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A reversed-phase high-performance liquid chromatographic method to analyze retinal isomers

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Abstract

A high-performance liquid chromatographic (HPLC) procedure was developed to separate all-*trans*-, 13-*cis*-, 11-*cis*- and 9-*cis*-retinal isomers. Two reversed-phase Vydac C_{18} columns in series were used with an isocratic solvent system of 0.1 *M* ammonium acetate–acetonitrile (40:60, v/v) as mobile phase and all-*trans*-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraene-1-ol (TMMP) as internal standard. Prior to HPLC, the retinal isomers were efficiently extracted in their original isomeric conformation using dichloromethane–*n*-hexane in the presence of formaldehyde. This technique is suitable for the assay of 11-*cis*- and all-*trans*-retinal isomers in retina.

Keywords: Retinal isomers; Vitamin A

1. Introduction

Vitamin A is a major component of the visual cycle. Geometric isomers of retinal (vitamin A aldehyde) (Fig. 1) play an important role as the chromophoric group of the visual pigment rhodopsin [1–3]. All-*trans*-retinal is released from rhodopsin after light bleaching and is then reprocessed to the 11-*cis* configuration necessary for the visual pigment regeneration. Methods for the separation and quantification of retinal isomers in their endogenous geometric configuration are therefore essential in visual process studies, and high-performance liquid chromatographic (HPLC) methods have been widely used for this purpose [4,22].

The HPLC techniques described in the literature for the separation of the various conformations of retinal sometimes use a reversed-phase mode [5–7], but mostly a normal-phase mode [8–22]. Many isocratic or gradient elution normal-phase HPLC methods have been described for the separation of retinal isomers. However, normal-phase chromatography has disadvantages including problems with gradients, varying retention times and need for more clean-up [4].

Only a few reports refer to reversed-phase HPLC

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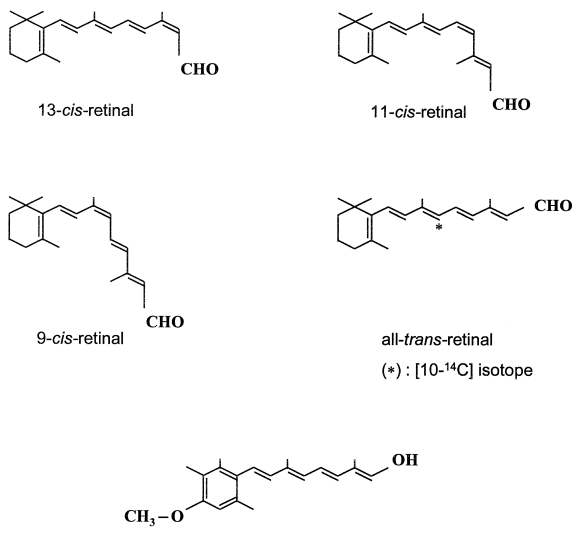

TMMP: all-*trans*-9-(4-methoxy-2, 3, 6-trimethylphenyl)-3, 7-dimethyl-2, 4, 6, 8-nonatetraene-1-ol

Fig. 1. Structures of retinal isomers and TMMP (internal standard).

methods for analyzing retinal isomers, and only all-*trans*-, 13-*cis*- and 9-*cis*-isomers have been separated [6,7]. No study of the separation of the major isomers 11-*cis*- and all-*trans*-retinal was reported in the reversed-phase mode. Accordingly, we investigated whether reversed-phase HPLC could be used to separate all-*trans*-, 13-*cis*-, 11-*cis*- and 9-*cis*-retinal isomers.

Prior to HPLC analysis, retinal isomers must be extracted, and a major problem during the extraction of retinal is its nonspecific isomerization. To overcome this, we used the formaldehyde-based extraction procedure of Suzuki et al. [16].

In this paper, we describe a new isocratic reversed-phase HPLC technique allowing the separation and quantitative analysis of all-*trans*-, 13-*cis*-, 11-*cis*- and 9-*cis*-retinal isomers.

2. Experimental

2.1. Instrumentation and equipment

HPLC analysis was carried out on a Waters system equipped with a pump (Waters 610 Fluid Unit), a regulator (Waters 600 Controller), an autosampler (Waters 717 plus) and a UV–Visible photodiode-array detector (Waters 996). Millennium software (version 2.1) was used for programmation and calculations. Detection was performed by scanning from 200 to 500 nm. Absorption spectra of pure authentic standards (all-*trans*, 13-*cis* and 11-*cis*) were built between 200 and 500 nm using the diode-array detector. These spectra were stored in the software spectral library and used for peak identification, together with retention times. Radioactivity of samples was measured using a Packard 4550 scintillation counter when [^{14}C]-all-*trans*-retinal was used as the internal standard.

A Vydac TP 54 C_{18} (5 μm, 250×4.6 mm, Vydac, Hesperia, CA, USA) column and a Vydac TP 54 C_{18} (5 μm, 150×4.6 mm, Vydac, Hesperia, CA, USA) column were used in the reversed-phase mode for the separation of the retinal isomers. The mobile phase was a mixture of 0.1 *M* ammonium acetate–acetonitrile (40:60, v/v). The flow-rate was set at 1.6 ml min^{-1}.

