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SEPARATION AND IDENTIFICATION OF GEOMETRIC ISOMERS OF RETINOIC ACID AND METHYL RETINOATE*

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SUMMARY

Geometric isomers of retinoic acid and methyl retinoate were separated by high-performance liquid chromatography on bonded, reversed-phase, octadecylsilane columns using methanol–water mixtures as solvents. The isomers have been identified by nuclear magnetic resonance, infrared, ultraviolet and mass spectral analyses as 9,11,13-tri-*cis*-, 11,13-di-*cis*-, 13-*cis*-, 9,13-di-*cis*-, 11-*cis*-, 9-*cis*- and all-*trans*-methyl retinoate. Seven isomers were observed simultaneously upon chromatography of retinoic acid isomerates, while eight isomers were observed when methyl retinoate was chromatographed.

INTRODUCTION

The metabolism of retinoic acid has been studied in this laboratory for several years. The vitamins A are sensitive to light, thermally unstable, readily oxidized and easily isomerized, and their separation and handling is difficult. Thus, a need exists for routine methods of purification and analysis of the starting materials as well as unknown metabolites.

The advent of high-performance liquid chromatography (HPLC) and the development of high-performance columns have made such analytical procedures more readily available. Examples of this particular use of HPLC have been provided by Vecchi *et al.*[1], who demonstrated the separation of some of the geometric isomers of retinyl acetate using both adsorption and normal-phase columns, and by Tsukida *et al.*[2], who separated geometric isomers of retinaldehyde. The present paper is concerned with the separation of the geometric isomers of retinoic acid and methyl retinoate on reversed-phase octadecylsilane (ODS) columns.

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EXPERIMENTAL

Glass-distilled residue-free solvents (Burdick & Jackson Labs., Muskegon, Mich., U.S.A.) were used throughout. The water used was deionized, filtered over charcoal and distilled from glass.

All-*trans*-retinoic acid was a gift from Dr. W. E. Scott, Hoffmann-La Roche Inc. (Nutley, N.J., U.S.A.). Isomerates of retinoic acid or methyl retinoate, in solution, were prepared by irradiation using a fluorescent lamp (GE F-15 T8 CW). The irradiations were carried out in either 4-ml specimen vials (flint glass) or 22-ml liquid scintillation vials (borosilicate glass) held at a distance of *ca.* 5 cm from the lamp. Methyl esters were prepared using diazomethane in methanol–diethyl ether solutions, essentially as described by Schlenk and Gellerman[3].

Separations were performed on 0.46 $\times$ 25 cm, bonded, octadecylsilane (ODS) columns (Partisil 10-ODS; Whatman, Clifton, N.J., U.S.A.). Two high-pressure pumping systems were used: a DuPont Model 830 liquid chromatograph (E. I. Du Pont de Nemours & Co., Wilmington, Del., U.S.A.) equipped with a single-beam 254-nm photometer, and a Model 314 pump (Isco, Lincoln, Nebr., U.S.A.) operated through a DuPont gradient-elution accessory. The second pumping system was coupled to a Model 25 spectrophotometer (Beckman, Fullerton, Calif., U.S.A.) fitted with a set of Model LC-25 microcells (Waters Assoc., Milford, Mass., U.S.A.). The detectors were readily interchangeable to serve with either pumping system or, if desired, could be placed in series to operate simultaneously with a single pumping system. Sample application in both systems was via six-port sampling valves (Valco, Houston, Texas, U.S.A.). If not already in an appropriate solvent, samples were dried under a gentle stream of nitrogen with the aid of a warm water-bath. The samples were then redissolved in methanol and diluted with water until the solvent strength was less than or equal to that being used for elution at that time.

Nuclear magnetic resonance (NMR) spectral analyses were performed on a Varian XL-100(15) instrument (Varian, Palo Alto, Calif., U.S.A.) using tetramethylsilane as the internal standard, and infrared (IR) spectra were obtained on a Model 457 grating spectrophotometer (Perkin-Elmer, Norwalk, Conn., U.S.A.). Low-resolution mass spectral analyses were obtained via the direct inlet probe as described by Lin *et al.*[4] and Reid *et al.*[5] for vitamin A and related compounds.

