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PREPARATIVE THIN-LAYER CHROMATOGRAPHY OF THE OXIDATION PRODUCTS OF RETINYL ACETATE

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SUMMARY

Preparative thin-layer chromatography was applied to the separation of retinyl acetate degradation products obtained in the accelerated lipid oxidation test (Schaal method). Fourteen substances were separated by repeated developments using silica gel HR plates (3 mm) and the mobile phase hexane–diethyl ether in ratios 95:5 to 10:90. The isolated substances were characterized by their UV spectra, R_F values and the colours during detection with a 366 nm lamp.

INTRODUCTION

The oxidation products of vitamin A (retinyl acetate and retinol) have a lower biological activity than the original substance. However, they absorb or emit radiation in the analytical spectral range and therefore interfere in the spectral (UV and visible absorption spectroscopic and fluorimetric) determination of vitamin A. Chromatography must therefore often be used as a preliminary technique to isolate the active substance for further spectral evaluation.

Degradation of retinol and retinyl esters during the chromatographic separation caused by the effect of adsorbent and solvents[1], light[2,3] and oxidation[4] limits the application of paper and thin-layer chromatography. The recovery of retinol is only *ca.* 85%[5], even from thin layers impregnated with a phenolic antioxidant. The degradation is slower on columns. Retinol was thus separated for its fluorimetric[6] and colorimetric[7] determination. Nevertheless, both column and gel permeation chromatography[8] failed in the separation of the oxidation products of retinol. High-performance liquid chromatography (HPLC) can doubtlessly offer interesting results. The method has been applied to the separation and determination of retinol and its esters in the mixtures of lipophilic vitamins in drugs[9,10] and foodstuffs[10,11]. Vitamin A compounds pertaining to the visual cycle (geometric isomers of retinyl esters, retinal and retinol[12] and *cis–trans* isomeric retinals[13]) have been determined by HPLC. They could not be separated by other chromatographic methods. Geometric isomers of retinoic acid and methyl retinoate[14] were analysed successfully in the presence of aromatic retinoic acid analogue and retinoic acid isomers[15] in biological fluids after intravenous administration of 13-*cis*-retinoic acid. Reversed-phase HPLC[16] yielded a rapid separation of substances with vitamin A activity in mixtures (retinol–retinoic

acid). However, no fractionation of retinol or retinyl ester oxidation products by HPLC has been described so far.

We have therefore concentrated on the selection of optimal procedures for the isolation of oxidation products resulting in a standard preparation containing 50% of retinyl acetate, commonly used for the enrichment of foodstuffs with vitamin A, using the conditions of model lipid oxidation, *i.e.*, the Schaal method. We used preparative thin-layer chromatography for this purpose.

EXPERIMENTAL

Chemicals

Retinyl acetate was a 50% oily preparation (Société de Chimie Organique et Biologique, Commentry, France). Silica gel HR (reinst nach Stahl) was obtained from Merck (Darmstadt, G.F.R.). Solvents were of either reagent grade or UV spectrometry grade.

Apparatus

A Unicam SP-700 spectrophotometer (Pye Unicam, Cambridge, Great Britain) and a Camag universal UV lamp (366 nm) (Camag, Muttenz, Switzerland) were used.

Model oxidation of retinyl acetate

Retinyl acetate was oxidized in a 1-cm layer in the dark at 60° with free access of air until the concentration of the active substance was reduced to 5–10% of the original value. The course of oxidation was followed by UV spectrometry[17] and colorimetry[15,19].

Chromatography

Plates (20 × 20 cm) with 3-mm layers of silica gel HR were dried for 24 h in air and activated for 1 h at 120°. The sample (1–1.5 g in chloroform) was applied and the plate was immediately (before complete drying of the solvent) placed in the developing chamber and developed with *n*-hexane–diethyl ether (95:5 to 1:9, depending on the polarities of the separated substances) for 50–60 min in the dark. Detection was carried out before the solvents had evaporated. The separated zones were transferred into flasks with chloroform or chloroform–methanol, again depending on the polarities of the substances. Extraction from the silica gel was performed by stirring for 10 min in the dark and by filtration and washing of the silica gel with chloroform–methanol. The isolated substances, after the evaporation of the solvents at 40° in the dark, were used for further chromatographic purification or for spectral analysis. Multiple development of one plate was impossible owing to degradation on the dry adsorbent.

RESULTS AND DISCUSSION

The pure fractions of oxidized products were obtained by the system shown schematically in Fig. 1 and Table I. The optimal procedure was as follows. The oxidized retinyl acetate was first separated into fractions 1–5 by preparative thin-layer chromatography using *n*-hexane–diethyl ether (9:1). The R_F values and UV

characteristics of these main fractions are summarized in Table I. Although the zones of these fractions were not well resolved, the efficiency of this separation was apparent from the UV spectra (unseparated oxidized retinyl acetate, maximum at 285 nm, shoulder at 325 nm; fractions 1, 2 and 3, $R_F = 0.00–0.10$, 0.10–0.30 and 0.30–0.55, respectively, maximum at 285 nm; fraction 4, $R_F = 0.55–0.85$, maximum at 325 nm; fraction 5, $R_F = 0.85–1.00$, triplet at 351, 372 and 392 nm).

