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Short communication

Single-run analysis of retinal isomers, retinol and photooxidation products by high-performance liquid chromatography

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Abstract

A high-performance liquid chromatographic (HPLC) procedure was developed to allow the rapid separation, in a single run, of a mixture of the main retinal isomers (all-*trans*, 13-*cis*, 9-*cis*), all-*trans*-retinol, and of the two major photooxygenated photoproducts (5,8-peroxyretinal and 5,6-epoxyretinal). The mixture was separated by HPLC on an octadecyl (ODS) column with 16% (v/v) diethyl ether in hexane as mobile phase and anthracene as the internal standard. A commercial type cosmetic formulation containing 0.05% all-*trans*-retinal was analyzed successfully for this analyte. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

Retinoids play an important role in a great range of biological processes: such as vision [1,2], differentiation of epithelial tissues [3–5]. They are used in oncology [6], dermatology [7] and recently in cosmetology [8].

The photoreactivity of retinal has been extensively studied. Photoisomerisations [9,10] and photooxidations [11–13] have been reported. So the photoreactivity of retinoids in skin must be considered. This led us to undertake a study of the photochemistry of retinoids in organized media which can be considered as simple membrane models.

The study required a technique allowing the identification and quantitative analysis of the photoproducts. High-performance liquid chromatography (HPLC) methods have been widely used in studies concerning retinoids [14]. Normal-phase chromatography offers good resolution of retinal isomers [15] and all-*trans*-retinol [16]. In order to overcome the problems arising from contaminating detergent, reversed-phase HPLC has been used to separate retinal isomers, but the resolution was not as good as that obtained with normal-phase HPLC. Pepe and Schwemer [17] used an ODS column with 16% (v/v) diethyl ether in hexane as mobile phase, giving a satisfactory separation of retinal isomers. We intended to determine whether this method could be used to separate, in a single run, mixtures of retinoids containing both closely related compounds that differ only by configuration and compounds that differ markedly in polarity: retinal and its isomers, all-*trans*-retinol, 5,8-peroxyretinal and 5,6-epoxyreti-

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nal. A quantitative evaluation of all-*trans*-retinal is also described.

2. Experimental

2.1. Instrumentation and equipment

HPLC was performed using a system consisting of a Millipore Waters unit (Model 510) with an automatic gradient controller and a Waters 991 UV–Visible photodiode array detector (Water, Saint-Quentin-en-Yvelines, France). Detection was performed by scanning from 250 to 400 nm. The detection wavelength was set at 330 nm where all retinal derivatives absorb. The analyses were carried out on a Spherisorb ODS 1 C_{18} (5 μm, 250×4.6 mm, part No.: 2546 1825, serial No. 031 15B27, Bischoff Chromatography, Leonberg, Germany) reversed-phase column eluted with 16% (v/v) diethyl ether in hexane. The flow-rate was 1 ml/min. All procedures were carried out in subdued light.

UV spectra were recorded on a Hewlett-Packard 8452A spectrophotometer (Waldbronn, Germany). ^{1}H Nuclear magnetic resonance (NMR) spectra were recorded in deuteroacetonitrile on a Brucker 400 MHz apparatus Wissembourg, France).

A LiChrospher RP18 (5 μm, 125×4 mm, Bischoff Chromatography) reversed-phase column was used for the preparative separation of photooxidation products of retinal using 1% ammonium acetate–acetonitrile (1:4, v/v) as eluent. Photooxidation reactions were carried out in a Rayonet-type reactor (The Southern New England Ultraviolet Company, Hamden, USA) at 350 nm using 10-ml Pyrex tubes on a rotating rack.

2.2. Chemicals and reagents

Standards of all-*trans*-, 13-*cis*- and 9-*cis*-retinal, all-*trans*-retinol and anthracene were purchased from Sigma (Saint-Quentin Fallavier, France). Hexane (Prolabo, Gradignan, France), diethyl ether (Carlo Erba Reagent, Nanterre, France), acetonitrile and dioxane (Merck–Clevenot, Salon de Provence, France) were HPLC grade. Millipore-filtered (5 μm), double-distilled water was also used.

Retinal was photooxidized by irradiation of oxygenated hexane solutions. The progress of photooxidation was followed by UV spectrophotometry. The all-*trans*-retinal absorption at 368 nm decreased whereas the 5,8-peroxyretinal and the 5,6-epoxyretinal absorption appeared at 330 nm and 365 nm, respectively. The photooxidation products were separated by preparative HPLC and identified by ^{1}H NMR on comparison of the chemical shifts with those reported by Baron et al. [13] and Ito et al. [18].