A LiChrosorb Si 60 (5 μm, 250×4.6 mm, Touzart and Matignon, Vitry-sur-Seine, France) normal-phase column was used to validate the retinal extraction using hexane–diethyl ether (98:2, v/v) as eluent at a flow-rate of 2 ml min^{-1}.

2.2. Chemicals and reagents

Methanol, isopropanol, acetonitrile, *n*-hexane and ethyl ether were obtained from Carlo Erba (Chaussée-du-Vexin, France) and dichloromethane from Mallinckrodt (Deventer, Holland). All the solvents were HPLC grade. Formaldehyde (aqueous solution, 37% w/v) was purchased from Prolabo (Fontenay-sous-Bois, France). Ammonium acetate 7.5 *M* came from Sigma Chemical Co. (St. Louis, USA).

2.3. Standards and samples

Retinoids used as standards were obtained as follows: Pure standards of 13-*cis*- and all-*trans*-retinal were purchased from Sigma Chemical Co (St. Louis, USA). Pure standard of 11-*cis*-retinal was synthesized according to Wada et al. [23] (Pharmaceutical University, Kobe, Japan). The [10-^{14}C]-all-*trans*-retinal (9 mCi $mmol^{-1}$) was synthesized ac-

cording to Azim et al. [24] (INSERM U484, Clermont-Ferrand, France). The all-*trans*-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraene-1-ol (TMMP) was a gift from Hoffmann–La Roche (Basel, Switzerland).

A mixture of retinal isomers was prepared from the all-*trans*-isomer by photoisomerization: Under white light, a solution of all-*trans*-retinal in acetonitrile was irradiated for 48 h with a 60 W lamp. Using 200 MHz nuclear magnetic resonance (NMR) spectroscopy, we made sure we had obtained a mixture of 13-*cis*-, 11-*cis*-, 9-*cis*- and all-*trans*-retinal according to Liu et al. [25] and Lugtenburg [26].

The retinas were prepared from eyes of adult Long–Evans rats. Animals were sacrificed after one night dark adaptation and eyes were enucleated quickly. The retinas were then isolated, suspended in phosphate buffer saline, sonicated and stored at −80°C until extraction. All the operations were carried out under dim red light (λ>620 nm).

2.4. Extraction

Retinal isomers were extracted by dichloromethane–*n*-hexane in the presence of formaldehyde according to the procedure of Suzuki et al. [16]. The extract was then evaporated to dryness under nitrogen, whereafter the residue was dissolved in 200 μl of hexane, and transferred into an autosampler vial; 180 μl was injected into the chromatographic system. All the operations were carried out under dim red light (λ>620 nm).

3. Results and discussion

The extraction procedure, using dichloromethane–*n*-hexane in the presence of formaldehyde [16], had been validated by normal-phase HPLC on the mixture of retinal isomers obtained from the photoisomerized retinal (for the validity of using this kind of mixture, see e.g., Bridges et al. [2] and Nöll [22]). To make sure the retinal isomers remained in their original conformation during the extraction, we compared the percentages of the isomers obtained with and without extraction. No significant differences were observed in the ratios of 13-*cis*, 11-*cis*, 9-*cis*, all-*trans* and di-*cis* conformations, which were 21, 16, 20, 38 and 5% respectively. The absence of nonspecific isomerization during the extraction was also confirmed by comparing the UV absorption spectra of the isomers obtained with and without extraction. The recovery of retinal was measured with [^{14}C]-all-*trans*-retinal (Fig. 1), which gave an average extraction yield of 92±7% (mean±SD, n=6).

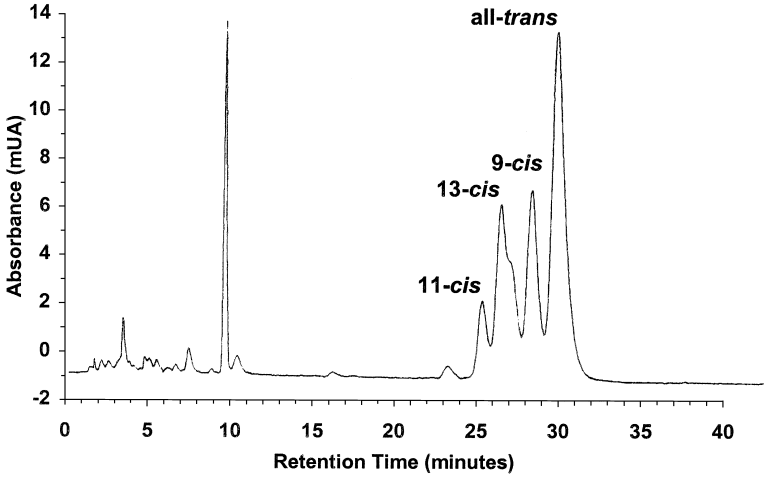

Fig. 2. HPLC separation of photoisomerized retinal in a reversed-phase mode. Columns, Vydac C_{18} (5 μm, 150×4.6 mm) in series with Vydac C_{18} (5 μm, 250×4.6 mm); detection absorption at 380 nm; mobile phase, 0.1 *M* ammonium acetate–acetonitrile (40:60, v/v); flow-rate, 1.6 ml min^{-1}.

