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High-performance liquid chromatography of non-polar retinoid isomers

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Abstract

Normal-phase high-performance liquid chromatograms of retinal, retinol and retinyl palmitate isomers using *n*-heptane–*tert*.-butyl methyl ether as mobile phase are presented. Methods for the synthesis of various isomers of these retinoids are described. This enables one to produce standard chromatograms and to select various isomers for cochromatography and the identification of the various peaks under study. Assignment of the peaks is based on chromatograms published previously in this journal. For the main isomers it is possible to get baseline separation of the commonly occurring isomeric forms in a reasonably short analysis time. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Retinal, retinol and retinyl palmitate isomers play an eminent role in visual transduction and regeneration processes [1–3], and in the bacteriorhodopsin cycle [4], these retinoids (e.g. the amount of vitamin A) are the subject of research in different fields such as in the analysis of samples of food [5,6], human serum and liver [6,7], human skin [8] or corpus luteum [9]. Especially the isomeric forms of retinal and retinol are important in vision, and a tool to distinguish such retinoids is high-performance liquid chromatography (HPLC). This paper will show how to prepare the more common isomers and how to analyse them and produce standard chromatograms for one's own use.

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2. Experimental

2.1. Preparation of retinoid samples

Only a few retinoids are commercially available, i.e. all-*trans*-, 9-*cis*-, 13-*cis*-retinal, further all-*trans*- and 13-*cis*-retinol and all-*trans*-retinyl palmitate (Sigma, Fluka, Aldrich). Therefore a method is described on how to prepare other isomers and to calibrate the own chromatograms. For further details see Nöll [10].

2.2. Retinal isomers

About 2 mg all-*trans*-retinal are dissolved in 2 ml ethanol or 2,2,2-trifluoroethanol and illuminated in an optical cuvette on ice for 40 min with white light (e.g. 60 W Osram desk bulb). Then 100 μl of this irradiated sample are dried under nitrogen gas and are redissolved in 1 ml *n*-heptane. In order to get a

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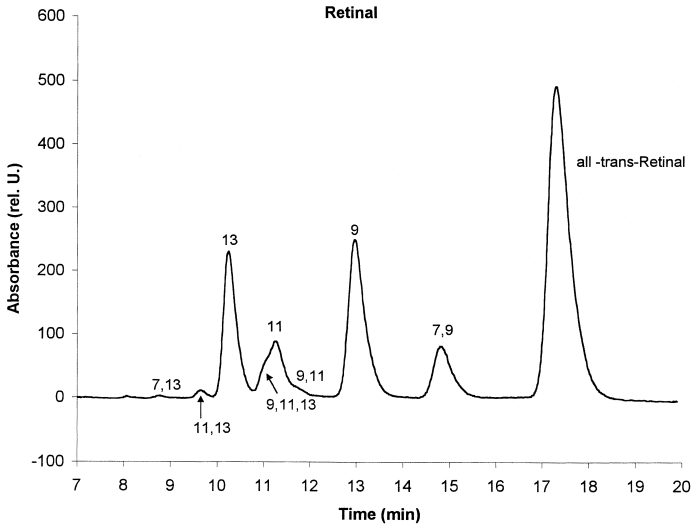

Fig. 1. Chromatogram of retinal isomers illuminated in 2,2,2-trifluoroethanol with the mobile phase *n*-heptane–*t*BME (98:2, v/v), flow-rate 2 ml/min, 109 bar, measuring wavelength 371.34 nm on a DuPont column Zorbax SIL, 25 cm×4.6 mm I.D., packed with Si60 porous spherical particles of 5–6 μm in diameter. The elution order (retention time in min) is 7,13-di-*cis*- (8.74), 11,13-di-*cis*- (9.65), 13-*cis*- (10.27), 9,11,13-tri-*cis*- (11.08), 11-*cis*- (11.28), 9,11-di-*cis*- (11.65), 9-*cis*- (12.98), 7,9-di-*cis*- (14.83), all-*trans*-retinal (17.33). Peak detection is in the range of 0.1 ng for all-*trans*-retinal.

well separated chromatogram as shown in Fig. 1, 5–10 μl injection volume is sufficient. The samples are then dried and stored in a freezer at −25°C.

