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Note

Comparison between straight and reversed phases in the high-performance liquid chromatographic fractionation of retinol isomers

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The importance of the analysis of retinol and retinyl esters and their geometric isomers, in order to evaluate their activity, has been recognized by many workers in food science and various biological fields.

Many studies of these compounds have been carried out by means of high-performance liquid chromatography (HPLC). McKenzie *et al.*[1] and Halley and Nelson[2] fractionated and identified eleven geometric isomers of methyl retinoates. As far as retinol isomers are concerned, Tsukida *et al.*[3], Paanakker and Groenendijk[4] and Bridges *et al.*[5] separated four geometric isomers, *i.e.*, all-*trans*-, 9-*cis*-, 13-*cis*- and 11-*cis*-retinol. However, the complete and rapid resolution of all possible isomers of retinol still remains a problem.

In this paper a rapid separation of five common isomers of retinol (all-*trans*-, 9-*cis*-, 9,13-di-*cis*-, 13-*cis*- and 11,13-di-*cis*-retinol) is described. In addition HPLC separation of two di-*cis*-isomers of retinol is reported for the first time. 2-Nitrofluorene was used as internal standard[6]. Two different modes (straight and reversed phase) of HPLC have been compared, in order to determine the best system for solving this analytical problem.

EXPERIMENTAL

Apparatus

The liquid chromatographic apparatus consisted of a Series 3 pumping system (Perkin-Elmer, Norwalk, CT, U.S.A.) connected to a column oven (P.E. LC 100). The spectrophotometric detector (P.E. variable-wavelength LC 55B) was equipped with a digital scanner (P.E. LC 55S), which permitted the stop flow recording of the UV-visible spectra of the eluted peaks. Spectra and chromatograms were recorded with a P.E. Model 56 recorder.

Materials and methods

The columns used were 5-μm Si 60 (250 × 4 mm) (E. Merck, Darmstadt, G.F.R.) for straight-phase and 5-μm Supelcosil LC 18 (150 × 4.6 mm) (Supelco, Bellefonte, PA, U.S.A.) for reversed-phase chromatography. A Supelco precolumn filled with 40-μm pellicular packing (ODS or silica) was always used in order to protect the analytical columns.

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All-*trans*-retinol (puriss.) and 2-nitrofluorene (purum) were purchased from Fluka (Buchs, Switzerland). 9-*cis*-Retinal was obtained from Sigma (St. Louis, MO, U.S.A.).

Silver nitrate and all the solvents (*n*-hexane, *n*-heptane, dioxan, ethyl methyl ketone, methanol and ethanol) were of analytical reagent grade (E. Merck) and were used without further purification.

Test mixture

In order to compare the two selected chromatographic modes, a mixture of retinol isomers, starting from the two available pure standards of all-*trans*-retinol and 9-*cis*-retinal, was prepared as described below.

All-*trans*-retinol in heptane was submitted to controlled photoisomerization as suggested by Halley and Nelson[7]. The obtained solution of retinol isomers was evaporated under a gentle stream of nitrogen and the retinols then redissolved in ethanol.

The starting solution of 9-*cis*-retinal had previously been tested by HPLC and found to be contaminated (about 10%, w/w) with 9,13-di-*cis*-retinal. Thus, the corresponding retinol solution, obtained by reduction with sodium borohydride according to Hubbard *et al.*[8], contained 9-*cis*- and a minor quantity of 9,13-di-*cis*-retinol. This solution was added to that obtained by photoisomerization. After standing, an ethanol solution containing five major retinol isomers and three other isomers in small quantity was obtained. This test solution was stored under nitrogen in the dark at −20°C. Test solutions remained stable for over 2 months under such conditions.

RESULTS AND DISCUSSION

Isomer identification

Isomer identification was achieved as follows.

Spectral data. The spectrum in the region of 300–350 nm was recorded for each major eluting peak. The spectral shapes and wavelengths of the absorbance maxima are as expected[8,9] (Table I). There is practically no shift of the absorbance maxima of the retinols nor of the spectral shapes in the different solvent systems used (water–methanol or hexane–dioxan). However, a shift of λ_{max} and a change in shape were observed for 2-nitrofluorene.

Internal standard. Coelution of the test mixture with the two known pure isomers all-*trans*- and 9-*cis*-retinol was performed. All-*trans*- and 9-*cis*-retinols are the

TABLE I

UV SPECTRAL DATA FOR RETINOL ISOMERS

Compound	λ_{max} *(nm)*		
	In ethanol[8]	*In hexane–dioxan (75:25) at 20°C*	*In water–methanol (20:80) at 20°C*
11,13-Di-*cis*-retinol	312	312	311.5
13-*cis*-Retinol	328	328.5	328
9,13-Di-*cis*-retinol	324	324.5	—
9-*cis*-Retinol	323	323.5	—
All-*trans*-retinol	325	326	325
2-Nitrofluorene	—	323.5	330

last to be eluted and their separation is difficult. Our 9-*cis*-compound was slightly contaminated with 9,11-di-*cis*-retinol, and its coelution with the test mixture permitted the positive identification of the 9-*cis*- and 9,13-di-*cis*-peaks.

