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Normal phase LC–MS determination of retinoic acid degradation products

DANIEL K. BEMPONG,† IRWIN L. HONIGBERG*† and NOEL M. MELTZER‡

†*College of Pharmacy, University of Georgia, Athens, GA 30605, USA*
‡*Astro-Merck Group, Wayne, PA, formerly Hoffman-La Roche, Nutley, NJ 07011, USA*

Abstract: The degradation products formed when 13-*cis* retinoic acid (13-*cis* RA) and all-*trans* RA were exposed to fluorescent light and air were investigated. These retinoids are known to undergo *Z-E* isomerization (due to the existence of four unsaturated double bonds) and oxidation when exposed to light and air. Analysis by LC was carried out on a 25 cm × 4.6 mm Zorbax Rx-SIL (5 μm) with a mobile phase (1.4 ml min^{-1}) of heptane–THF–acetic acid (96.5:3.5:0.015) and an in-line UV (365 nm) detector. The LC eluate was coupled through a Vestec universal interface to a Finnigan 4023 mass spectrometer. EI–mass spectra were obtained at 77 eV from *m/z* 200 to 350 with multiplier voltage of 1200 V. Solid samples of 13-*cis* RA and all-*trans* RA exposed to light and air and also solutions of these retinoids in the mobile phase exposed to the same conditions were used for the analysis. Tentative identities of the degradation products from the mass spectra suggest the isomerization of the retinoids (*Z–E* isomerism) and the formation of the 5,6-epoxides of these isomers. Identities of the 5,6-epoxides were confirmed with chromatographic and mass spectral data from synthetic samples of the epoxides. Isomerization occurred more readily in solution than in the solid form and the 13-*cis* RA isomer oxidized more readily than the all-*trans* isomer.

Keywords: *IC–MS; retinoic acid; degradation; isomerization; autoxidation.*

Introduction

Retinoic acid and other retinoids are an important class of compounds which are involved in the regulation and control of diverse physiological processes. These processes include epithelial cell growth and differentiation [1] and vision [2]. These retinoids have found wide application in the treatment of skin disorders and in cancer prevention and therapy [3, 4]. For example, all-*trans* retinoic acid (tretinoin) is used for local treatment of acne and 13-*cis* retinoic acid (isotretinoin) is administered orally for severe acne [5].

Retinoids are unstable compounds — they are sensitive to heat, oxygen and light. Their stability is, therefore, of pharmaceutical interest and from their molecular structures, the decomposition is expected to be complex [6]. Theoretically, each of the double bonds in the conjugated polyene chain portion (Fig. 1), can undergo isomerization to give both mono-*cis* and multiple *cis* isomers but due to steric hindrance, some of these isomers may not be thermodynamically stable at room temperature and therefore will isomerize to more stable forms [7, 8]. Some of the effects of retinoic acid depends on the geometry of the polyene chain (Fig. 1). For example, all-*trans* retinoic acid and 13-*cis* retinoic acid display differenential effects on mRNA levels of RA receptors [9]. Also there are differences in the receptor binding of the various isomers [10]. Furthermore, some retinoic acids are able to act as teratogens [11], hence efficient separation and determination of degradation products of these retinoids is needed.

There are several reports on the use of LC for the separation of photoisomers of retinoic acid usually after exposing the products in solution to light for a short period of time [6, 7, 10, 12, 13]. We have reported a capillary zone electrophoresis (CZE) and a micellar electrokinetic capillary chromatography (MECC) methods for the separation of the degradation products of retinoic acid [14]. In this paper, the degradation products formed after prolonged exposure of solution and solid samples of all-*trans* retinoic acid and 13-*cis* retinoic acid to light and air have been investigated. Analytical

* Author to whom correspondence should be addressed.