In ethanol mainly mono-*cis*- (13-, 11-, 9-) and di-*cis*- (7,9-) isomers will appear, in 2,2,2-trifluoroethanol preferentially the more unstable di-*cis*- (13,11-, 9,11-) and tri-*cis*-isomers (9,11,13-) will arise. The most unstable isomer is 7-*cis*-retinal, which decays within minutes (see Fig. 2 in [10]). One can obtain this isomer by illuminating all-*trans*-retinal for 30 min in the solvent acetonitrile as described above (see Fig. 2).

2.3. Retinol isomers

Illuminating all-*trans*-retinol in the above mentioned solvents is not very effective, since only some 13-*cis*- and 9-*cis*-retinol-isomers will arise. A better way is to create the various retinal isomers as described in the previous section and reduce them to the corresponding retinol isomers. For instance, 1 mg retinal is exposed to white light in 1 ml ethanol, then about 10 mg $NaBH_4$ and 2 ml petroleumbenzine are added, and the sample is gently shaken by hand, cooled on ice, the upper benzine layer is drawn off and washed twice with 2 ml water, and finally dried with nitrogen gas. The sample is again resuspended in 1 ml *n*-heptane, 10–20 μl are taken for chromatography (see Fig. 3).

On the other hand, it was not possible to isolate 7-*cis*-retinol, neither by illumination of all-trans-retinol nor by reduction of a 7-*cis*-retinal sample. This isomer is obviously very unstable.

2.4. Retinyl palmitate isomers

Two methods allow the synthesis of all-*trans*-retinyl palmitate isomers, and other retinol esters:

2.4.1. Synthesis by illumination

About 3 mg all-*trans*-retinyl palmitate are dissolved in 3 ml ethanol (or in 2,2,2-trifluoroethanol or in acetonitrile) and are illuminated for 40 min as described above. Using this method, 4 (in ethanol) or 5 isomers can be detected in small amounts. *n*-heptane is not suitable as solvent in this case.

2.4.2. Synthesis from retinal

Twenty mg of retinol samples, originating from the retinal sample under study, are dissolved in a

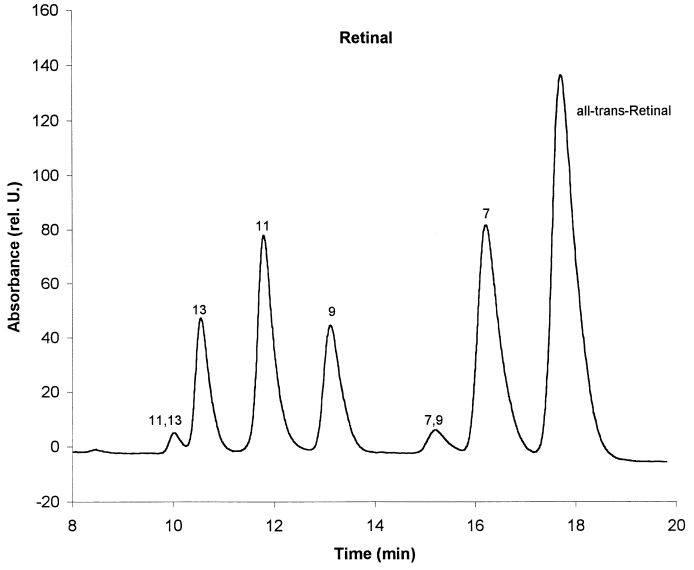

Fig. 2. Chromatogram showing the 7-*cis*-retinal with a flow-rate of 1.5 ml/min (*n*-heptane–*t*BME, 97:3, v/v), 83 bar. About 8 times 7-*cis*-retinal fractions of illuminated all-*trans*-retinal in the solvent acetonitrile were collected. Other conditions as in Fig. 1. The elution order (retention time in min) is 11,13-di-*cis*- (10.03), 13-*cis*- (10.55), 11-*cis*- (11.80), 9-*cis*- (13.14), 7,9-di-*cis*- (15.22), 7-*cis*- (16.24), all-*trans*-retinal (17.73).

mixture of 4 ml dichloromethane and 0.12 ml palmitoyl chloride in a two neck distilling flask under argon atmosphere. After 10 min, a mixture of 20 ml double-distilled water and 80 ml diethyl ether are added. The ether phase is drawn off in a separatory funnel and washed twice with 60 ml aqua bidest, then 100 ml distilled light petroleum (b.p. 40–60°C) are added and finally dried for 3 h with dehydrated sodium sulfate. After filtering and evaporation 5 ml *n*-heptane are added and about 10 μl used for chromatography. Again it was not possible to create the 7-*cis* isomer.

