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Note

Capillary gas chromatography of retinoids (vitamin A compounds) and apo-retinoids: determination of Kováts retention indices

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Early attempts at gas chromatography (GC) of vitamin A compounds (retinoids) were hindered by these compounds' lability in the presence of hot injector surfaces and incompletely inactivated column packings. For example, Dunagin and Olson [1] found that pre-treatment of SE-30-packed columns with large amounts of β-carotene was necessary for successful chromatography of retinol and retinyl acetate; anhydroretinol, methyl retinyl ether, retinaldehyde, and methyl retinoate could be chromatographed without column pretreatment. Ninomiya et al. [2] had earlier reported that retinol, retinyl acetate, and retinyl palmitate are dehydrated to anhydroretinol under conditions of conventional packed-column gas chromatography. Cullum et al. [3] took advantage of the on-column dehydration of retinol to anhydroretinol for gas chromatographic–mass spectrometric (GC–MS) analysis of deuterated retinol. Vecchi et al. [4] found it necessary to make the trimethylsilyl derivative of retinol for chromatography on QF-1-packed columns. Taylor and Ikawa [5]

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have summarized the GC of catalytically hydrogenated carotenoids on packed columns.

Kováts retention indices (retention relative to that of *n*-alkanes) are useful for the identification of compounds by GC [6]. However, because only limited studies of GC of retinoids and carotenoids have been carried out to date, retention indices for these compounds have not been determined previously.

Smidt et al. [7] achieved a major advance in the GC of retinoids by chromatographing underivatized retinol on methylsilicone capillary columns by using cold on-column injection. We now have extended their observations by chromatographing apo-retinoids and retinoids having variations in chain length and functional group, and by determining retention indices of these compounds.

EXPERIMENTAL

Geranial and neral (isomers of 3,7-dimethyl-2,6-octadien-1-al), α-ionone [4-(2,6,6-trimethyl-1-cyclohexen-2-yl)-3-buten-2-one], β-ionone [4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-3-buten-2-one], and retinaldehyde [3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4,6,8-nonatetraen-1-al] were purchased commercially (Aldrich, Milwaukee, WI, U.S.A.; Sigma, St. Louis, MO, U.S.A.). The C_{15} aldehyde [3-methyl-5-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4-pentadien-1-al], C_{15} acid ethyl ester, and C_{18} ketone [6-methyl-8-(2,6,6-trimethyl-1-cyclohexen-1-yl)-3,5,7-octatrien-2-one] were synthesized by Dr. Robert Bergen [8]. Structures of the parent compounds are shown in Fig. 1. The corresponding alcohols were prepared by reduction with sodium borohydride and were then acetylated by using acetic anhydride in triethylamine. Methyl retinoate was prepared by diazomethane methylation of retinoic acid (Sigma), and anhydroretinol by acid-catalyzed dehydration of retinol [9]. Short-chain and medium-chain retinyl esters were prepared from retinol and the corresponding acid anhydrides (Sigma). β-Apo-12'-carotenal [2,7,11-trimethyl-13-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4,6,8,10,12 undecahexen-1-al] was the gracious gift of Hoffman-La Roche (Basel, Switzerland). Axerophthene [vitamin A hydrocarbon; 3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4,6,8-nonatetraene] was generously provided by BASF (Ludwigshafen, F.R.G.). Only chromatography of all-*trans* compounds was studied.

GC was carried out on a Carlo Erba Model 4130 chromatograph (Carlo Erba Instruments, Saddle Brook, NJ, U.S.A.), equipped with an on-column injector (J & W Scientific, Folsom, CA, U.S.A.) and flame-ionization detector (Erba). Samples (1 μl of 100–400 ng/μl in hexane or methanol) were injected onto the head of the column at room temperature, and the column segment was then lowered into the chromatograph oven to begin chromatography. An electronic integrator (Model 3390A, Hewlett-Packard, Palo Alto, CA, U.S.A.) was used to determine peak retention times. Most studies were performed with a bonded-phase methylsilicone wall-coated open-tubular capillary column (DB-1, 15