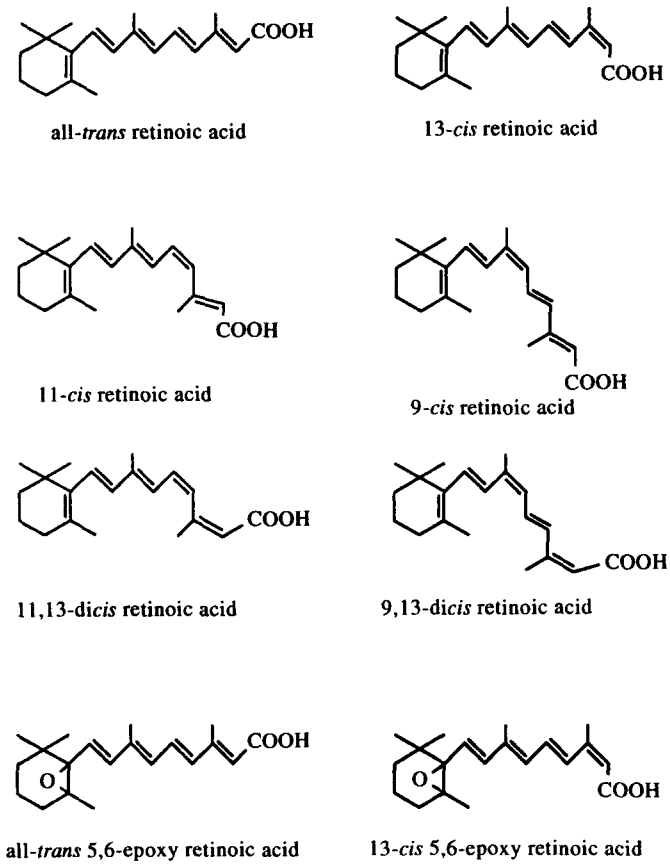

Figure 1
Retinoic acid isomers from photoisomerates [10] and 13-*cis*/all-*trans* 5,6-epoxy retinoic acid.

methodology for the separation and determination of the breakdown products of these retinoids is also reported.

Experimental

Chemicals and reagents

All-*trans* retinoic acid and 13-*cis* retinoic acid were kindly supplied by Hoffmann-La Roche (Nutley, NJ, USA). Heptane was purchased from Baker (Phillipsburg, NJ, USA), THF from Fisher (Fair Lawn, NJ, USA), 3-chloroperoxybenzoic acid was obtained from Aldrich (Milwaukee, WI, USa).

LC–MS System

The LC–MS system consisted of: a Finnigan 4023 mass spectrometer (Finnigan MAT, San Jose, CA, USA) equipped with a particle beam interface — Vestec Universal interface model 700 (Vestec, Houston, TX, USA), Waters LC pump model 510 and U6K injector (Waters, Milford, MA, USA), a 25 cm × 4.6 mm Zorbax Rx-Sil (5 μm) column (Mac-Mod, Chadds Ford, PA, USA), an in-line Beckman UV detector model 160 (Beckman, Berkeley, CA, USA) with detection wavelength of 365 nm and a Hewlett–Packard HP 3390A Integrator (Avondale, PA, USA). The mobile phase consisted of heptane–THF–acetic acid (96.5:3.5:0.015) with flow rate of 1.4 ml min^{-1}. The LC eluate was transferred via the particle beam interface — without splitting, for MS detection. Data was acquired in electron impact mode at 77 eV with scanning from *m/z* 200 to 350 (positive ion) at 1 s per scan. Multiplier voltage and ion source temperature were 1200 V and 300°C, respectively.

Samples

Solutions of all-*trans* RA acid and 13-*cis* RA (1 mg ml^{-1}) in the mobile phase were exposed to light from a fluorescent lamp and air for four weeks at room temperature. Solid samples of these retinoids were also exposed to these conditions for a period of 8 weeks. Solutions of these solid samples and the solutions exposed

to degradation conditions were used in this study.

Synthesis of 5,6-epoxy RA

5,6-Epoxy derivatives of all-*trans* RA and 13-*cis* RA were synthesized according to a literature procedure [15, 17].

Results and Discussion

*Mass spectra of all-*trans *RA and 13-*cis *RA*

EI-Mass spectra of freshly prepared solutions of all-*trans* RA and 13-*cis* RA were recorded using the LC–MS system. The spectra had the features shown in Fig. 2. As expected the two isomers gave similar mass spectra. From Fig. 2, the molecular ions together with fragments arising from the loss of a carboxyl and methyl groups were recorded.