3. Chromatograms

We applied adsorption chromatography at 25°C in deep red light in the isocratic mode using a HPLC System 400 (Kontron Instruments). All components and details are described in a previous publication [10]. Here we stress only the eluent system *n*-heptane–*tert*.-butyl methyl ether (*t*BME), because other commonly used eluent systems are toxic and are not recommended for standard routines.

3.1. Retinal isomers

Fig. 1 shows a chromatogram of 8 retinal isomers with the mobile phase *n*-heptane-*t*BME (98:2, v/v) prepared as described in Section 2.1 above. The assignment of peaks was done according to Nöll [10]. Separation of the 7-*cis*-retinal isomer is possible by lowering the flow-rate to 1.5 ml/min at an eluent ratio of 97:3 (v/v). Fig. 2 shows for the first time a 7-*cis*-chromatogram, obtained by collection of several retinal fractions that were illuminated for 30 min in acetonitrile and measured immediately.

The mixing ratio of 98:2 (v/v) is near the limit of some gradient formers, which work reliably in a middle range. How stable and even higher (e.g. 99.5:0.5, v/v) ratios can be used is described in Section 3.3.

In order to get most isomers in one run, a ratio of 98:2 (v/v) should be chosen and elution of all-*trans* takes about 19 min. If one is interested only in the main isomers 13-*cis*-, 11-*cis*-, 9-*cis*- and all-*trans*-retinal a ratio of 92:8 (v/v) makes baseline separation possible for these isomers, the run takes only 5.9 min (maximum of all-*trans*-retinal). At 90:10 (v/v) they are not separated any more. At a ratio of