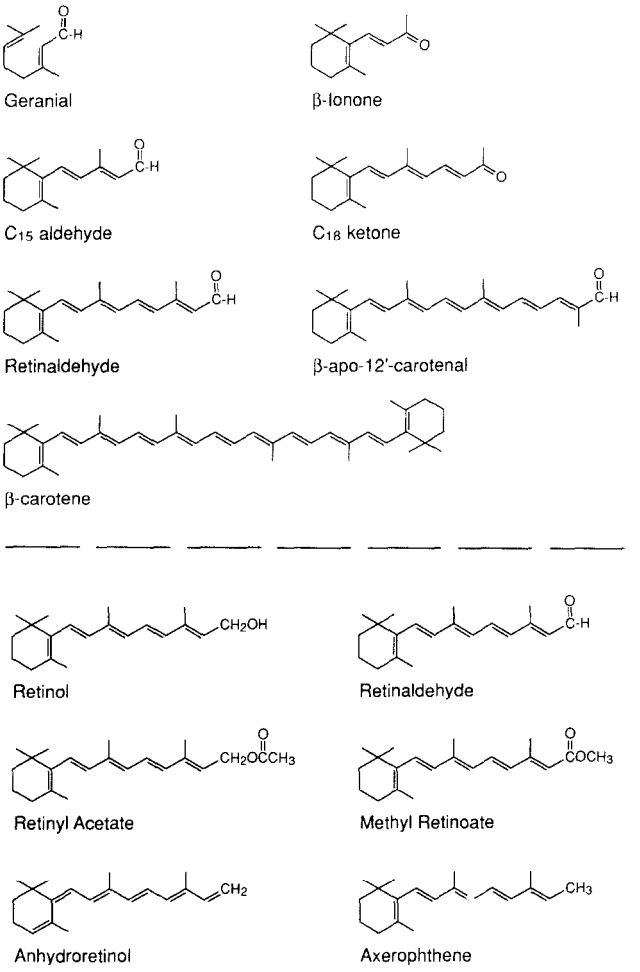

Fig. 1. Structures of representative apo-retinoids and retinoids studied by capillary GC with on-column injection.

m×0.25 mm I.D., 0.25 μm film thickness; J & W Scientific); for some studies, a short, thin-film methylsilicone column (DB-1, 5 m×0.25 mm I.D., 0.1 μm film thickness) or a cyanopropyl-phenyl-methylsilicone column (DB-225, 30 m×0.25 mm I.D., 0.15 μm film thickness; J & W Scientific) was used. Helium was used as carrier gas, at 34-kPa (5-p.s.i.) column inlet pressure (2.5 ml/min). To confirm peak identity in some studies a SpectrEL quadrupole mass spectrometer (EXTREL, Pittsburgh, PA, U.S.A.) was used for detection with the same chromatograph and columns.

Kováts retention indices [6] were determined under appropriate isothermal conditions for each compound ($1 < k' < 10$) by comparison of compound reten-

tion times with those of *n*-alkane standards (Alltech, Deerfield, IL, U.S.A.), by using a computer program to calculate chromatographic dead times and retention indices [10].

RESULTS AND DISCUSSION

Room temperature on-column injection of these compounds resulted in sharp chromatographic peaks, with some peak-broadening observed only for compounds requiring high operating temperatures (as discussed below). Total ion current chromatograms and mass spectra from capillary GC of several retinoids are presented in Fig. 2.

Kováts retention indices (retention times relative to *n*-alkane standards) are presented in Table I. All values were determined in triplicate; all coefficients of variation (standard deviation divided by mean value) were $<0.36\%$. Retention indices for some compounds determined on a second DB-1 column were within 1% of those presented here (data not shown). There is good agreement between the values found on this methylsilicone capillary column for neral, geranial, and β-ionone and those measured on OV-101 glass capillary columns [11].

As can be seen in Fig. 3, retention indices increase linearly with chain length for the apo-retinoid compounds. (The number of carbon atoms in each family of cited analogues is: β-ionone, 13; C_{15} aldehyde, 15; C_{18} ketone, 18; retinaldehyde, 20; β-apo-12′-carotenal, 25; β-carotene, 40.) The order of elution is anhydro < alcohol < aldehyde/ketone < acetate for a given alkyl chain length (excluding the carbon atoms in the ester moiety). As expected for these compounds with conjugated polyene systems, retention indices for several representative compounds on a more polar column (DB-225, cyanopropyl-phenyl-methylsilicone) are greater than those on a methylsilicone column (Table I).

Retinyl esters exhibit linearly increasing retention indices with increasing fatty acyl chain length (Table I). Interestingly, the effect of an increasing number of saturated carbons (carbon atoms of the fatty acyl moiety of retinyl esters) on the retention index is less than that of unsaturated carbons (carbon atoms of the apo-retinoid series), in contrast to what we have observed in reversed-phase liquid chromatography (unpublished observations). The difference in retention index (1.8%) of retinyl acetate observed on the two methylsilicone columns may be due to differences in film thickness between the columns; we have not studied this effect further.