Normal phase HPLC separation of the degradation products

Normal phase HPLC chromatograms of the degradation product mixture obtained from all-*trans* RA and 13-*cis* RA solutions after exposure to air and light with UV detection at 365 nm are shown in Figs 3(i) and 4(i), respectively. From these chromatograms, it is realized that 8–9 breakdown products were resolved (in addition to the original retinoid). The corresponding total ion current chromatograms are shown in Figs 3(ii) and 4(ii).

Normal phase HPLC analysis of solid samples of 13-*cis* RA exposed to similar conditions for a longer period of time yielded only four degradation products as shown in the chromatograms in Fig. 5. Solid all-*trans* retinoic acid yielded fewer peaks. Table 1 also shows that more than 88% of the solid sample remained in the original form (13-*cis* RA) after the prolonged exposure to the degradation conditions. On the other hand, more than 75% of the sample in solution degraded to other forms of the retinoid. The solid sample, therefore, appeared to be more stable under the degradation conditions used in the experiment than solution of the retinoid in the mobile phase. 13-*cis* RA (solution and solid samples) is converted to all-*trans* RA (peak 3) more readily than any other degradation product (Table 1).

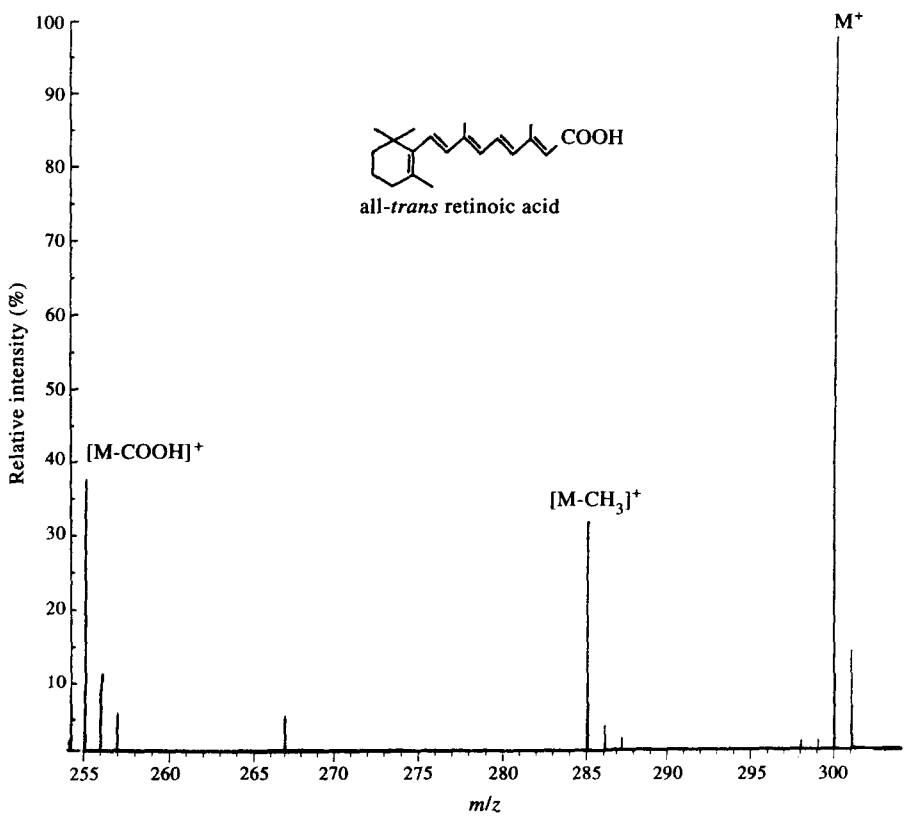

Figure 2
EI–Mass spectra of freshly prepared 13-*cis*/all-*trans* retinoic acid (M^+) — peak 1/peak 3 in Fig. 3.