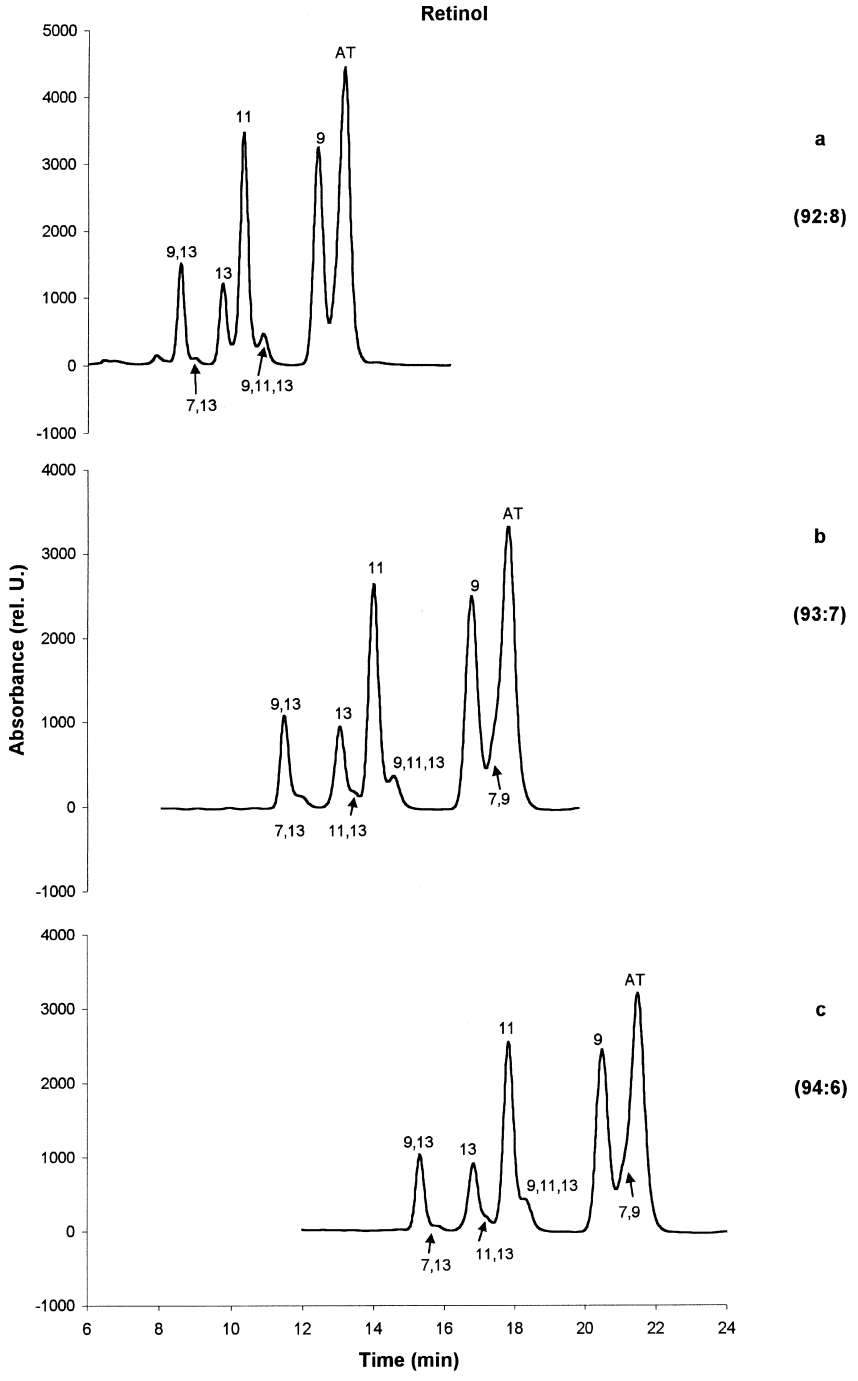

Fig. 3. Chromatogram of retinol isomers with the mobile phase *n*-heptane–*t*BME in (a) (92:8, v/v); flow-rate 3 ml/min; 139 bar; 325 nm, in (b) (93:7, v/v); 3 ml/min; 136 bar, and in (c) (94:6); 3 ml/min; 140 bar, DuPont column. Elution order [retention time in min for (a); (b); (c)]: 9,13=9,13-di-*cis*-retinol (8.55; 11.50; 15.30), 7,13=7,13-di-*cis*-retinol (8.96; 11.90; 15.79), 13=13-*cis*-retinol (9.70; 13.06; 16.83), 11,13-di-*cis*-retinol (–; 13.41; 17.14), 11=11-*cis*-retinol (10.26; 14.00; 17.82), 9,11,13=9,11,13-tri-*cis*-retinol (10.78; 14.60; 18.28), 9=9-*cis*-retinol (12.31; 16.80; 20.48), 7,9-di-*cis*-retinol (12.75; 17.33; 21.07, hidden by AT) and AT=all-*trans*-retinol (13.07; 17.82; 21.50).

93:7 (v/v) (resp. 96:4) the maximum of all-*trans*-retinal elutes at 7.6 (resp. 11.8) min.

3.2. Retinol isomers

Separation of retinol isomers is shown for three eluent ratios in Fig. 3. The best separation is achieved at *n*-heptane–*t*BME (94:6, v/v), but it takes 23 min at a flow-rate of 3 ml/min. If one does not need all the di-*cis*- and tri-*cis* isomers and wants shorter elution times, one can increase either the *t*BME fraction (Fig. 3(b) and (a)) or the flow-speed, but then a relatively high pressure is needed (188 bar) (cf. Fig. 3B in [11]).

3.3. Retinyl palmitate isomers

In Fig. 4 up to 6 retinyl palmitate isomers can be detected, but here the separation is not down to the baseline. The run takes 5 min at *n*-heptane–*t*BME (99:1, v/v). Retinyl palmitate chromatograms are very difficult to establish. In order to handle such extreme elution ratios which lead to mixing problems of the HPLC pumps, we recommend not to use the clean solvent but rather a mixture of the two eluents for such a small portion in the second delivering system. That means feeding the first solvent line e.g. 96 parts of *n*-heptane and the second solvent line a mixture of 3 parts of *n*-heptane and one part *t*BME [96:(3+1)] results in a final eluent ratio *n*-heptane–*t*BME of 99:1 (v/v). This technical mixing ratio 96:4 (v/v) is then easier to handle for the apparatus. But one has to be aware that the composition of the mixed solvents in the second line has to be constant and does not change in time by evaporating one of them slowly, which would cause slowly changing chromatograms.