Comparison of relative retention times. The elution order of retinol compounds is already known. Methyl retinoates follow the order reported by Halley and Nelson[2]. Retinols follow the same order but, as pointed out by Paanakker and Groenendijk[4], the order of the 13-*cis*- and 11-*cis*-isomers is inverted compared to the correspondent esters or aldehydes. However, 11-*cis*-retinol (as well as the more hindered 7-*cis*-retinol) was not expected to be present in detectable amounts in our test mixture, owing to the conditions used in the photoisomerization[3,7]. Therefore, in our sample solution, the elution order was expected[2–4] to be as follows: 2-nitrofluorene (internal standard); 11,13-di-*cis*-; 13-*cis*-; 9,13-di-*cis*; 9-*cis*-; all-*trans*-retinol. Three other minor peaks were observed. According to Halley and Nelson[7] one would expect to find in such a mixture of photoisomerized retinols also the 9,11,13-tri-*cis*- and the 13-*cis*-(5 → 10)-photocyclized-retinol. However, we did not try to identify these minor peaks.

Reversed-phase HPLC

This chromatographic mode has previously been used for resolving all-*trans*- and 13-*cis*-retinol[10]. The same eluent system of water–methanol mixtures was adopted, several different percentages of methanol (from 65 to 95%, v/v) being investigated. The best chromatogram obtained in this mode is presented in Fig. 1. Efforts to improve the resolution in this elution mode included the use of silver ions. Silver nitrate ($58.9 \cdot 10^{-3}$ *M*) dissolved in water used in the mobile phase had no appreciable effect on the chromatogram. De Ruyter and De Leenheer[11] observed an increase in selectivity of the reversed-phase system for retinyl esters when using silver ions. They also noticed no effect of silver ions on the retention times of retinyl esters with a saturated fatty acid chain, although the retinyl moiety contains five double bonds; they used the all-*trans*-retinyl moiety. This lack of any effect of silver ions in the case of retinol isomers was confirmed.

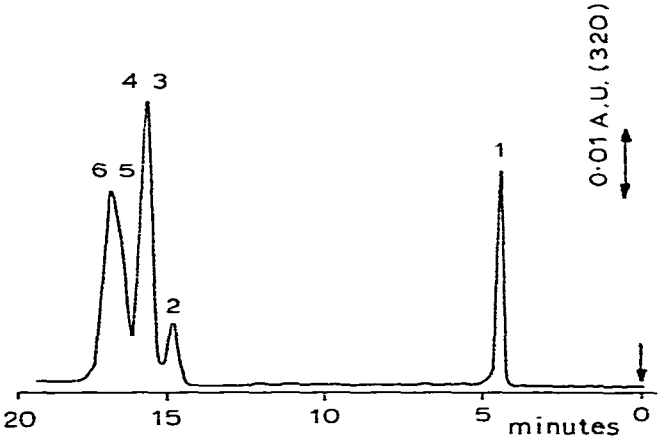

Fig. 1. Chromatogram of a text mixture of five *cis-trans*-isomers of retinol. Column: Supelcosil LC 18 (150 × 4.6 mm). Mobile phase: water–methanol (20:80); pressure 3.8 MPa; flow-rate 1 ml/min. Temperature 40 C. Wavelength: 320 nm. Peaks: 1 = 2-nitrofluorene (internal standard); 2 = 11,13-di-*cis*-retinol; 3 = 13-*cis*-retinol; 4 = 9,13-di-*cis*-retinol; 5 = 9-*cis*-retinol; 6 = all-*trans*-retinol.

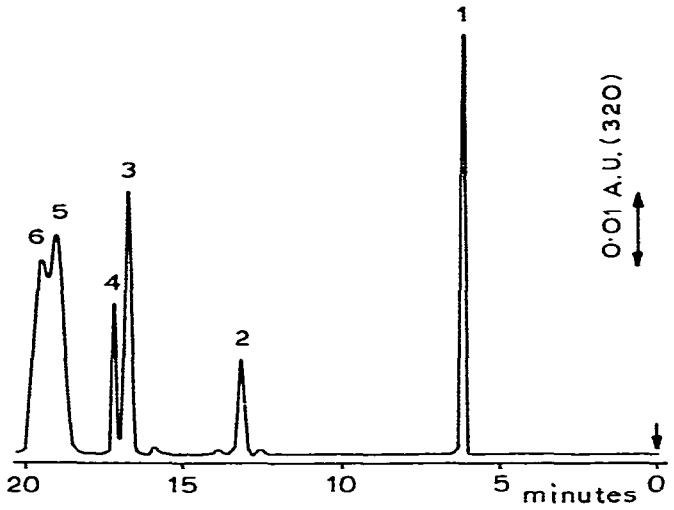

Fig. 2. Chromatogram of a test mixture of five *cis-trans*-isomers of retinol. Column: Merck Si 60 (250 × 4 mm). Mobile phase: *n*-hexane–dioxan (92:8); pressure 4.1 MPa: flow-rate 1 ml/min. Other details as in Fig. 1.