2.3. Sample preparation

All solutions were stored in brown vessels under an argon atmosphere.

Internal standard solution: in a 20-ml volumetric flask, 0.0313±0.0001 g of anthracene was dissolved with hexane.

All-*trans*-retinal standard solution: in a 100-ml brown volumetric flask, 0.0142±0.0001 g of all-*trans*-retinal was dissolved with hexane.

Qualitative analysis: the concentration of all solutions was about 10^{-3} *M* except for the solutions of 5,8-peroxyretinal and the 5,6-epoxyretinal in which the concentration was unknown. We injected 5 μl of each solution three times.

Calibration curve solutions: several volumes (100, 200, 300, 500 μl) of all-*trans*-retinal standard solution were added to 100 μl of the internal standard solution and the volume completed to 10 ml with hexane in order to obtain concentrations (from $4.992 \cdot 10^{-5}$ *M* to $2.496 \cdot 10^{-5}$ *M*). One hundred μl of each sample was then run through the entire system under the conditions described above.

Assay 1: in a 100-ml volumetric flask, 0.0162±0.0001 g of all-*trans*-retinal was dissolved with hexane. A 350-μl volume of this solution was mixed to 50 μl of each of the sample solution of 9-*cis*-retinal, 13-*cis*-retinal, 5,8-peroxyretinal and 5,6-epoxyretinal, then 100 μl of the internal standard solution were added and the volume completed to 10 ml with hexane. A 100-μl sample of this solution was injected three times.

Assay 2: in a 50-ml volumetric flask, 0.150 g of a freshly prepared commercial cosmetic formulation (Pierre Fabre Dermo-Cosmétique) containing 0.05% (w/w) retinal was mixed with 1.5 ml of 1,4-dioxane, after stirring, 0.2 ml of isopropanol was added, the volume made up to 50 ml with hexane. A 10-μl

volume of the internal standard solution was added to 990 μl of the above solution. A 100-μl aliquot of this mixture was injected three times.

3. Results and discussion

At the present time, no HPLC studies have been carried out with the intention of separating, in a single run, the main geometric isomers (all-*trans*, 13-*cis*, 9-*cis*) of retinal, all-*trans*-retinol, the products of photooxidation and a compound used as internal standard.

As synthetic retinoids are not stable enough to be used as internal standards, β-ionone, nitrobenzaldehyde, naphthalene and anthracene were tested and anthracene gave the best results.

A solution containing a mixture of anthracene and of the various retinoids studied was injected under the conditions described above. The chromatogram shown in Fig. 1 demonstrates the separation of retinal isomers (all-*trans*, 9-*cis*, 13-*cis*), all-*trans*-retinol, 5,8-peroxyretinal, 5,6-epoxyretinal and anthracene. The retention times were 3.26±0.01, 4.44±0.11, 5.02±0.08, 6.48±0.02, 7.60±0.02, 8.06±0.02, 8.98±0.04, 9.78±0.03, 10.42±0.02,