In this study we did not attempt to determine limits of detection for GC of these compounds. A lower limit of detection of 3.5 ng for retinol by capillary GC with on-column injection and flame-ionization detection was reported by Smidt et al. [7]. Although we did not specifically study the separation of *cis* isomers of these polyene compounds, we did observe in GC–MS studies that

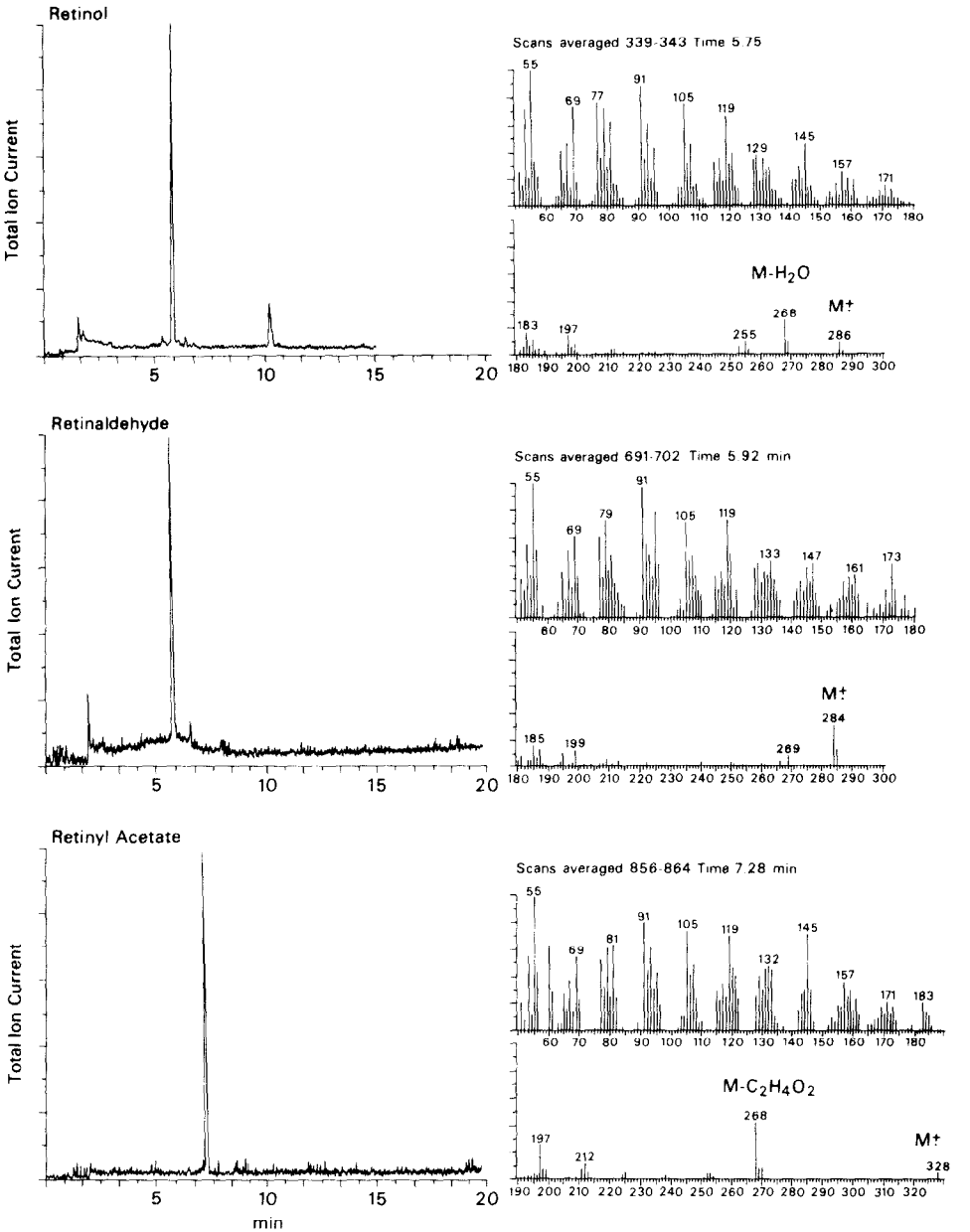

Fig. 2. Capillary GC–MS of retinoids, with on-column injection. Upper panel, retinol; center panel, retinaldehyde; lower panel, retinyl acetate. Column: wall-coated open-tubular methylsilicone (DB-1, 15 m × 0.25 mm I.D., 0.25 μm film thickness), linear temperature gradient from 200 to 300 °C at 5 °C min. Carrier gas, helium at 2.5 ml/min (5 p.s.i.). Detection by electron-impact MS (SpectrEL quadrupole mass spectrometer), 70 eV; scan range 50–300 a.m.u. or 50–350 a.m.u. Left side: Total ion current chromatogram; right side; mass spectra of retinoid peaks. (The peak at 10.3 min in the chromatogram of retinol is identified as a phthalate ester.)