RESULTS AND DISCUSSION

The separation of an isomerate of methyl retinoate by HPLC on a reversed-phase column is shown in Fig. 1. The separation of eight peaks between 35 and 70 min is readily apparent in the trace of the 254-nm spectra. Peak 5 was only a shoulder on the trace made at 350 nm, and peak 0 was not seen at all at this wavelength. The resolution of peaks 3–5 was the most difficult part of this separation. The observation of the three isomers depended upon their relative abundance, the detector wavelength utilized and the maintenance of peak column performance.

The separation of the free acid forms of these isomers is shown in Fig. 2. This separation was more demanding due to the broader peaks obtained. The situation was further complicated by an inversion in the elution order of two of the isomers.

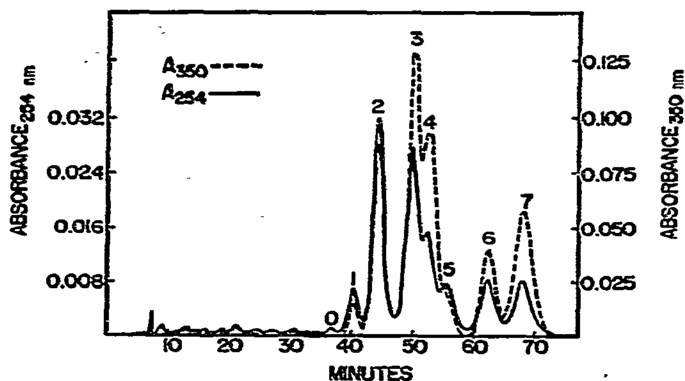

Fig. 1. The HPLC elution profile of an isomerate resulting from irradiation for 3 h of all-*trans*-methyl retinoate in heptane–diethyl ether (9:1). Elution was with methanol–water (70:30) at 20° and a flow-rate of 0.55 ml/min. Approximately 100 μg of methyl retinoate were applied to the column. The identity of peak 0 has not been obtained. Peaks 1–7 have been identified as 9,11,13-tri-*cis*-, 11,13-di-*cis*-, 13-*cis*-, 9,13-di-*cis*-, 11-*cis*-, 9-*cis*- and all-*trans*-methyl retinoate, respectively.

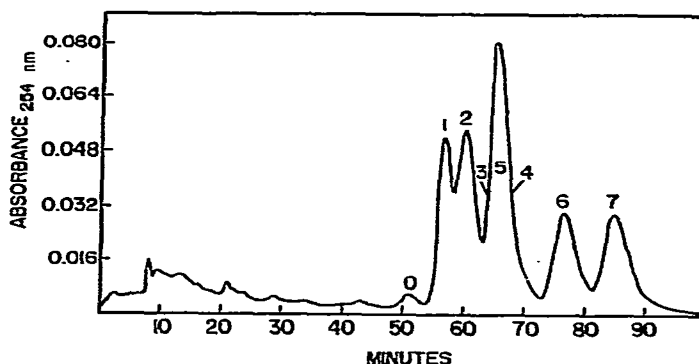

Fig. 2. The elution profile of an isomerate resulting from the irradiation for 1 h of all-*trans*-retinoic acid in methanol–water (70:30). Elution was with methanol–water (65:35) at 20° and a flow-rate of 0.4-ml/min. Approximately 250 μg of retinoic acid were applied to the column. Isomer identification is as in Fig. 1.

Retaining the peak identifications used in Fig. 1, peaks 4 and 5 were found to be reversed when chromatographed as the corresponding acids.

The minimum number of peaks observed in an isomerate of retinoic acid containing all of the eight isomers was six, as represented in Fig. 2. It was possible, given the appropriate relative isomer balance, to observe seven peaks. In such instances, peaks 3 and 5 or peaks 3 and 4 were resolved, while either 4 or 5 seemed to be absent. The resolution of peaks 4 and 5 in this system was insufficient to permit their simultaneous demonstration. However, the actual presence of all the eight isomers in acid isomerates could be demonstrated by methylation of the isomerates and chromatography of the resulting methyl retinoate isomers.

