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SEPARATION AND IDENTIFICATION OF RETINOIC ACID PHOTOISOMERS

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SUMMARY

Retinoic acid was isomerized in ethanol–water (90:10) with fluorescent light. Reversed-phase high-performance liquid chromatography (HPLC) on a 3-μm ODS-2 column with a highly specific mobile phase allowed simultaneous determination of ten retinoic acid isomers that were produced during the photoisomerization. Nine of the isomers were isolated by HPLC and characterized by spectroscopic methods (^{1}H NMR, mass spectrometry and UV). The variation of product distribution with time was determined over the course of the reaction (21 h).

INTRODUCTION

Retinoic acid [**1**, (all-*E*)-3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4,6,8-nonatetraenoic acid, tretinoin] and its analogues (retinoids) are an important class of biomolecules. Retinoids are involved in many biological functions including such processes as epithelial cell growth and differentiation[1] and vision[2]. Retinoids are also used therapeutically for the treatment of acne[3] and photo-damaged skin[4] and have shown potential use as cancer chemotherapeutic agents[5].

In general, retinoids are know to isomerize by chemical methods, with heat, and, most importantly, by the action of light[6,7]. In particular, the photoisomerization of retinoids plays an important role in vision (11-*cis*-retinal isomerization in retinyl opsins) and in some bacterial proton transport (13-*cis*-retinal isomerization in bacteriorhodopsin)[2]. Due to the importance of retinal in these biological systems, photoisomerization of this molecule has been very well documented in the literature[6–8] along with the analytical methodology for the separation and identification of retinal photoisomers[7,9,10]. However, the photoisomerization of retinoic acid has not been as well documented. Previous literature reports of retinoic acid photoisomerization[11,12] did not contain analytical methodology capable of separating all of the retinoic acid isomers. To overcome this deficiency, the isomer mixtures were derivatized to give the methyl retinoates which were more easily resolved by high-performance liquid chromatography (HPLC). After derivatization, eight isomers of retinoic acid were observ-

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ed and characterized as their methyl esters by McKenzie *et al.*[11] and seven isomers by Curley and Fowble[12]. In both papers, chromatography of the retinoic acid mixtures themselves gave poor resolution with much peak overlap. This paper presents an improved reversed-phase method for the separation of retinoic acid isomers, the identification of these isomers by isolation and spectroscopic characterization and an analysis of the time-dependence of photoisomerization.

EXPERIMENTAL

Retinoic acid and 13-*cis*-retinoic acid were purchased from Eastman Kodak (Rochester NY, U.S.A.) and used without further purification. Acetonitrile, methanol and isopropanol were HPLC grade and purchased from Burdick and Jackson (Muskegon, MI, U.S.A.). Acetic acid (glacial) was purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Water for HPLC was obtained using a Milli-Q water purification system from Millipore Corporation (Bedford, MA, U.S.A.). Absolute ethanol was purchased from Pharmco (Bayonne, NJ, U.S.A.).

Separations were performed on an Spherisorb 3-μm ODS-2 (150 mm × 4.6 mm) HPLC column (Alltech, Deerfield, IL, U.S.A.). We found that a minimum plate count of 60 000 plates/m, based on the manufacturer's test chromatogram, was necessary to achieve the desired separation. The HPLC system consisted of a Perkin-Elmer (Norwalk, CT, U.S.A.) Series-4 quaternary solvent gradient pumping system equipped with a Hewlett-Packard (Palo Alto, CA, U.S.A.) photodiode array (1040M) detection system, with the detection wavelengths set at 345, 290 and 235 ±2 nm. Integration was performed by the HP photodiode array system at each of the wavelengths. Sample injection was accomplished by using a programmable autosampler (WISP; Water Assoc., Milford, MA, U.S.A.).

Proton NMR spectra were obtained in C^2HCl_3 or [$^2H_6$]acetone on a Varian XL-400 (Varian, Palo Alto, CA, U.S.A.) or a GE QE-300 spectrometer (General Electric, Freemont, CA, U.S.A.). Mass spectra were obtained on a Finnigan MAT 8230 mass spectrometer (Finnigan, San Jose, CA, U.S.A.) by desorption electron impact (DEI) or desorption chemical ionization (DCI). DCI spectra were obtained with isobutane as reagent gas. UV spectra were recorded on the Hewlett-Packard 1040M diode array HPLC detector as the compounds were eluting from the HPLC column.

