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Short Communication

Separation of retinoic acid all-*trans*, mono-*cis* and poly-*cis* isomers by reversed-phase high-performance liquid chromatography

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ABSTRACT

An HPLC method using a reversed-phase Suplex-pKb-100 column that resolves photoisomerates of retinoic acid into nine peaks of products and the initial all-*trans* isomer is described. This separation is achieved with an isocratic mobile phase and a total elution time of <25 min. Amounts of the five main peaks (which together accounted for *ca.* 95% of the products detected at 350 nm) sufficient for identification by proton NMR spectroscopy as mono-*cis* (9-, 11- and 13-) and di-*cis* (9,13- and 11,13-) isomers of retinoic acid were isolated using a protocol with two chromatographic steps. The methods described in this paper may prove of value in the study of retinoic acid isomer metabolism.

INTRODUCTION

Retinoic acid is involved in cellular processes such as growth promotion and differentiation, and retinoic acid and other retinoids are of interest in cancer prevention and therapy and in the treatment of skin disorders (reviewed in refs. 1–5). Some of the observed effects of retinoic acid may depend on the geometry of its polyene chain (see Fig. 1), *e.g.* all-*trans*- and 13-*cis*-retinoic acid display differential effects on mRNA levels of retinoic acid receptors [6], and a family of retinoic acid receptors that binds 9-*cis*-retinoic acid but not other mono-*cis* isomers or all-*trans*-retinoic acid [7–9] has been characterized. The isomeric composition of these compounds has therefore become a topic of increasing interest.

Study of the metabolism and activities of the

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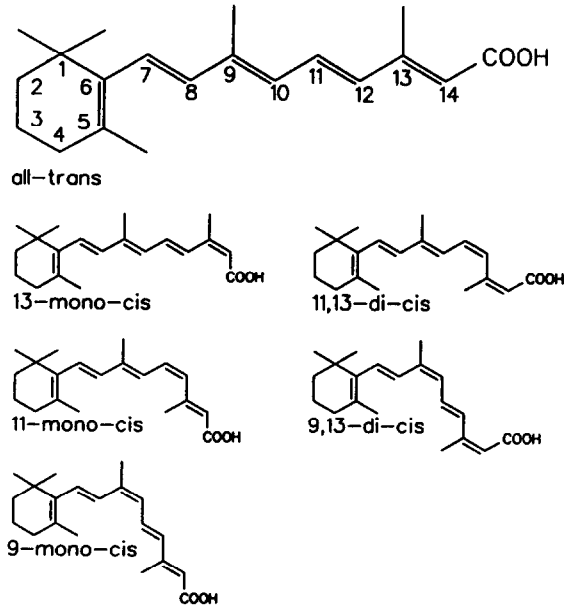

Fig. 1. Isomers isolated from photoisomerates of retinoic acid.

retinoic acid geometric isomers requires analytical methods capable of resolving the closely related compounds. HPLC methods based on reversed-phase octadecylsilane (ODS) columns and normal-phase bonded silica columns have been described and offer good but incomplete separations of the thermodynamically most stable isomers of retinoic acid, *i.e.* the all-*trans* and sterically unhindered mono- and di-*cis* isomers [7,10–13]. Greater success had been achieved with the isomers of methyl retinoate [10,12,14], but the separations are lengthy and would require a derivatization step prior to analysis of biological samples. A method using a small particle size ODS-2 column with an isocratic mobile phase and yielding a superior separation of retinoic acid photoisomerization products has recently been described [15].

In the present report we describe an HPLC methodology based on a reversed-phase Suplex-pKb-100 column that resolves photoisomerates of all-*trans*-retinoic acid into nine peaks of products. The main products, 9-mono-*cis*-, 11-mono-*cis*-, 13-mono-*cis*-, 9,13-di-*cis*- and 11,13-di-*cis*-retinoic acid, were isolated following two chromatographic steps and identified by proton NMR.

MATERIALS AND METHODS

Materials

All-*trans*-retinoic acid (>99% pure by HPLC) was generously provided by Dr. H.E. Keller (F. Hoffmann-La Roche, Basle, Switzerland). Mixtures of mono-*cis*- and poly-*cis*-retinoic acid were generated by photoisomerization of 1 m*M* solutions of all-*trans*-retinoic acid in a Rayonet photochemical reactor (Cat. No. RPR 100; Southern N.E. Ultraviolet, Hamden, CT, USA) fitted with sixteen UV lamps (350 nm emission maximum; Cat. No. RPR 3500A). In experiments to determine the time course of photoisomerization, the incubations were conducted in tightly capped quartz cuvettes (0.5 cm path length), which were chilled between periods of illumination; aliquots were diluted into eluent prior to HPLC analysis. For the purification of retinoic acid isomers, 20 ml of a 1 m*M* methanolic retinoic acid solution was illuminated in a graduated cylinder, into which a smaller cylinder filled with ice had been inserted both to control the temperature and increase light exposure; acetic acid was added to 0.05% prior to the first HPLC step.

