cis* and 9-*cis* retinoic acids were prepared in the mixture of methanol–tetrahydrofuran (84:16, v/v) (concentration ranges in Table 1). Triplicate injections for each standard solution were made and the peak-area was plotted against the corresponding concentration to obtain the calibration graphs.

2.6. Analysis of ''retinoid solution''

About 100 mg amount of a commercial sample were diluted to 5 ml with methanol–tetrahydrofuran (84:16, v/v). The solution was filtered with a 0.45-μm nylon, 25-mm filter and analysed by LC as was a standard solution containing all-*trans* retinoic acid (10 μg/ml), vitamin A palmitate (10 μg/ml), β-carotene (40 μg/ml), 13-*cis* retinoic acid (0.40 μg/ml) and 9-*cis* retinoic acid (0.40 μg/ml).

Table 1
Data for calibration graphs[a] and limits of detection ($S/N=3$) of retinoids and β-carotene

Drug	Detection	Correlation coefficient	Concentration range (μg/ml)	Detection limit (pmol)	λ $\lambda_{ex}/\lambda_{em}$ (nm)
13 *cis*-retinoic acid	UV–DAD	0.9999	0.32–6.4	12	350
	Fl[b]	0.9999	0.15–5.9	7	350/520
9 *cis*-retinoic acid	UV–DAD	0.9997	0.28–5.6	11	350
	Fl	0.9993	0.20–5.0	7	350/520
trans-retinoic acid	UV–DAD	0.9999	1.1–28	11	350
	Fl	1.0000	1.1–28	5	350/520
vitamin A palmitate	UV–DAD	1.0000	3.1–61	15	350
	Fl	1.0000	1.0–25	1	350/520
β-carotene	UV–DAD	0.9999	4.0–64	5	450
	Fl	0.9997	3.7–74	4	450/520

[a] Each data point ($n=6$) was generated from three injections.
[b] Fl, fluorimetric detection.

3. Results and discussion

3.1. Chromatography and detection

In order to improve a previously published HPLC fluorimetric method [2], in the present study a versatile liquid chromatographic system for the analysis of ''retinoid solution'' components, 13-*cis* and 9-*cis*-retinoic acids was developed. A representative separation of the ''retinoid solution'' components and retinoic acid isomers is shown in Fig. 1A.

For an unambiguous identification of the components post-column on-line photochemical derivatization with fluorescence detection was used; the column effluent was irradiated using two lamps with emission at 254 and 366 nm, respectively. Because of the photoderivatization, the chromatograms obtained with and without UV irradiation display different changes in the peak height (Figs. 1B and 2, respectively). The fluorescence intensity of the vitamin E acetate increases significatively after irradiation at 254 nm under isocratic conditions (Fig. 2A), while the peak is not detected under gradient conditions (Fig. 1B) at λ_{em}=330 nm with λ_{ex}=296 nm due to the interferences of the mobile phase. After UV irradiation at 366 nm under gradient elution (Fig. 1B) and isocratic (Fig. 2B) conditions an increasing and a reduction of the signal of vitamin A palmitate was observed, respectively. The emission (Fig. 3) as the excitation spectra, recorded on line with and without UV irradiation, displayed significant profile modifications, confirming the chromatographic behaviour.

The reproducibility of the photochemical derivatization reaction was satisfactory as indicated by the relative standard deviations (R.S.D. range 1.2–3.2%) of the peak-area obtained from replicate (n=5) analyses with photoreactor on (254 and 366 nm) of a single standard solution of *trans* retinoic acid (5.8 μg/ml), β-carotene (30 μg/ml), vitamin A palmitate (5.7 μg/ml) and vitamin E acetate (4.6 μg/ml).

3.2. Analysis of ''retinoid solution''

Eight formulations of ''retinoid solution'', a viscose mixture of vitamins prepared according to the ''Di Bella'' protocol, were purchased from different sources and stored at 4°C, in the dark for about 4–6 days.