Photoisomerization was carried out by irradiation of a solution of retinoic acid ($1.67 \cdot 10^{-3}$ *M*) in ethanol–water (90:10). The solution was placed in a quartz vessel and purged with argon. The vessel was then placed within the chamber of a Rayonet photochemical reactor (Southern New England Ultraviolet Co., Hamden, CT, U.S.A.) and irradiated with visible light (cool-white fluorescent F6T5/CW; Philips Lighting, Somerset, NJ, U.S.A.). Samples of the solution were periodically removed and analyzed by HPLC.

RESULTS AND DISCUSSION

Retinoic acid and related retinoids have been shown to isomerize in the presence of light to give a photostationary isomer mixture[6,7,11–14]. Theoretically, each of the double bonds in the chain portion of retinoic acid can undergo isomerization to

give both mono *cis* and multiple *cis* isomers resulting in a total of sixteen double-bond isomers. Eight additional isomers are possible due to cyclization of the 7-*cis* isomers of retinoic acid. Therefore a total of twenty-four photoisomers are possible. Due to steric hindrance, some of these isomers may be unstable at room temperature and thermally isomerize to more stable compounds, as was observed for the isomers of retinal[8,15]. In the present study, nine photoisomers of retinoic acid were isolated and characterized by spectroscopic methods and a tenth unidentified isomer was observed in the reversed-phase chromatogram. The structures of these isomers are shown in Fig. 1.

Reversed-phase HPLC separation of retinoic acid isomers

Separation of the retinoic acid photoisomers was accomplished on a 3-μm reversed-phase HPLC column at a flow-rate of 1.2 ml/min with an isocratic mobile phase consisting of 30% acetonitrile, 25% methanol, 15% isopropanol and 30% water each containing 1.2% (v/v) of acetic acid. This HPLC method was found to give an improved separation of the retinoic acid isomers when compared to literature method[11,12]. Typical chromatograms for the photostationary state mixture after 3 h irradiation and for a long term irradiation mixture (7 days) are shown in Figs. 2 and 3, respectively. Resolution of all eight double-bond isomers that were identified in this study was achieved and a minor product that was assumed to be a 7-*cis* isomer of retinoic acid was observed. Complete resolution of one of the two photocyclized isomers was obtained and selective wavelength monitoring allowed observation of the other photocyclized isomer (which overlapped the double-bond isomers). The photocyclized isomers were found to have a maximum absorption at *ca.* 290 nm and to have almost no absorbance at 345 nm. Therefore, quantitation of all of the retinoic acid double-bond isomers was achieved at 345 nm without interference from the photocyclized compounds.

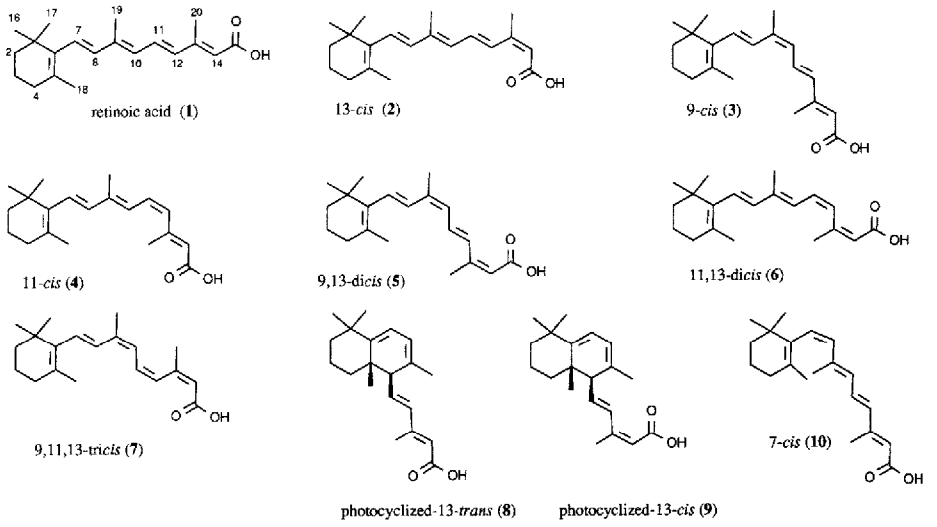

Fig. 1. Retinoic acid isomers observed in this study.