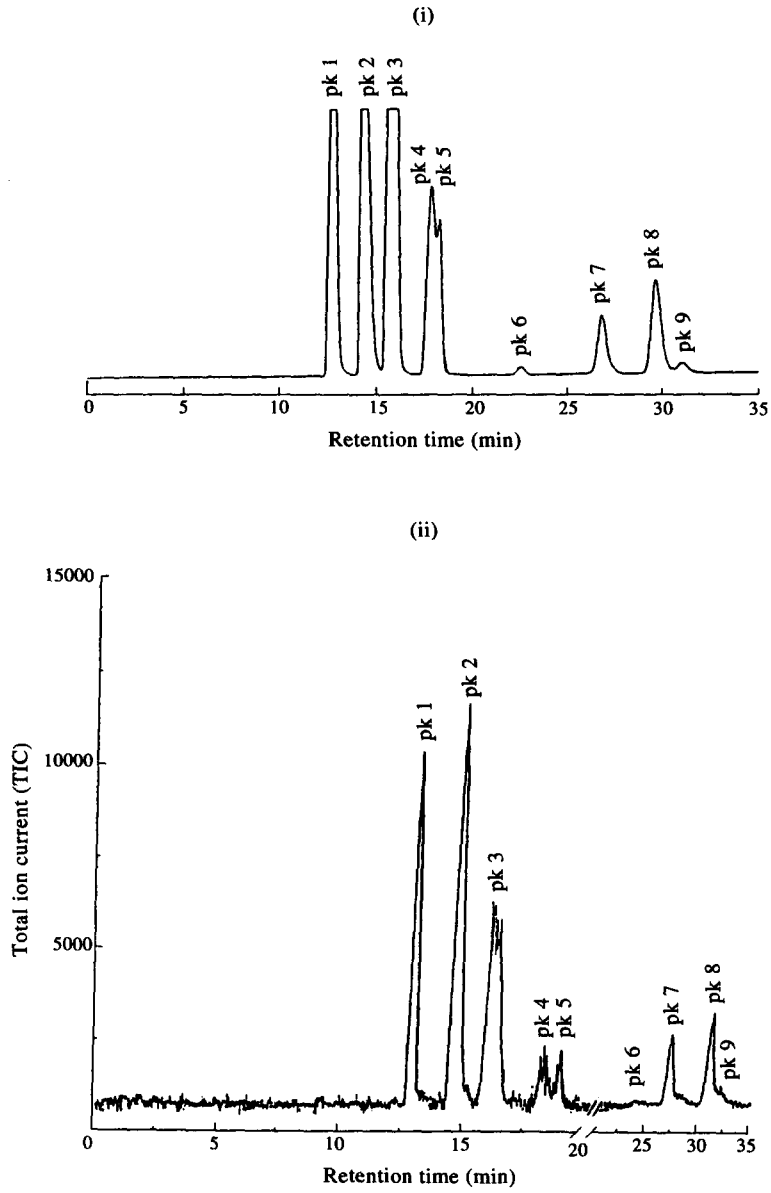

Figure 3
Normal Phase HPLC analysis (with UV detection) of all-*trans* retinoic acid in solution exposed to air and light for 4 weeks (i) and corresponding total ion current chromatogram under EI–MS conditions (ii). Peak 1 — 13-*cis* RA, peak 3 — all-*trans* RA, peaks 2, 4 and 5 — other *e/z* isomers of RA, peak 7 — 13-*cis* 5,6-epoxy RA, peak 8 — all-*trans* 5,6-epoxy RA, peaks 9 and 10 (Fig. 4) — *e/z* isomers of 5,6-epoxyRA.

Cis–trans *isomerization*

EI-Mass spectra of resolved peaks 1–5 in Figs 3(ii) and 4(ii) gave mass spectra which were similar to that obtained for freshly prepared all-*trans* RA or 13-*cis* RA (Fig. 2). This suggests that these are photoisomers of the retinoids since the retinoids are known to isomerize in the presence of light. Double bond isomers reported to have been isolated from photoisomerates of all-*trans* RA are 13-*cis* RA, 11-*cis* RA, 11,13-di*cis* RA, 9-*cis* RA and 9, 13-di*cis* RA [10]. Structures of these retinoids are shown in Fig. 1.

Autoxidation products

Peaks 7 or 8 in the total ion chromatograms (Figs 3(ii) and 4(ii)) gave mass spectra shown in Fig. 6. The molecular ion and its fragments suggest an autoxidation product of RA.