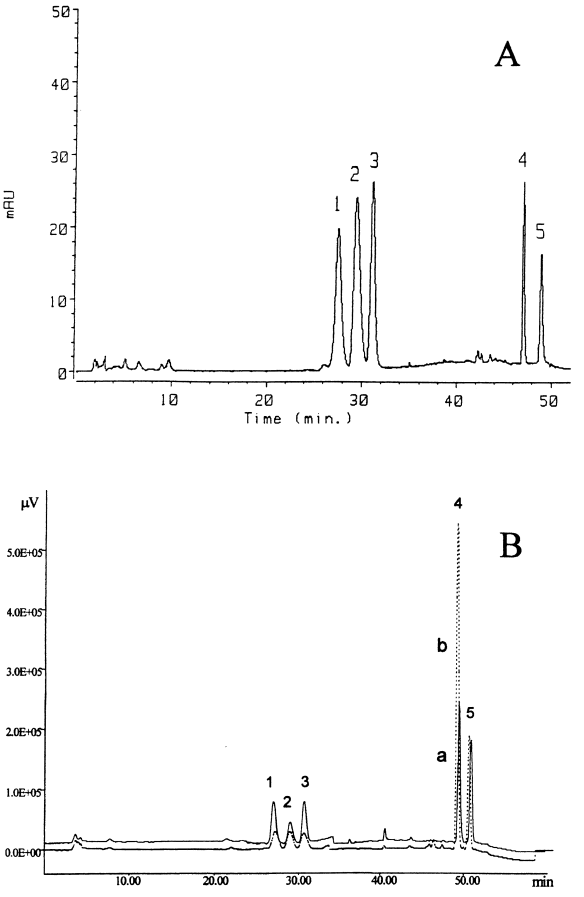

Fig. 1. Gradient LC separation of the ''retinoid solution'' components and retinoic acid isomers by (A) UV-DAD detection (λ=350 nm) and (B) fluorescence detection with on-line photoreactor switched (a) off and (b) on with irradiation at 366 nm. LC conditions: Phenomenex Luna 3μ C_{18} column (150×4.6 mm I.D.), with a mixture of A:B, where A is methanol–10 m*M* ammonium acetate (75:25, v/v) and B is methanol–tetrahydrofuran (84:16, v/v) as mobile phase; flow-rate, 0.8 ml/min. Peaks: 1=13-*cis* retinoic acid; 2=9-*cis* retinoic acid; 3=all-*trans* retinoic acid; 4=vitamin A palmitate; 5=β-carotene.

Routine analyses of the samples were performed by direct UV–DAD and fluorescence detection without photochemical derivatization. To this end, a linear relationship between the peak-area (y) and the analyte concentration was obtained for each compound (Table 1). Table 1 reports the limits of detection expressed in picomoles and evaluated as the concentration of compound that resulted in a

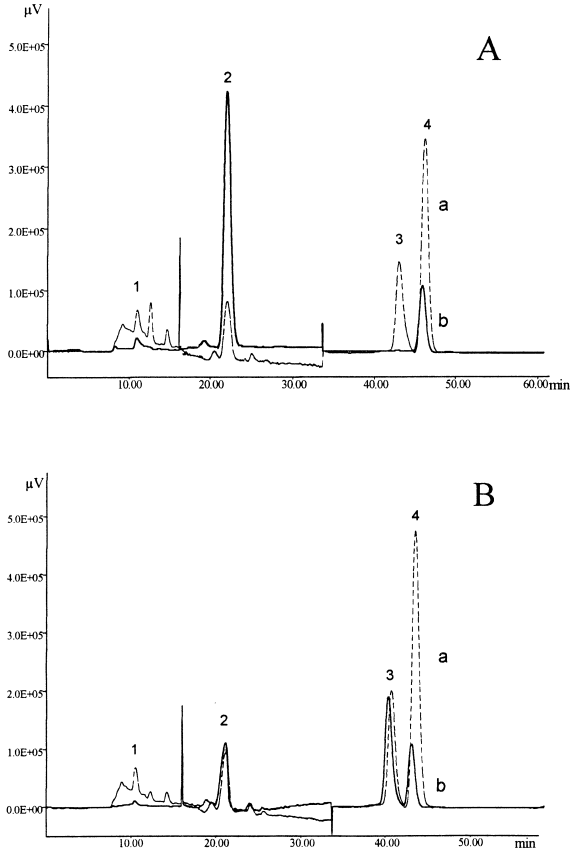

Fig. 2. LC chromatogram with fluorescence detection, obtained at 32±2°C, of ''retinoid solution'' components with on-line photoreactor switched (a) off and (b) on with irradiation at (A) 254 and (B) 366 nm. LC conditions: Phenomenex Prodigy $5ODS_3$ (250× 3.2 mm I.D.) column and acetonitrile–ethanol–tetrahydrofuran (73:25:2, v/v/v) at a flow-rate of 0.5 ml/min. Peaks: 1=all-*trans* retinoic acid; 2=vitamin E acetate; 3=β-carotene; 4=vitamin A palmitate.