TABLE I

SPECTRAL DATA FOR RETINOIC ACID ISOMERS

Compound	*UV*[a] λ_{max} *(nm)*	*NMR assignments (ppm)*[b]													*Coupling constants (Hz)*[c]		
		16,17-CH_3	*18-CH_3*	*19-CH_3*	*20-CH_3*	*2-CH_2*	*3-CH_2*	*4-CH_2*	*7-H*	*8-H*	*10-H*	*11-H*	*12-H*	*14-H*	$J_{7,8}$	$J_{10,11}$	$J_{11,12}$
1	357	1.032	1.716	2.010	2.367	1.471	1.619	2.025	6.299	6.147	6.157	7.046	6.313	5.798	16.0	11.4	15.0
2	360	1.035	1.719	2.004	2.102	1.472	1.622	2.030	6.29[j]	6.18[j]	6.264	7.026	7.737	5.655	16.1	11.5	15.3
3	352	1.044	1.751	2.008	2.349	1.489	1.644	2.053	6.293	6.651	6.064	7.128	6.248	5.797	16.0	11.5	15.0
4	347	1.027	1.715	1.973	2.357	1.467	1.616	2.018	6.285	6.142	6.533	6.588	5.913	5.897	16.1[g]	13.0[g]	11.2[g]
5[e]	353	1.049	1.752	1.99[d]	2.1[d]	1.496	1.641	2.0[d]	6.347	6.793	6.175	7.207	7.780	5.70	16.0	11.5	15.3
6	350	1.022	1.707	1.964	2.207	1.465	1.613	2.015	6.283	6.121	6.404	6.645	6.966	5.721	16.0	12.6	11.6
7	340	1.031	1.732	1.992	2.199	1.47[f]	1.62[f]	2.031	6.29[j]	6.671[f]	6.29[f]	6.729[f]	6.865	5.724	16.0	11.5[h]	12.0
8	265	1.126[k], 1.141[k]	0.977[k]	1.679	2.328	1.45[f]	1.45[f]	1.45[f]	5.768	5.836	2.795	6.253	6.201	5.7[i]	5.7[g]	10.5[g]	15.3[g]
9	268	1.121[k], 1.136[k]	0.972[k]	1.686	2.059	1.40[f]	1.40[f]	1.40[f]	5.758	5.825	2.889	6.237	7.609	5.660	5.6	10.7	15.7

[a] UV spectra were recorded by the HPLC detector as the peaks eluted from the column, spectral resolution was ±2 nm.

[b] Spectra were recorded at 400 MHz at 25°C for a C^2HCl_3 solution with tetramethylsilane (TMS) as an internal standard, chemical shifts are reported in ppm downfield of TMS with an estimated error of ±0.005 ppm.

[c] Estimated error ±0.3 Hz.

[d] Resonance overlapped with solvent.

[e] Solvent was [$^2H_6$]acetone with TMS as internal standard.

[f] Unresolved or overlapped resonances.

[g] Assignment confirmed by spectral simulation.

[h] Estimated from unresolved resonances.

[i] Estimated error ±0.1 ppm.

[j] Estimated error ±0.01 ppm.

[k] Assignments may be reversed.

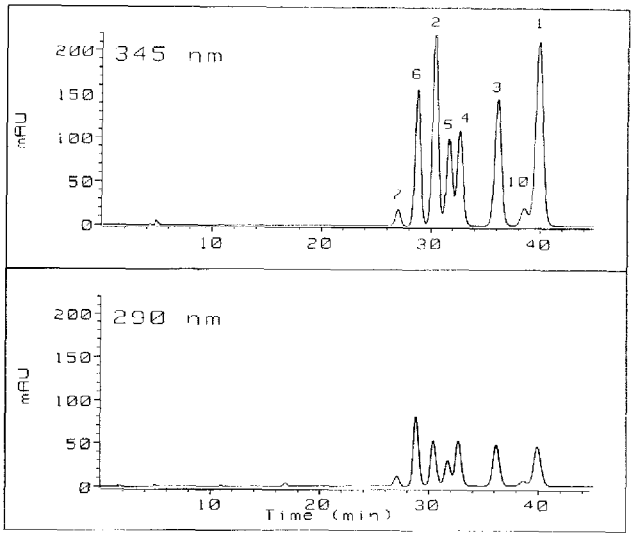

Fig. 2. Reversed-phase HPLC chromatogram of retinoic acid isomer mixture on an Altex Spherisorb ODS-2 3-μm column. The column was eluted with acetonitrile–methanol–isopropanol–water (30:25:15:30) containing 1.2% acetic acid at a flow-rate of 1.2 ml/min. The detection wavelengths were set at 345 and 290 nm. The isomer mixture was produced by irradiation of retinoic acid for 3 h in ethanol–water (90:10) with fluorescent light.