The absorption bands of fractions 1–5 were well resolved; those of the latter two corresponded to retinyl acetate and anhydroretinol, respectively. The combined recovery of the sample was 95–100%. The recovery of unoxidized retinyl acetate (control) that was developed and eluted from the plate was 93–97% (according to the absorptivity at 325 nm) under the above conditions (all operations carried out in the dark, no contact with dry adsorbent, high concentration of the substance on the plate).

Fractions 1–5 were then subfractionated and the subfractions purified by repeated preparative thin-layer chromatography. The solvent ratio was chosen so that the R_F value of the main substance in the final development was *ca.* 0.5. The number of developments depended on the weight of the fraction and its purity. The procedure was terminated when the eluted subfraction represented 90–95% of the sample applied to the plate and formed one clearly resolved zone with the R_F value given in Table I. The detailed subfractionation procedure was as follows.

Fraction 1 was subfractionated with *n*-hexane–diethyl ether (1:9). The first development yielded four substances, revealed under UV light (366 nm) as a greyish white zone at the start (1a, $R_F = 0.00–0.10$), a broad yellow-green zone (1b, $R_F = 0.10–0.45$), a narrow yellow zone (1c, $R_F = 0.45–0.65$) and a broad yellow-green front (1d, $R_F = 0.65–1.00$). Subfractions 1a and 1d were each purified once and subfractions 1b and 1c twice by development with *n*-hexane–diethyl ether (1:9).

Fraction 2 was first subfractionated with *n*-hexane–diethyl ether (1:9), yielding the same zones as fraction 1, but in different weight ratios. Subfraction 2a was developed once and subfraction 2b twice with *n*-hexane–diethyl ether (1:9), and subfractions 2c and 2d each twice with *n*-hexane–diethyl ether (2:8).

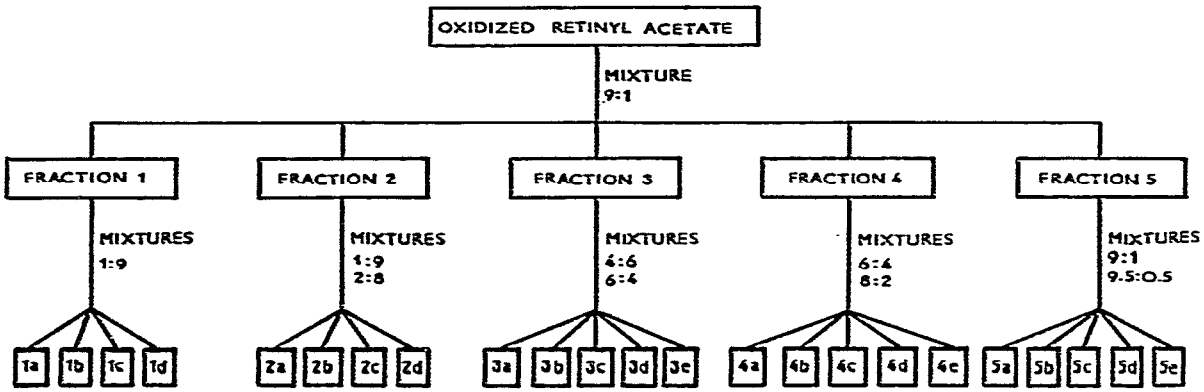

Fig. 1. Chromatography of oxidized retinyl acetate. Preparative layers of silica gel HR (3 mm); mobile phase, *n*-hexane–diethyl ether (indicated by "mixtures") in different proportions; UV detection at 366 nm.