HPLC analysis and purification of retinoic acid isomers

HPLC was done on either a Suplex-pKb-100 (5 μm, 250 × 4.6 mm main column 20 and 20 × 2.6 mm precolumn; Supelco, Bellefonte, PA, USA) or a LiChrospher 100 RP-18 end-capped (5 μm, 250 × 4 mm main column and 4 × 4 mm precolumn; Merck, Darmstadt, Germany) column, supported with a pump (L6210), UV–visible detector (L4250) and integrator (D2500) from Merck/Hitachi (Darmstadt, Germany), and a FRAC 100 fraction collector (Pharmacia-LKB, Uppsala, Sweden). Eluents A (acetonitrile–methanol–acetic acid, 95:5:0.6), B (methanol–0.05% acetic acid), and C (acetonitrile–methanol–water–acetic acid, 50:25:25:0.5) were first degassed in a sonicating water bath. A flow-rate of 1 ml min^{-1} was used throughout.

During the isolation, fractions containing iso-

mers were dried in a refrigerated and darkened lyophilizator. Addition of water (20% of final volume) to fractions in eluent B prior to lyophilization greatly diminished the degree of decomposition that can otherwise occur during this step. Purified isomers were stable when stored dry at −70°C.

Characterization of retinoic acid isomers

Proton NMR spectroscopy of the retinoic acid isomers was carried out at room temperature with a Varian VXR-300 instrument and thick-walled tubes containing 0.15–0.4 mg of isomer in 0.15 ml of C^2HCl_3.

RESULTS AND DISCUSSION

Retinoic acid readily undergoes isomerization upon exposure to light, and this represents a convenient method for generating mixtures of retinoic acid geometric isomers. The photoisomerization of all-*trans*-retinoic acid in methanol was followed using a new HPLC method based on a Suplex-pKb-100 column and eluent A. This column, which is composed of a "proprietary" reversed-phase matrix, was recently shown to be effective in resolving β-carotene and lycopene geometric isomers [16].

Significant product formation occurred after only 1 min of illumination, and extending the period to 30 min resulted in nine peaks of products (Fig. 2A). Illumination of the mixture an additional 30 min affected only slightly the relative amounts and yields of products detectable at 350 nm, indicating that a steady-state-like mixture of relatively stable products had been achieved. When the isomerization was repeated in *n*-hexane the same major products formed, but in different amounts, and three additional peaks eluting between 6 and 10 min were detected (Fig. 2B). The influence of solvent polarity on the course of photoisomerization has been previously described for methyl retinoate [14] and retinal [17,18].

The main products, which together accounted for *ca.* 95% of the decrease in the all-*trans* peak at 350 nm (Fig. 2), were isolated by HPLC for identification. Photoisomerates of all-*trans*-retinoic acid prepared in methanol were first

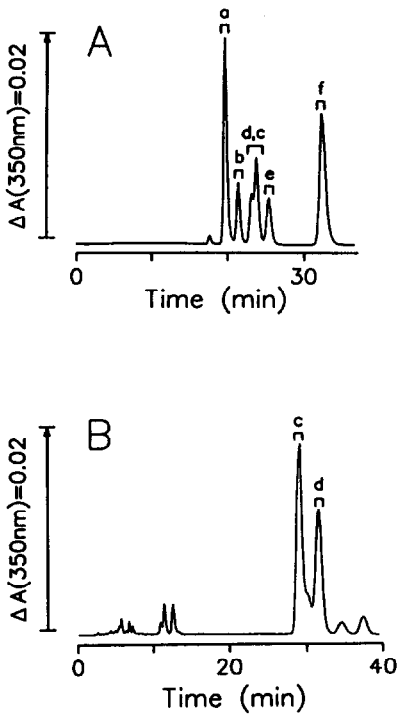

Fig. 2. Reversed-phase HPLC analysis of retinoic acid photoisomerization products. Solutions of all-*trans* retinoic acid in methanol (A) and *n*-hexane (B) were exposed to UV light (350 nm maximum) and analysed after different times of irradiation by HPLC with a Suplex-pKb-100 column and eluent A. The isomeric purity of the all-*trans*-retinoic acid prior to irradiation was *ca.* 99%. The lettered peaks were isolated and identified as follows: a = 13-mono-*cis*-retinoic acid; b = 11-mono-*cis*-retinoic acid; c = 11,13-di-*cis*-retinoic acid; d = 9,13-di-*cis*-retinoic acid; e = 9-mono-*cis*-retinoic acid; f = all-*trans*-retinoic acid (see Fig. 3).

separated with the Suplex column and eluent B (Fig. 3A). Using this eluent, three of the peaks of products (a, b and e) and the all-*trans*-retinoic acid peak (f) could be collected without need for further purification; the other two main peaks (c and d) were pooled and then resolved using a standard ODS column with eluent C (Fig. 3B). The purity of each of the six fractions was estimated by HPLC to be ≥95%.