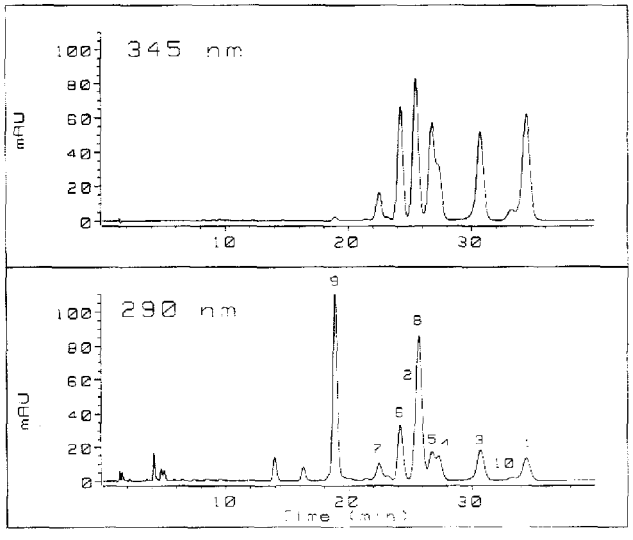

Fig. 3. Reversed-phase HPLC chromatogram of retinoic acid isomer mixture after long term irradiation with fluorescent light (7 days). HPLC conditions as in Fig. 2.

Identification of photoisomers of retinoic acid

Each of the compounds observed in the analytical HPLC chromatogram was isolated by preparative HPLC and its structure determined by a combination of spectral methods that included MS, UV and ^{1}H NMR. ^{1}H NMR spectroscopy, due to its sensitivity to retinoid stereochemistry, has been the method of choice for identifying retinoid isomers[7]. The proton chemical shift assignments and coupling constants and the observed UV maximum for each of the photoisomers are shown in Table I. The spectral data were consistent with those reported in the literature for similar retinoids (retinal[7,16] and methyl retinoate[13]). The stereochemistry of the cyclized isomers was determined by comparison of the NMR spectral data with that for similar compounds reported in literature[13,14,17]. Additionally, a small nOe (nuclear Overhauser enhancement) was observed for the 11-proton upon irradiation of the 18-CH_3 protons in compound **9**. This observation suggests that the 18-CH_3 and the side chain are in a *cis* configuration.

Time-dependence of retinoic acid photoisomerization

The time dependence of retinoic acid photoisomerization ($1.67 \cdot 10^{-3} M$ in 90% ethanol) with visible light was determined by reversed-phase HPLC. The concentrations of the double-bond isomers and the photocyclized isomers were calculated in mole percent based on the observed area counts at 345 and 290 nm, respectively. Calibration curves were obtained for retinoic acid and 13-*cis*-retinoic acid at 345 nm. The concentrations of the remaining double-bond isomers were based on the assumption that the responses for these compounds at 345 nm were similar to that of the 13-*cis* isomer. The response for each of the photocyclized isomers was calculated relative to that for the all-*trans* isomer at 290 nm. Table II gives the concentration of

TABLE II

RETINOIC ACID ISOMER CONCENTRATION AFTER 3 h IRRADIATION WITH VISIBLE LIGHT

Obtained by irradiation of a $1.67 \cdot 10^{-3} M$ solution of retinoic acid in ethanol–water (90:10).

Compound	*Mole-%*[a]
1	18
2	19
3	16
4	10
5	13
6	15
7	3.7
8	1.7
9	1.7[b]
10	2.5

[a] The concentrations of the double-bond isomers (**1**–**7**,**10**) were calculated in mole % based on the observed area counts at 345 nm. Calibration curves were obtained for **1** and **2** with the concentrations of the remaining double-bond isomers based on the assumption that the responses for these compounds at 345 nm were similar to **2**. The concentration for the cyclized compounds **8** and **9** were based on area counts at 290 nm and a response calculated for a pure sample **8** relative to a pure sample of **1**.