5,6-Epoxy retinoic acid is known to be an autoxidation product of RA [16]. Authentic samples of all-*trans* 5,6-epoxy RA and 13-*cis*

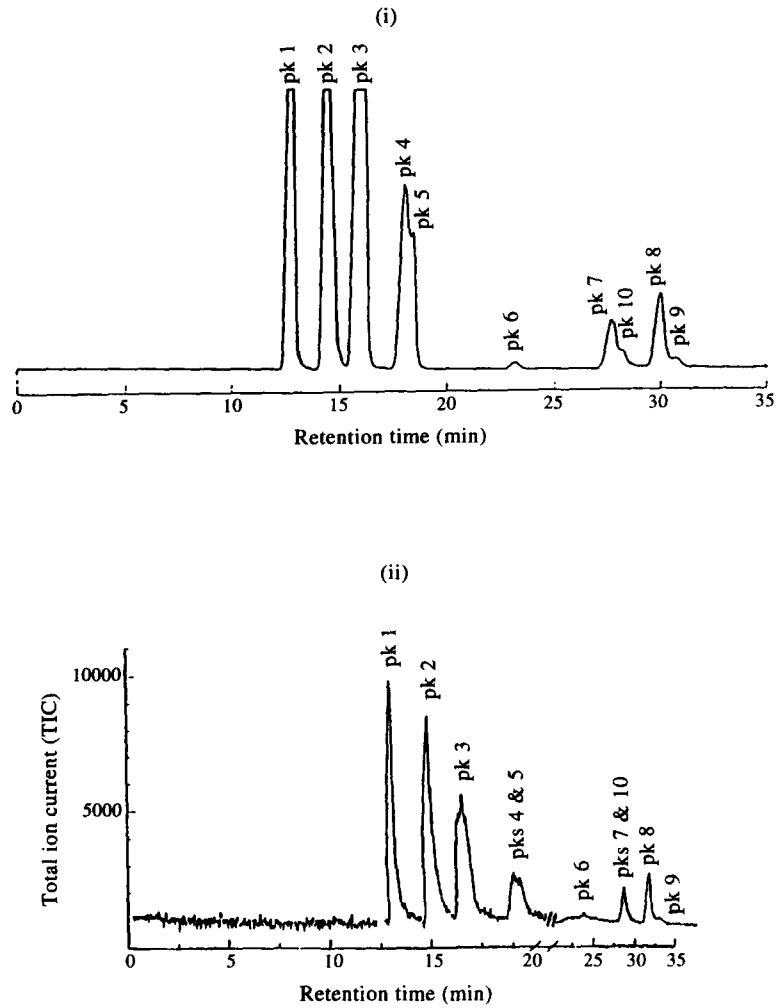

Figure 4
Normal Phase HPLC analysis (with UV detection) or 13-*cis* retinoic acid in solution exposed to air and light for 4 weeks (i) and corresponding total ion current chromatogram under EI–MS conditions (ii). Peak identities are same as in Fig. 3.

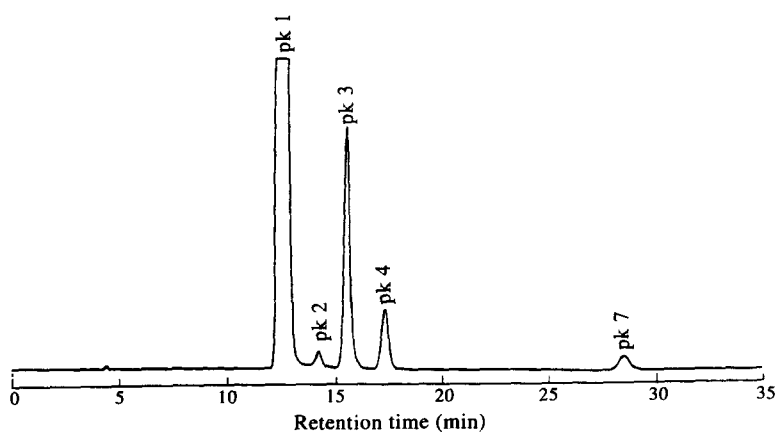

Figure 5
Normal Phase HPLC analysis of 13-*cis* retinoic acid after the solid sample have been exposed to air and light for 8 weeks. Peak identies same as in Fig. 3.