The products were identified as geometric isomers of retinoic acid based on a comparison of their proton NMR chemical shifts and coupling constants with values reported for isomers of the methyl ester derivative of retinoic acid [14,19] and retinoic acid [15], as follows: a = 13-

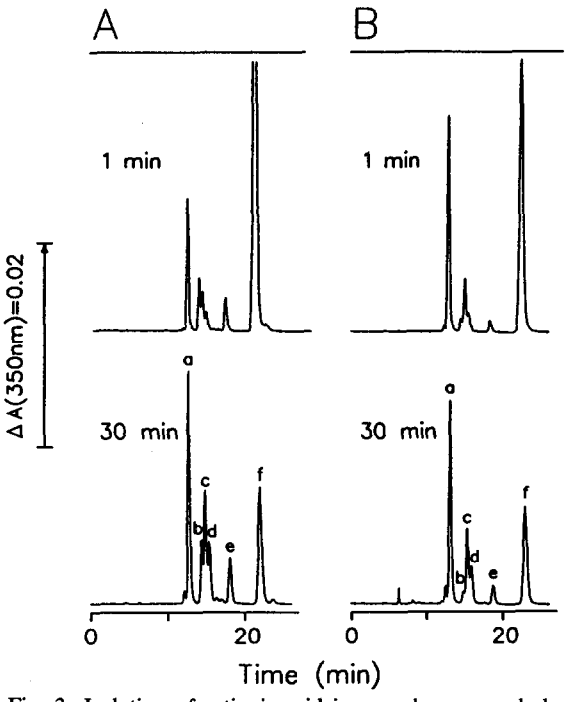

Fig. 3. Isolation of retinoic acid isomers by reversed-phase HPLC. (A) Photoisomerates of retinoic acid in methanol were resolved into five fractions on a Suplex-pKb-100 column with eluent B. Fractions a, b, e and f were not purified further. (B) Fraction d,c was chromatographed on a ODS column with eluent C to yield fractions c and d. The six final fractions were subjected to proton NMR spectroscopy and shown to be geometric isomers of retinoic acid (see Results and Discussion). Peak lettering corresponds to that of Fig. 2.

mono-*cis*-retinoic acid; b = 11-mono-*cis*-retinoic acid; c = 11,13-di-*cis*-retinoic acid; d = 9,13-di-*cis*-retinoic acid; e = 9-mono-*cis*-retinoic acid; f = all-*trans*-retinoic acid [note: the order of elution of the two di-*cis* isomers from the Suplex column is reversed with eluents A (Fig. 2) and B (Fig. 3A)].

The composition of the photoisomerates of retinoic acid in methanol is in agreement with some published values. In a study in which photoisomerates of retinoic acid isomers were generated in buffered ethanol and esterified with diazomethane prior to HPLC analysis, the formation and relative abundance based on detection at 340 nm of the following *cis* isomers were observed: 13-mono (1.00), 11,13-di (0.68), 11-mono (0.42), 9-mono (0.38), 9,13-di (0.37), 9,11,13-tri (0.24) [12]. The yield of the mono- and di-*cis* isomers coincide with those of the photoisomerates in methanol (see Fig. 2A). The reason for the lower level of 9,11,13-tri-*cis*-retinoic acid found in methanol is unclear, but may result from a slight solvent effect.

The structures of the eight or so minor photolysis products of retinoic acid (see Fig. 2) may correspond to some of those found in photoisomerates of methyl retinoate. In addition to the mono-, di- and tri-*cis* isomers seen with retinoic acid, the hindered 7-mono- and 7,13-di-*cis* isomers have been isolated from methyl retinoate photoisomerates [14]. Isomers of (5→10)-cyclized retinoic acid have ben shown to form upon long-term irradiation of retinoic acid [15], but since these products appear to be present in relatively low amounts (<2 mol% each) in photostationary state mixtures of the non-cyclized geometric isomers and to absorb only poorly at 345 nm compared with the non-cyclized isomers [15], they are possibly not among those detected with the present method (see Fig. 2).

To summarize, a rapid and simple HPLC protocol for the analysis of the geometric isomers of retinoic acid has been developed using a Suplex-pKb-100 column. In comparison with this method, a previously described separation based on a small particle size ODS-2 column better resolved the two di-*cis* isomers from 11-mono-*cis* retinoic acid but required significantly longer sample elution times (*i.e.* 40 min *vs.* 25 min). When used in combination with extraction techniques already developed for tissue and plasma [13,20–23], such HPLC methods may prove of use as the research in retinoic acid metabolism and function focuses more closely on the activity and interconversions of the all-*trans* and individual *cis* isomers of retinoic acid.

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