TABLE I

KOVÁTS RETENTION INDICES OF APO-RETINOIDS AND RETINOIDS

Compound	Column temperature (°C)	Kováts index
DB-1 column (15 m×0.25 mm I.D.; 0.25 μm film thickness)		
Neral	100	1216 (Lit. 1227 [11])
Geranial	100	1244 (Lit. 1252 [11])
α-Ionone	145	1416
β-Ionol	145	1406
β-Ionone	145	1469 (Lit. 1474 [11])
β-Ionyl acetate	150	1525
Anhydro C_{15} alcohol	150	1569
C_{15} Alcohol	165	1732
C_{15} Aldehyde	165	1754
C_{15} Acetate	180	1857
C_{15} Acid ethyl ester	180	1885
C_{18} Alcohol	180	2029
C_{18} Ketone	180	2089
C_{18} Acetate	180	2161
Axerophthene	220	2148
Anhydroretinol	240	2233
Retinol	240	2453
Retinaldehyde	240	2466
Methyl retinoate	240	2528
Retinyl acetate	240	2578
DB-1 column (5 m×0.25 mm I.D.; 0.10 μm film thickness)		
Retinyl acetate	190	2531
Retinyl butanoate	190	2738
Retinyl hexanoate	220	2970
Retinyl octanoate	220	3157
Retinyl decanoate	220	3359
Retinyl dodecanoate	240	3577
β-Apo-12′-carotenal	240	3040
DB-225 column (30 m×0.25 mm I.D.; 0.15 μm film thickness)		
Anhydroretinol	220	2468
Methyl retinoate	220	3200

small peaks with similar mass spectra (presumably *cis* isomers) elute before all-*trans*-retinoids (data not shown).

The flame-ionization detector used in these studies could not be heated above 250°C; therefore it was necessary to use a thin-film column to allow chromatography of the retinyl esters and of β-apo-12′-carotenal. Attempts to chromatograph longer-chain retinyl esters (retinyl tetradecanoate, hexadecanoate,

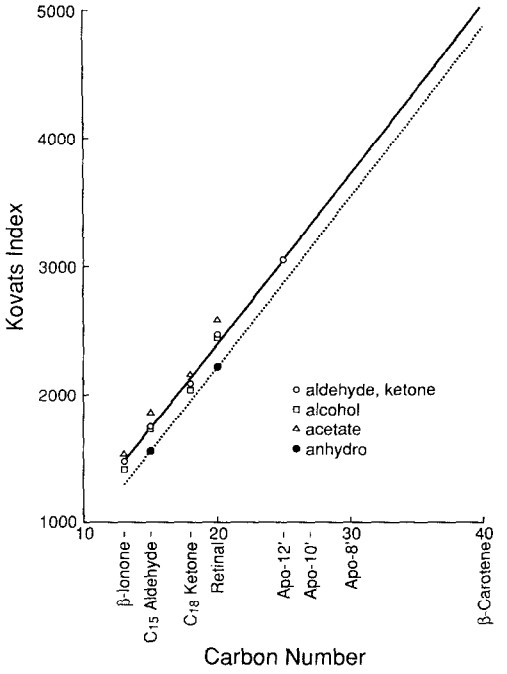

Fig. 3. Relationship of Kováts retention index to carbon number for apo-retinoids and retinoids (alcohol, aldehyde/ketone, acetate forms).

and octadecanoate) at higher temperatures resulted in severely broadened peaks, presumably caused not by degradation of the compounds, but rather by condensation at the detector. Thus the upper limit for GC of these compounds in this study was determined by the operating limits of the instrumentation, not by the thermal stability of the compounds themselves. This observation is in marked contrast to early experience in GC of retinoids and carotenoids, when thermal degradation limited attempts at GC. The hydrocarbon axerophthene, a 'half-β-carotene', chromatographed cleanly, confirming thermal stability of the carotene structure on the methylsilicone bonded-phase column under these conditions (data not shown). Extrapolation from retention indices of apo-retinoids and apo-carotenoids suggests that β-carotene should have a Kováts retention index of approximately 5000, a value now within reach of high-temperature capillary GC.

We have now extended the previous observation that underivatized retinol can be analyzed readily by capillary GC by use of on-column injection [7] to show that a variety of apo-retinoid and retinoid compounds, including retinyl esters, and an apo-carotenal can be analyzed by this technique. These compounds can be identified by comparison of their retention times (by use of retention indices) and/or by coupled GC–MS. The resolution of analogues

with the same carbon number (alcohol from aldehyde; ketone from acetate ester) and between analogues with different carbon numbers (e.g., β-ionone from C_{15} aldehyde from C_{18} ketone from retinaldehyde) is greater than we have yet achieved by reversed-phase high-performance liquid chromatography [12]. The universal nature and sensitivity of the flame-ionization detector or mass spectrometer as detector are also advantages over the more specific detectors available for liquid chromatography. Thus capillary GC with on-column injection can be a valuable tool in the study of apo-retinoids, apo-carotenoids, and, possibly, carotenoids.

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