TABLE I

SUBFRACTIONS OBTAINED FROM OXIDIZED RETINYL ACETATE

Fraction No.	*n-Hexane–diethyl ether ratio*	R_F	*UV detection (366 nm)*
1	9:1	0.00–0.10	Pale yellow
1a	1:9	0.00–0.10	Greyish white
1b	1:9	0.10–0.45	Yellow-green
1c	1:9	0.45–0.65	Yellow
1d	1:9	0.65–1.00	Yellow-green
2	9:1	0.10–0.30	Yellow
2a	1:9	0.00–0.10	Greyish white
2b	1:9	0.10–0.45	Yellow-green
2c	2:8	0.30–0.45	Yellow
2d	2:8	0.50–0.80	Yellow-green
3	9:1	0.30–0.55	Yellow-orange
3a	4:6	0.00–0.20	Yellow-green
3b	4:6	0.20–0.30	Yellow
3c	6:4	0.30–0.45	Yellow
3d	6:4	0.45–0.65	Orange-yellow
3e	6:4	0.70–1.00	Yellow-green
4	9:1	0.55–0.85	Yellow-green
4a	6:4	0.15–0.40	Yellow
4b	6:4	0.60–0.75	White
4c	8:2	0.50–0.60	Yellow
4d	8:2	0.60–0.90	Yellow-green
4e	8:2	0.90–1.00	Yellow, brown
5	9:1	0.85–1.00	Brown
5a	9:1	0.20–0.30	Yellow
5b	9:1	0.30–0.55	Yellow-white
5c	9:1	0.55–0.80	Yellow-green
5d	95:5	0.60–0.75	Yellow
5e	95:5	0.70–1.00	Brown

Fraction 3 was developed with *n*-hexane–diethyl ether (4:6). Five zones resulted: a pale yellow-green zone representing fraction 3a (R_F = 0.00–0.20), a bright yellow narrow zone (3b, R_F = 0.20–0.30) followed by another darker yellow zone (3c, R_F = 0.40–0.65) and an orange-yellow zone (3d, R_F = 0.65–0.85) which was yellow in daylight. The front was formed by a yellow-green compound (3e, R_F = 0.85–1.00). Subfractions 3a and 3b were developed once with *n*-hexane–diethyl ether (4:6) and subtractions 3c, 3d and 3e twice with *n*-hexane–diethyl ether (6:4) (R_F values in Table I).

Fraction 4 was first developed with *n*-hexane–diethyl ether (8:2), yielding five subfractions: a yellow zone at the start (4a, R_F = 0.00–0.10), then a narrow pale white zone (4b, R_F = 0.15–0.30), which was followed by a yellow narrow zone (4c, R_F = 0.35–0.45). The bright broad yellow-green zone (4d, R_F = 0.60–0.90) corresponded to unoxidized retinyl acetate that migrated with the bulk of the oil. The front (4e, R_F 0.90–1.00) separated into two badly resolved (yellow and brown) zones. Their UV spectra corresponded to that of anhydroretinol (fraction 5). Fractions 4e and 5

were therefore combined and subfractionated as follows. Subfractions 4a and 4b were purified by another development with *n*-hexane–diethyl ether (6:4), and fractions 4c and 4d by two developments with *n*-hexane–diethyl ether (8:2) (R_F values in Table I).

Fractions 5 + 4e were developed with *n*-hexane–diethyl ether (9:1), yielding a yellow narrow zone (5a, $R_F = 0.20–0.30$), a yellow-white broad zone (5b, $R_F = 0.30–0.55$) and a bright yellow-green zone of the residual unoxidized retinyl acetate (5c, $R_F = 0.55–0.80$). The yellow and brown zones near the front (5d, 5e) separated well and each was finally purified by double development with *n*-hexane–diethyl ether (95:5). Subfraction 5a was developed once and subfractions 5b and 5c twice with *n*-hexane–diethyl ether (9:1) (R_F values in Table I).

After the separations were completed, the solvents were evaporated and pure fractions 1a–5e (Fig. 1) were obtained. Table I gives their colours on detection at 366 nm and R_F values in the mobile phase that was used for the final development. The subfractions were characterized by UV spectra, fluorimetry and the visible spectra of their complexes with antimony(III) chloride. The results showed that some subfractions represented substances with the same spectral properties (Table II), and they were therefore combined.

TABLE II

OXIDATION PRODUCTS OF RETINYL ACETATE OBTAINED BY PREPARATIVE THIN-LAYER CHROMATOGRAPHY

Substance No.	*Fraction No.*	*UV maximum* (nm)*	*Relative intensity***
1	1a, 2a	262, 272	1:0.953
2	1b, 2b, 3a	242, 278	1:0.965
3	1c, 2c, 3b	242, 283	0.965:1
4	1d, 2d	240, 285	0.968:1
5	3c	285	—
6	3d	242, 285	0.882:1
7	3e	237, 298	0.800:1
8	4a	290, 328, 350, 370	1:0.905:0.778:0.495
9	4b	270, 285(s)	1:0.945
10	4c, 5a	285	—
11	4d, 5c	325	—
12	5b	292	—
13	5d	313, 325(s)	1:0.738
14	5e	351, 371, 392	0.690:1:0.870

* The UV spectra were measured in *n*-hexane. s = shoulder.

** Relative intensities of the UV absorption maxima, the highest peak having a relative intensity = 1.

The procedure therefore yielded 14 oxidation products of retinyl acetate. The detailed interpretation of their spectral characteristics will be the subject of another paper.

Preparative thin-layer chromatography represents the least time-consuming technique for the isolation of these relatively unstable compounds in a pure state.

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