Initially, peak 0 was ignored. It was thought to be a minor contaminant generated during the isomerization process since its spectral characteristics ($\lambda_{max.}$ ≈

263 nm, ratio of absorbances at 263 and 350 nm ≈ 10:1) were not typical of known methyl retinoate isomers. However, this peak, more so than any of the other unidentified, faster eluting, material, became even more prominent with increasing irradiation time (Fig. 3). A small quantity of this material was isolated and analyzed by mass spectroscopy. As was the case with all of the other isomers of methyl retinoate, the spectrum contained the molecular ion, *m/e* 314, and all of the fragment ions found in the mass spectrum of all-*trans*-methyl retinoate[4]. The geometry of this isomer has not been elucidated.

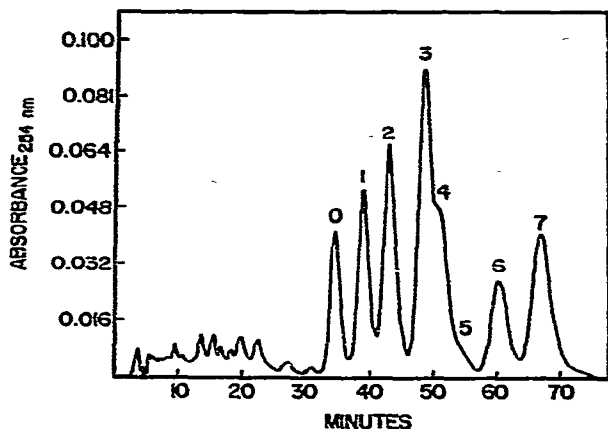

Fig. 3. The elution profile of a methyl retinoate isomerate resulting from irradiation for 72 h in heptane–diethyl ether (9:1). Elution was with methanol–water (70:30) at 25° and a flow-rate of *ca.* 0.6 ml/min. Approximately 250 μg of methyl retinoate were applied. Isomer identification is as in Fig. 1.

The vinyl regions of the NMR spectra of methylated retinoic acid isomers are shown in Fig. 4. The varying quality of the spectra was due to the quantity of isomers collected from the HPLC separations. Peaks 3 and 7 were obtained by single scans while the other peaks required 200-scan time-averaged spectra. Chemical shifts (δ) for singlets further upfield are listed in Table I.

In the high-field region of the NMR spectra (Table I) the C-20 singlets of peaks 1 and 2 are between the usual positions for the 13-*cis* and 13-*trans* isomers. Considering the assignments made to peaks 3–7 below, the most reasonable explanation for this anomalous behavior is that it is a peculiarity of the combination of 11-*cis* and 13-*cis* geometry. It does not happen in 11,13-di-*cis*-retinaldehyde[6], where the C-20 methyl resonates at δ 2.07 in $CDCl_3$. However, the additional steric requirements of the $-OCH_3$ group in the ester could cause this region of the molecule to twist so that the C-20 methyl is positioned closer to a deshielding region. IR spectroscopy (see below) seems to support the 11-*cis* geometry. The slightly higher δ value for C-18 (Table I) in peak 1 suggests 9-*cis* geometry by analogy with the other isomers (only the 9-*cis* isomers give $\delta \geqslant 1.70$), but these differences are marginal. In the vinyl regions of the spectra there is more evidence of a downfield shift for the C-8 doublet (characteristic of 9-*cis*) in peak 1. The small absorption near δ 6.1 could be the C-10 doublet shifted slightly upfield (also characteristic of 9-*cis*). The UV spectra of peak