Straight-phase adsorption HPLC

Adsorption HPLC has been applied to retinol isomers using hexane-dioxan as eluent. The results are presented in Fig. 2. Comparing the chromatograms of Figs. 1 and 2, it is clear that adsorption chromatography gives better resolution of retinol isomers than the reversed-phase system. Several different percentages of the dioxan

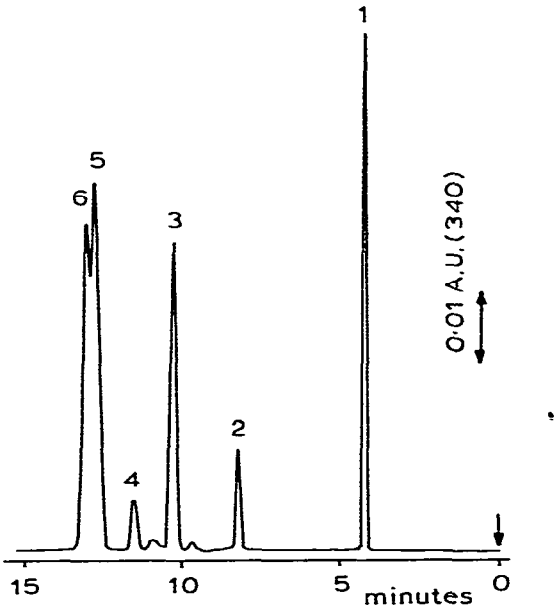

Fig. 3. Chromatogram of a test mixture of five *cis-trans*-isomers of retinol. Column as in Fig. 2. Mobile phase: *n*-hexane–methyl ethyl ketone (85:15); pressure 3.8 MPa; flow-rate 1 ml/min. Temperature: 40°C. Wavelength: 340 nm. Peaks as in Fig. 1.

modifier (from 6 to 15%, v/v) have been tried in order to improve resolution. Peak 4 (Fig. 2) shifted quickly from the cluster of peaks 5 and 6 towards peak 3 on decreasing the percentage of dioxan. However, with this elution system it has not been possible to obtain a resolution factor greater than about 0.4 between the 9-*cis*- and the all-*trans*-isomers.

Methyl ethyl ketone was also adopted as a modifier. The UV cut-off of this compound occurs at 330 nm, therefore the detection of the eluting peaks was shifted to 340 nm. This resulted in a loss of sensitivity of about 20%. There are two advantages in choosing methyl ethyl ketone: the low viscosity [0.30 *versus* 1.54 cP (20°C) of dioxan] allows better mass transfer; the low adsorptive energy (0.51 *versus* 0.56 $E^{\circ}Al_2O_3$, where $E^{\circ}Al_2O_3$ is the adsorptive energy of the considered eluent referred to Al_2O_3) allows the use of higher and a wider range of percentages of the modifier and a more reproducible pumping system. Fig. 3 shows a chromatogram obtained using methyl ethyl ketone–hexane as mobile phase. A better resolution between peaks 3 and 4 was obtained with this system, and there was no shift of relative retention times.

It is concluded that straight-phase HPLC, relative to reversed-phase HPLC, enables a rapid and better resolution of retinol isomers.

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REFERENCES

1 R. M. McKenzie, D. M. Hellwege, M. L. McGregor, N. L. Rockley, P. J. Riquetti and E. C. Nelson, *J. Chromatogr.*, 155 (1978) 379.
2 B. A. Halley and E. C. Nelson, *J. Chromatogr.*, 175 (1979) 113.
3 K. Tsukida, A. Kodama, M. Ito, M. Kawamoto and K. Takahashi, *J. Nutr. Sci. Vitaminol.*, 23 (1977) 263.
4 J. E. Paanakker and G. W. T. Groenendijk, *J. Chromatogr.*, 168 (1979) 125.
5 C. D. B. Bridges, S. L. Fong and R. A. Alvarez, *Vision Res.*, 20 (1980) 355.
6 F. Zonta, B. Stancher and C. Calzolari, *Rassegna Chimica*, 6 (1980) 301.
7 B. A. Halley and E. C. Nelson, *J. Vit. Nutr. Res.*, 49 (1979) 347.
8 R. Hubbard, P. K. Brown and D. Bownds, *Methods Enzymol.*, 18C (1971) 615.
9 C. D. Robeson, J. D. Cawley, L. Weisler, M. H. Stern, C. C. Eddinger and A. J. Chechak, *J. Amer. Chem. Soc.*, 77 (1955) 4111.
10 A. M. McCormick, J. L. Napoli and H. F. Deluca, *Anal. Biochem.*, 86 (1978) 25.
11 M. G. M. de Ruyter and A. P. de Leenheer, *Anal. Chem.*, 51 (1979) 43.