For retinyl palmitate the correction factors for retinol absorbance maxima should be used.

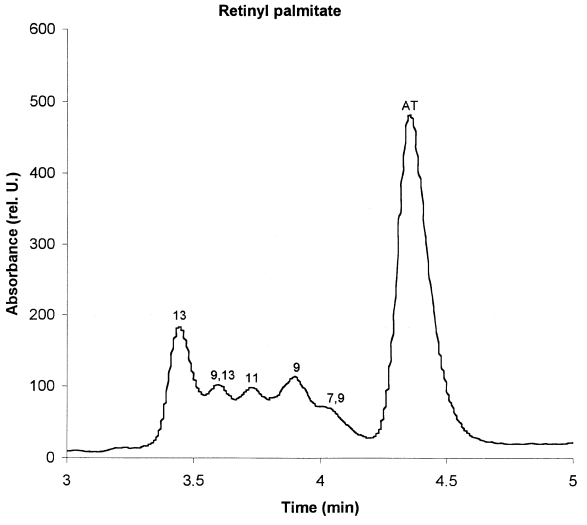

Fig. 4. Chromatogram of 6 retinyl palmitate isomers with the mobile phase *n*-heptane–*t*BME (effective 99:1, v/v), flow-rate 2 ml/min, 108 bar, 325 nm, DuPont column. Elution order (retention time in min): 13=13-*cis*- (3.44), 9,13=9,13-di-*cis*- (3.60), 11=11-*cis*- (3.73), 9=9-*cis*- (3.90), 7,9-di-*cis*- (3.99) and AT= all-*trans*-retinyl palmitate (4.36).

4. Discussion

The two critical parameters for separation of retinoid isomers are the choice of a proper column and of the eluent mixture. A comparison of the DuPont column with a Merck column is given in [11].

Many papers dealing with HPLC analysis of retinoids use various solvent systems such as freon 113–methyl *tert*.-butyl ether, hexane–diethyl ether, hexane–dioxane. A comparison of 52 publications dealing with retinol chromatography (mainly separation of 13-*cis*-, 11-*cis*-, all-*trans*-retinol) is given by Nöll and Kalinowski [11]. Due to its higher vapour pressure we preferred *t*BME to diethyl ether. Since 1,4-dioxane is supposedly carcinogenic and *n*-hexane is toxic [12] we used only *n*-heptane for safety reasons. We therefore recommend only this eluent mixture for further studies. It allows the analysis of many isomeric forms of retinal, retinol and retinyl palmitate and produces standard chromatograms. We used the isocratic mode because otherwise the results would not be reproducible, especially near the limit of the working range of the pump system. The relatively simple preparation of many rather stable isomers described in Section 2 allows calibration of the chromatographic runs. We

refer to Ref. [10] for detailed information on identification and elution orders for retinal and retinol isomers and on different mobile phases at various eluent ratios. The present paper extends the measurements given there to baseline separation of 7-*cis*-retinal which was not shown in literature so far and separation of isomers of retinyl palmitate, which should be an example for retinyl esters in general.

The information given here should help researchers working in the field of vision and vitamin A analysis to choose the parameters which suit best the purpose of the study in question. If peak resolution and baseline separation are wanted or specific isomers have to be separated, the choice of mobile phase and eluent ratio might be different from a situation where only a rough estimation of the complete vitamin A content of a sample in clinical application is needed. In the first case, long elution times and high solvent consumption are the consequence (e.g. Fig. 3). Since 13-*cis*-, 9-*cis*- and all-*trans*- are the main representatives of isomeric forms, the amount of the many di-*cis*- and tri-*cis*- can be neglected under such circumstances and a very fast run can be achieved. The data presented here could help to find a good compromise for all the different parameters which influence each other. This is also a simple method for producing many isomers, in order to identify unknown samples in an easy way with own standard chromatograms.

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