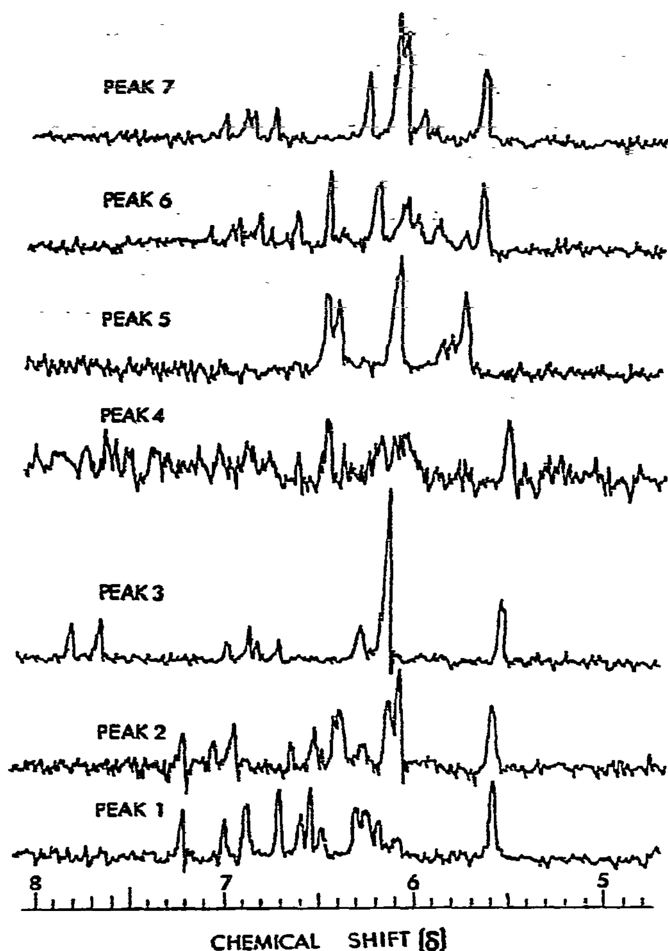

Fig. 4. The vinyl regions of the NMR spectra of geometric isomers of methyl retinoate. Peak numbers refer to compounds collected from the HPLC separations as shown in Fig. 1.

TABLE I

CHEMICAL SHIFT (δ) VALUES FOR THE VARIOUS METHYL SINGLETS IN THE NMR SPECTRA OF THE HPLC PEAKS OBTAINED FROM LIGHT ISOMERATES OF METHYL RETINOATE

Peak	*C-16,17*	*C-18*	*C-19*	*C-20*	*–OCH_3*
1	1.01	1.70	1.96	2.15	3.59
2	1.00	1.67	1.95	2.16	3.60
3	1.01	1.69	1.97	2.05	3.63
4	1.02	1.71	1.98	2.04	3.62
5	1.00	1.68	1.94	2.30	3.64
6	1.03	1.72	1.98	2.30	3.63
7	1.00	1.68	1.97	2.31	3.63

1 displayed a $\lambda_{max.}$ of 333 nm, about 10 nm less than the $\lambda_{max.}$ values of peaks 2–7 (342–353 nm). Hence, the evidence is consistent with peak 1 being probably 9,11,13-tri-*cis*- and peak 2 being 11,13-di-*cis*-methyl retinoate.

The NMR spectra of peaks 3 and 7 (Fig. 4) were obtained on samples collected by preparative HPLC of a mixture produced by isomerization of all-*trans*-methyl retinoate in the presence of iodine in the dark. Peak 7 is the starting material, all-*trans*-methyl retinoate, and peak 3 is 13-*cis*-methyl retinoate[7]. Notable changes from the all-*trans* spectrum for peak 3 include a shift in the C-12 doublet down to δ *ca.* 7.7, and the expected upfield shift of the C-20 singlet caused by the removal of that methyl group from the deshielding region of the carbonyl group in the 13-*cis* configuration.

The NMR absorption for the C-20 methyl group in peak 4 indicates 13-*cis* geometry. The vinyl region (Fig. 4) of the spectrum is very weak because of the small sample size, but there is a strong suggestion of a doublet ($J = 16$ Hz) centered between δ 6.5 and δ 6.6, which could be C-8 in a 9-*cis* isomer. Therefore, peak 4 is the 9,13-di-*cis* isomer.