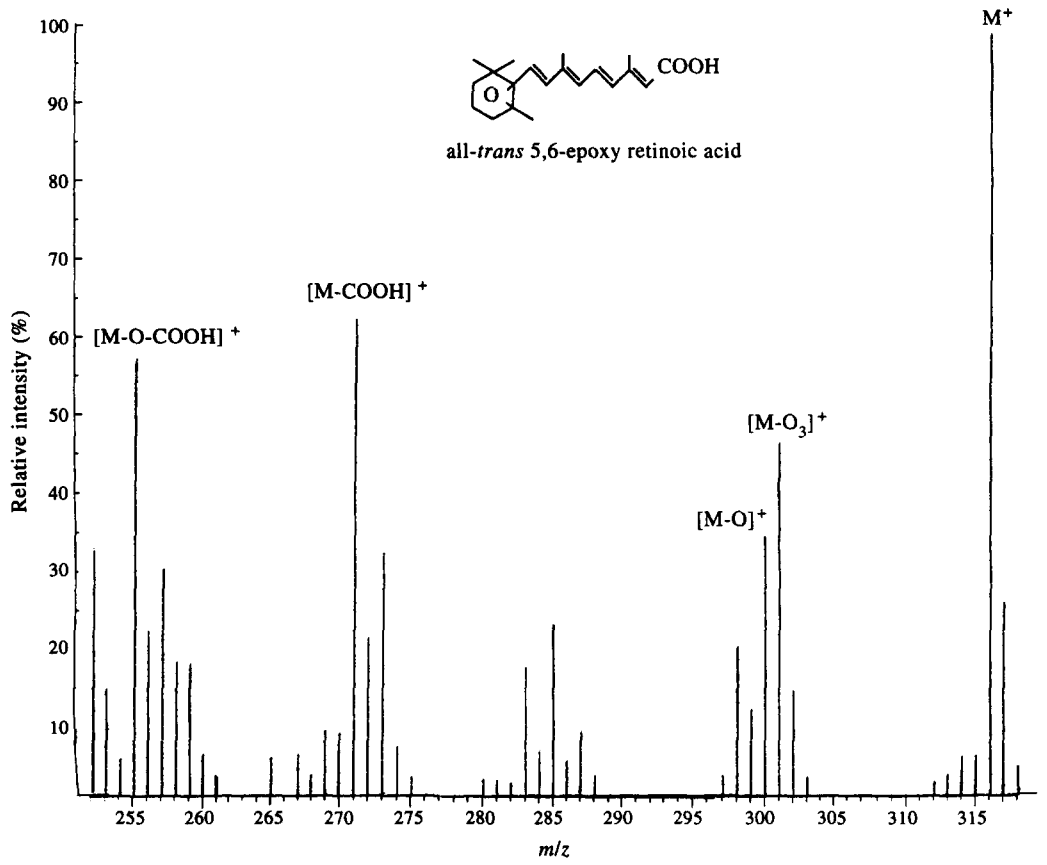

Figure 6
EI–Mass spectrum of peaks 7 or 8 recorded in Figs 3(ii) and 4(ii), M^+ = 5,6-epoxy retinoic acid.

Table 1
Percentage area* for degradation peaks after 13-*cis* retinoic acid solution and solid samples have been exposed to light and air for periods of 4 and 8 weeks, respectively†

	Solution (peak area %)	Solid (peak area %)
Peak 1	24.14	88.55
Peak 2	22.78	0.41
Peak 3	29.10	7.60
Peaks 4 and 5	15.64	2.56
Peak 6	0.29	0
Peaks 7 and 10	2.94	0.88
Peak 8	4.47	0
Peak 9	0.64	0

*Mean of two readings.
†Normal phase LC with a 25 cm × 4.6 mm Zorbax Rx-Sil (5 μm) column, mobile phase — heptane–THF–acetic acid (96.5:3.5:0.015), flow rate 1.4 ml min^{-1}, UV detection at 365 nm.

5,6-epoxy RA were synthesized for comparison. Chromatographic data and mass spectral data confirmed that the peaks of the degradation spectra and the synthetic products were the same. The normal phase degradation products agree with the oxidation products already reported by Oyler and co-workers in benzene [17].

It has been determined that all-*trans* Ra and 13-*cis* RA whether in solution or in solid form undergo *cis–trans* isomerization on prolonged exposure to light and air. Isomerization proceeded faster in solution than in the solid sample. Another degradation reaction is autoxidation of these isomers to their respective 5,6-epoxy derivatives.

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