[b] The percentage of **9** was approximated due to some peak overlap.

each of the photoisomers present in the retinoic acid solution at the photostationary state.

When retinoic acid was irradiated with visible light at room temperature under an argon atmosphere it quickly isomerized to give a mixture of ten isomers. The mole-percent of each of the isomers is shown in Fig. 4. Within the first 10 min of irradiation all of the double-bond isomers were formed and observed by HPLC. Initially, the 13-*cis* and the 11-*cis* isomers increased rapidly to a point (about 30 min irradiation) where they each made up about 18% of the product mixture. After that time, the remaining isomers increased and the 11-*cis* isomer decreased. The eight double-bond isomers reached a photostationary state mixture after about 3 h of irradiation. This mixture remained essentially unchanged over the next few hours of irradiation. The only subsequent change observed was an increase in the two photocyclized isomers and a concurrent decrease in all the double-bond isomers. After prolonged irradiation, the photocyclized isomers were the two major compounds remaining in the product mixture (Fig. 3). A similar observation has been reported by Halley and Nelson[14] for the methyl ester of retinoic acid. In the cited paper and in a previous paper[13], the authors concluded that the photocyclized isomers are formed by cyclization of the 7-*cis* and 7,13-di*cis* isomers of methyl retinoate. This conclusion was also supported by studies with other similar polyenes[17]. None of the 7-*cis* isomers were isolated in this work. These isomers may be present in the mixture at very low levels, and lead to the formation of the cyclized products. The low level peak (**10**) seen in the reversed-phase chromatogram is believed to be a 7-*cis* isomer. Peak **10** elutes just before the retinoic acid peak and has a UV maximum at 345 nm consistent with a double-bond isomer of retinoic acid.

The HPLC method shows the power of using small particle size colums for the analysis of closely related molecules. This high resolution column and the use of the

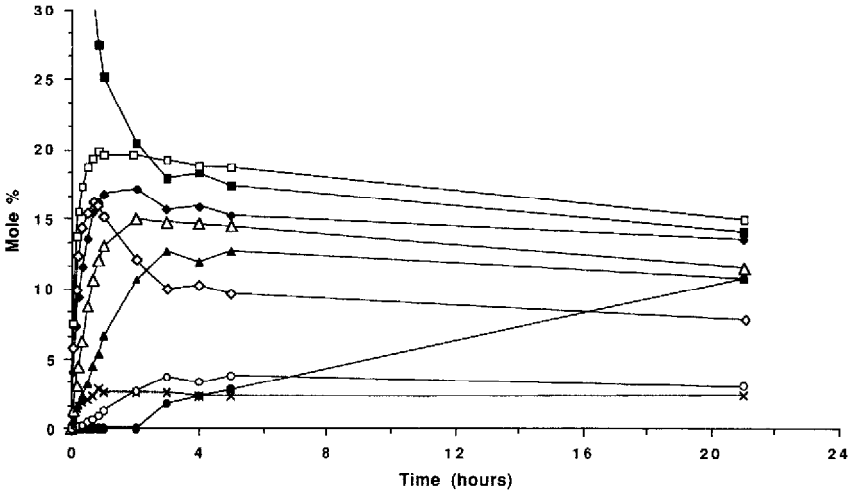

Fig. 4. Time dependence of retinoic acid photoisomerization over 21 h. Retinoic acid ($1.67 \cdot 10^{-3} M$) in ethanol–water (90:10) irradiated with cool-white fluorescent light. ■, retinoic acid; □, 13-*cis*; ◆, 9-*cis*; ◇, 11-*cis*; ▲, 9,13-di*cis*; △, 11,13-di*cis*, ○, 9,11,13-tri*cis*; ●, cyclized isomers (*cis* and *trans*); ×, 7-*cis* (tentative assigment).

specified mobile phase has enabled us to obtain complete separation of the ten isomers observed in this study. We found that the mobile phase selection was extremely important, as small variations in composition caused considerable loss of resolution as well as changes in elution order. In our studies we found that increasing the percentage of acetonitrile relative to the percentage of methanol caused a reversal in the elution order of peaks associated with compounds **4** and **5** and resulted in deteriorated resolution. However, this modified mobile phase gave higher resolution between the peak for retinoic acid and that of compound **10**. The selectivity achieved by small variations in mobile phase can be used as an advantage during the isolation of compounds by HPLC and was employed to help with the isolation of pure retinoic acid isomers.

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