peak height of three times the noise level ($S/N=3$). Six replicate injections for the same standard solution (drug concentrations as indicated in Section 2.6.: ''Analysis of retinoid solution'') were carried out to determine the precision of response. The repeatability of the peak-area, expressed as R.S.D., was between 0.48 and 3.2% and between 0.57 and 3.1% for UV–DAD and fluorescence detection, respectively. The mobile phase proved to be suitable for sample dissolution and LC analysis of the all-*trans*, 13-*cis*, 9-*cis* retinoic acids, vitamin A palmitate and β-carotene. It is not considered necessary to determine

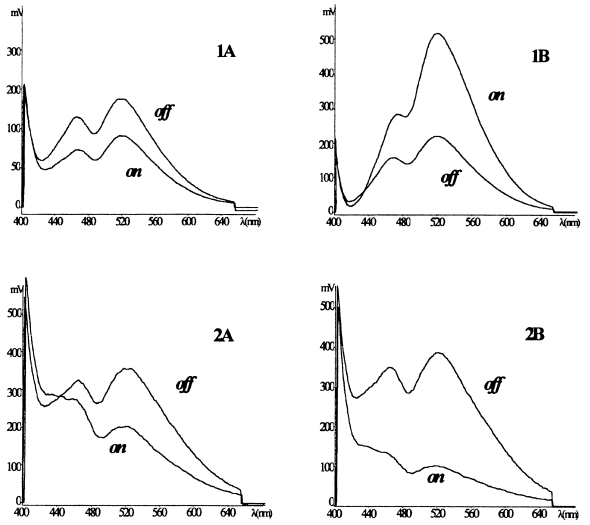

Fig. 3. Emission spectra of vitamin A palmitate, with on-line photoreactor switched off and on, obtained under gradient (1A and 1B) and isocratic (2A and 2B) elution. Fluorescence detection. Irradiation at (A) 254 nm and (B) 366 nm.

the vitamin E acetate, owing to the fact it is present in the formulation in remarkable amounts in respect to other ingredients. In general, the drug concentration found in each galenical agreed with the claimed content (Table 2), exhibiting good between-run precision (reproducibility) (R.S.D.=0.26–4.6%); three samples only presented low values of vitamin A palmitate and β-carotene. As regards the determination of impurities, two samples did not contain *cis* isomers; in others the 13-*cis* was found to be the main isomer (1.55÷13.2%) while 9-*cis* reti-

Table 2
Results of HPLC analysis of retinoids and β-carotene in eight samples[a] of ''retinoid solution''

Compound	Found (UV–DAD)	Found (Fl)
13-*cis* retinoic acid[b]	0.00–13.2	0.00–12.7
9-*cis* retinoic acid[b]	0.00–4.03	0.00–4.55
all *trans*-retinoic acid[c]	95.3–108	91.3–110
β-carotene[c]	74.4–95.8	73.5–95.7
Vitamin A palmitate[c]	67.9–111	65.3–112

[a] Other ingredients: vitamin E acetate.
[b] Mean of five determinations of impurity and expressed as a percentage of the drug.
[c] Mean of five determinations and expressed as a percentage of the claimed content.

noic acid (1.09÷4.03%) could be considered present at trace levels. The accuracy of the fluorescence and DAD methods was verified by analysing samples fortified with 20% and 50% of the claimed drug content; the recoveries were 98.9–101%. According to USP XXIV [22], the recovery was also determined by assaying samples of matrix (vitamin E acetate) spiked with amount of the analytes (concentration levels at 80–100–120% of the nominal concentration) and comparing the obtained results to those of standard solutions (without matrix). The recoveries ranged from 97.2 to 101%.

In summary, the reported methods proved to be selective, precise, linear and sensitive, being adequate for the simultaneous determination of retinoic acid isomers, vitamin A palmitate and β-carotene and can be conveniently employed for the routine quality control of these drugs. The combination of fluorescence detection with post-column online photoderivatization constitutes a simple and effective approach to enhancing the intrinsic selectivity of the HPLC method.

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