Peaks 5 and 6 were isolated by preparative HPLC of a 30-min light isomerate in 70% methanol, in which these isomers were at their highest concentrations. The vinyl region of the NMR spectrum for peak 5 corresponds to a 100-MHz theoretical spectrum of 11-*cis*-retinoic acid, drawn from data at 220 MHz[6]. The absence of any significant absorption downfield from δ 6.5 together with the C-12 absorption at $\delta < 6.0$ seems adequate to enable peak 5 to be assigned as the 11-*cis* isomer. Peak 6 is the 9-*cis* isomer since the δ value for C-20 (Table I) shows 13-*trans* geometry and the double doublet for C-11 in the vinyl region (Fig. 4) appears in its usual position, just below δ 7.0, indicating 11-*trans* geometry. In addition, the doublet centered at δ 6.54 ($J = 16$ Hz) is highly characteristic of 9-*cis* geometry. A 100-MHz theoretical spectrum was drawn, based again on the 220-MHz data for 9-*cis*-retinoic acid found in Schwieter *et al.*[6], and it revealed a vinyl pattern very similar to that in Fig. 4. Thus, there is little doubt that peak 6 is the 9-*cis* isomer.

The NMR spectrum of peak 7 (Fig. 4) clearly indicates that it is the all-*trans* isomer and compares well with the spectrum of this isomer given by Korver *et al.*[7]. The primary differences in our spectrum are a slight downfield shift due to the use of CCl_4 instead of $CDCl_3$ as the solvent, and an increased resolution due to the use of a 100-MHz (instead of a 60-MHz) instrument.

In IR spectra of the retinaldehydes it has been demonstrated that the absorption at 10.3–10.4 μm (*ca.* 960–975 cm^{-1}, C–H deformation in a *trans*-$R_1CH{=}CHR_2$ system) decreases in intensity for the 11-*cis* isomers[8]. In our spectra (Fig. 5) this band is much more intense than the nearby bands in isomers 3, 6 and 7 (11-*trans* isomers), but not in peak 5 which was previously assigned as 11-*cis*. Thus, the IR spectra are consistent with a 11-*cis* geometry in peaks 1 and 2 as well as in other isomers. Isomers 6 and 7 exhibit spectra so nearly alike that it may be argued that geometric isomerism at $C_9{=}C_{10}$ has practically no effect on the IR absorption.

Based on these data, peaks 1–7 have been identified as 9,11,13-tri-*cis*-, 11,13-di-*cis*-, 13-*cis*-, 9,13-di-*cis*-, 11-*cis*-, 9-*cis*- and all-*trans*-methyl retinoate, respectively.

The present study has not been fully extended to other vitamin A compounds. However, some observations on the behavior of the all-*trans* isomers of the vitamin A group are perhaps of some interest. Using methanol–water isocrats (70:30), the