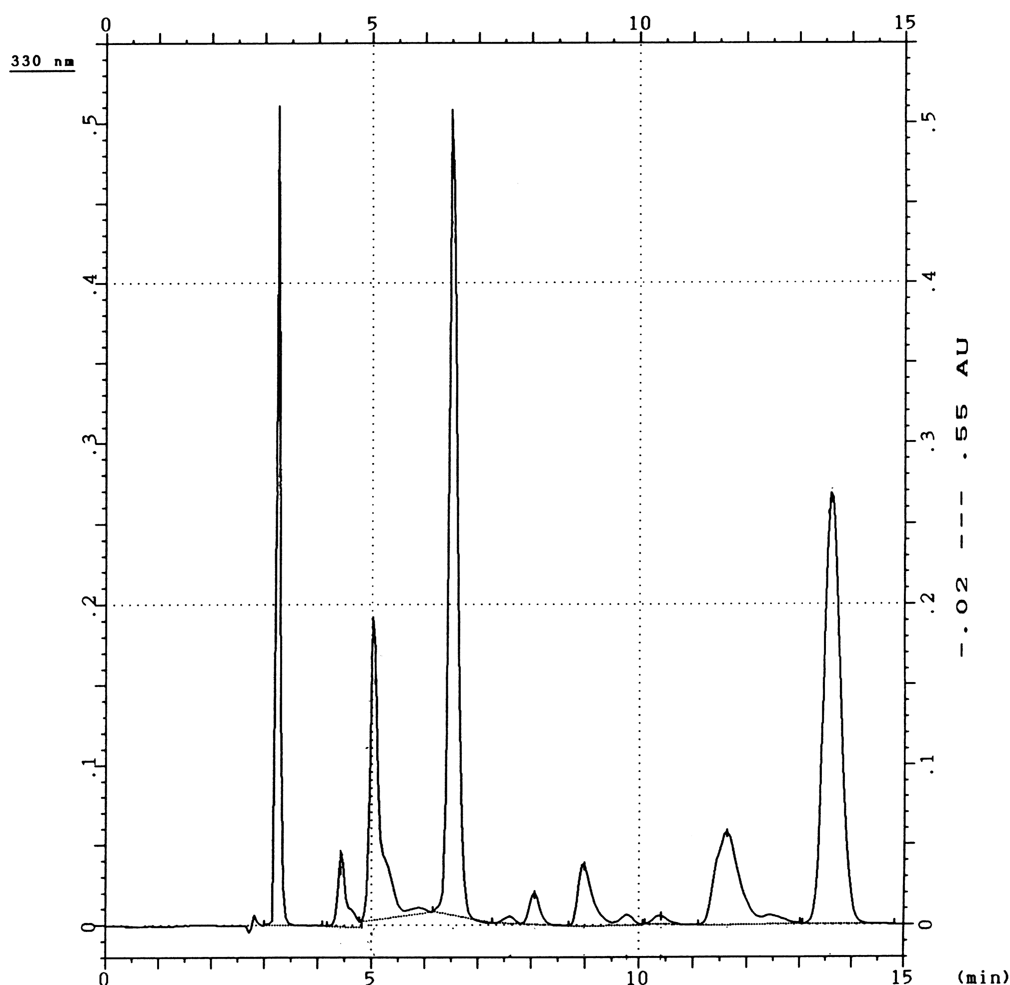

Fig. 1. HPLC separation of retinal isomers (all-*trans*, 9-*cis*, 13-*cis*), all-*trans*-retinol, 5,8-peroxyretinal isomers, 5,6-epoxyretinal isomers and anthracene. Column, Spherisorb ODS 1 C_{18} (5 μm, 250×4.6 mm); detection, absorption at 330 nm; mobile phase, hexane–diethyl ether (84:16); flow-rate, 1 ml/min; injection, 5 μl. Values on the *y*-axis are absorbance units.

11.64±0.05, 13.62±0.01 min for anthracene, 13-*cis*-retinal, 9-*cis*-retinal, all-*trans*-retinal, unidentified oxidation product, one 5,8-peroxyretinal isomer, one 5,6-epoxyretinal isomer, unidentified oxidation product, unidentified oxidation product, one 5,8-peroxyretinal isomer, all-*trans*-retinol, respectively.

A calibration curve of all-*trans*-retinal was established for a concentration range of $5 \cdot 10^{-6}$ *M* to $2.5 \cdot 10^{-5}$ *M*. We made triplicate injections of 100 μl of each solution. The detection wavelength selected was 360 nm. For each concentration, the average of ratios of all-*trans*-retinal (A_{ret}) peak area to anthracene peak area ($A_{I.S.}$), $R_S = \langle A_{ret}/A_{I.S.} \rangle$, was calculated. Linear regression was performed by least squares analysis R_S versus mass ratio. The relation obtained was: $y = 6.681x - 0.0185$ ($r = 0.995$).

The precision of the method was assessed by the coefficients relative standard deviation (R.S.D.) and the degree of repeatability of R_S in a series of ten injections during a single session. A good repeatability of the retention times for all-*trans*-retinal $t_R = 6.35 \pm 0.02$ min and anthracene $t_R = 3.26 \pm 0.01$ min was observed. The coefficients of variation for the retention times were respectively: 0.31% for all-*trans*-retinal and 0.37% for anthracene. The precision of the method is estimated at 1.03%.

The quantitative analysis of all-*trans*-retinal was tested with an artificial hexane solution containing a precise amount of all-*trans*-retinal determined by weighing (0.0162±0.0001 g in 100 ml) and approximate quantities of his major photoproducts. The quantity of all-*trans*-retinal determined by HPLC was 0.01605±0.00017 g (value±S.D. for four measures).

This HPLC method was then tested using a commercial type formulation containing 0.05% (w/w) retinal and several compounds including surfactants. The formulation was worked up as indicated in Section 2.3 and the quantity of retinal determined by HPLC was (97±2)% of the theoretical value.

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