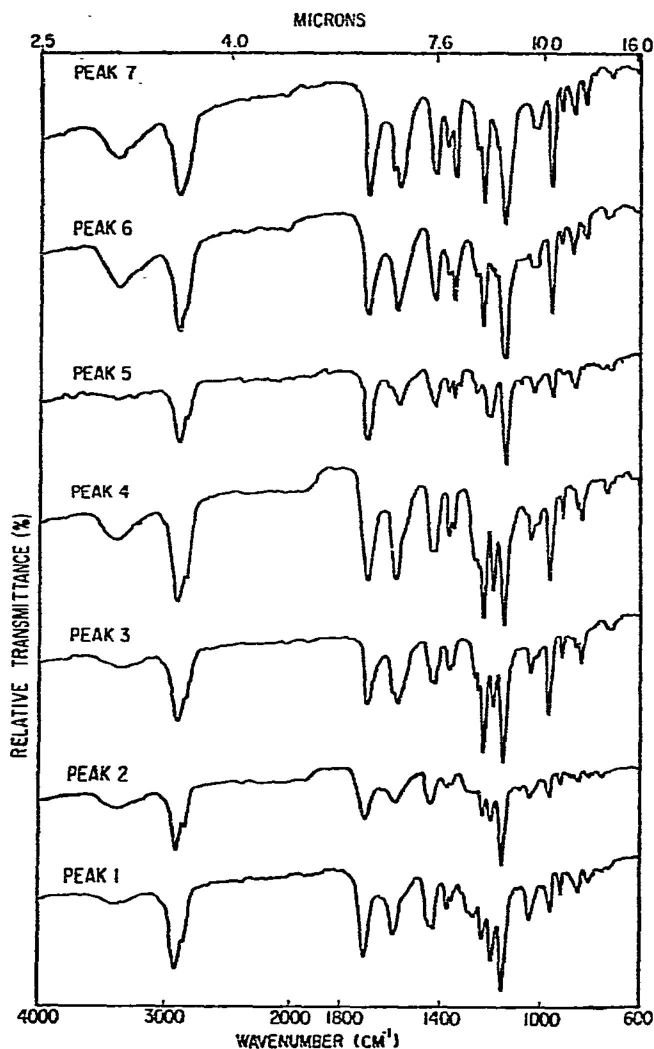

Fig. 5. IR spectra of geometric isomers of methyl retinoate. Peak numbers refer to compounds collected from the HPLC separation as shown in Fig. 1.

retention time of all-*trans*-retinyl acetate was found to be essentially the same as that of all-*trans*-methyl retinoate. All-*trans*-retinaldehyde and all-*trans*-retinol had retention times intermediate between those of all-*trans*-retinoic acid and all-*trans*-methyl retinoate. The approximate relative retention times of these three compounds with respect to the all-*trans*-methyl ester were retinoic acid 0.5, retinol 0.55 and retinaldehyde 0.6.

All-*trans*-retinyl palmitate cannot be recovered from ODS columns when methanol–water solvents are used. Since 100% methanol does not elute high-molecular-weight non-polar compounds, retinyl palmitate would probably go unobserved if these separation methods were carelessly applied to unknown mixtures. With re-

spect to the possible loss of compounds on ODS columns, it is important to emphasize that such columns can be readily fouled. The performance of a highly efficient column can be significantly impaired by a single injection of an inappropriately screened sample. The recommended procedure for regenerating the reversed-phase columns was to elute with a solvent such as a chloroform–methanol mixture. In the present study the compounds having the most dramatic adverse effect on column performance were not readily removed by use of chloroform–methanol. Excellent results were obtained by regenerating fouled columns with 5% aqueous acetic acid.

Owing to differences in the biopotencies of the isomers of retinol and related compounds, the isomeric purity as well as the general chemical purity of these compounds is of importance in biological studies. The procedures outlined here provide a rapid and accurate means of determining the isomeric and chemical purity of retinoic acid and methyl retinoate as well as a procedure for purifying most of the isomers in question. If desired, the procedures permit quantitation on a relative basis. Absolute quantitation will not be possible until all of the required spectral values are acquired. The time required for the analysis is entirely dependent upon the composition of the samples and the degree of separation desired. If only qualitative results are required, the analysis time can be reduced by increasing the methanol concentration and/or flow-rate.

An effort was made to decrease the analysis time, while retaining resolution, through operation at elevated temperatures. However, as the column efficiency improved, the gains were nullified by declines in the relative differences between the partition coefficients. Using the same isomers and operating conditions reported in Fig. 1, but at a temperature of 54°, the column operating efficiency (number of theoretical plates) was approximately doubled. All-*trans*-methyl retinoate eluted with a retention time of 30–35 min. The isomerate separation was similar to that in Fig. 3 except for a compressed time scale. However, the separation of peaks 3–5 was borderline on a column which was operating at maximal efficiency. An even more efficient column might permit the total separation of the isomerate in 30 min on a routine basis. However, with the columns described, those adjustments in the flow-rate and solvent strength that were necessary to assure the separation seen in Fig. 1 increased the retention times to the extent that any advantage gained by operating at elevated temperature was insufficient to justify the inconvenience of doing so. An equally effective, decreased analysis time could be more conveniently obtained by an increase in methanol concentration and operation at ambient temperature.

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