To our knowledge, no HPLC studies had been carried out for the separation, in the reversed-phase mode, of the four geometric isomers of retinal: all-*trans*, 13-*cis*, 11-*cis* and 9-*cis*. We set out to determine whether reversed-phase HPLC could be used to separate these isomers. Isocratic 0.1 *M* ammonium acetate–acetonitrile was chosen as the mobile phase for the separation of the retinal isomers. Isocratic elution was preferred to gradient elution because it gave precise and reproducible results [22]. Thus, several columns were tested using the composition ammonium acetate–acetonitrile. We obtained the best separation with a system of two Vydac C_{18} columns in series and a mobile phase 0.1 *M* ammonium acetate–acetonitrile (40:60, v/v; 1.6 ml min^{-1}; 200 bar) at pH 7.5. The isomers all-*trans*, 13-*cis*, 11-*cis* and 9-*cis* of retinal were separated in less than 40 min, as shown by the chromatogram in Fig. 2.

Isomers of the photoisomerized retinal were identified by comparing the retention times and UV absorption spectra of the various peaks with those of known standards. Pure standards of all-*trans*-, 13-*cis*- and 11-*cis*-retinal allowed the identification of the first, second and fourth peak respectively as the 11-*cis*-, 13-*cis*- and all-*trans*-isomer, and the deduction of the third peak as the putative 9-*cis*-isomer

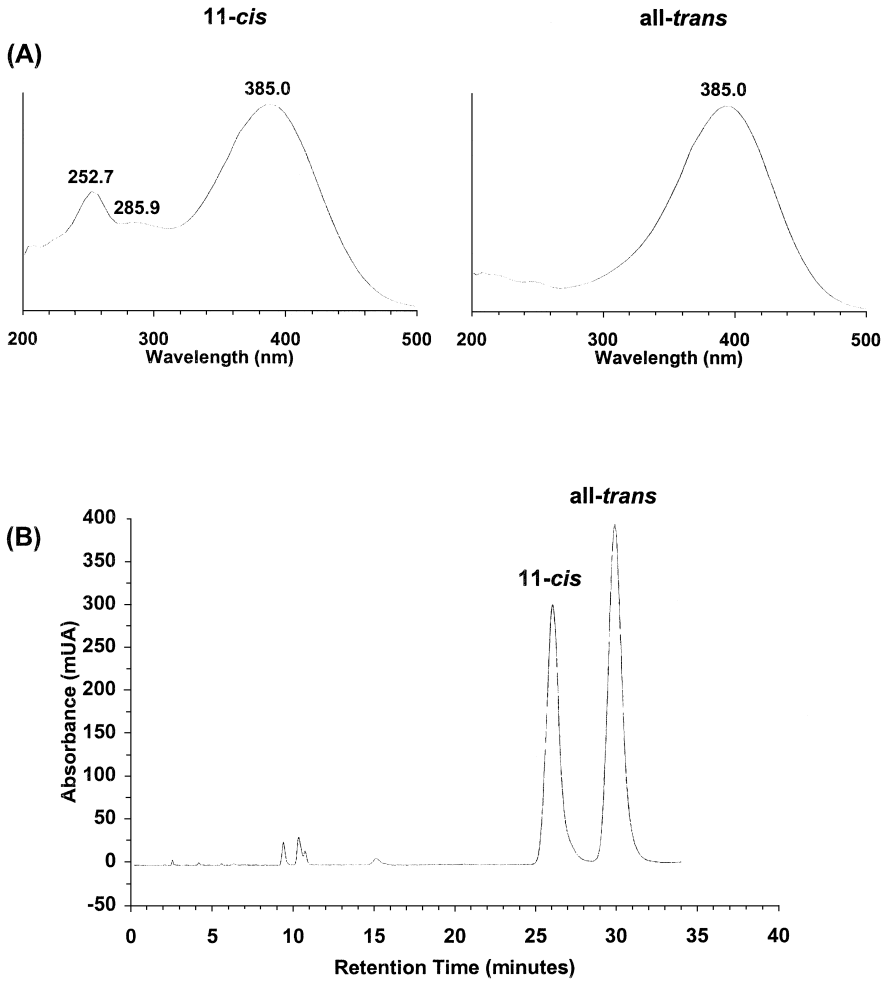

Fig. 3. HPLC separation of pure standards of 11-*cis*- and all-*trans*-retinal in a reversed-phase mode. Columns, Vydac C_{18} (5 μm, 150×4.6 mm) in series with Vydac C_{18} (5 μm, 250×4.6 mm); mobile phase, 0.1 *M* ammonium acetate–acetonitrile (40:60, v/v); flow-rate, 1.6 ml min^{-1}. (A) Absorption spectra corresponding to the 11-*cis*- and all-*trans*-retinal. (B) Detection absorption at 380 nm.

(Fig. 2). The shoulder observed on the second peak could be due to a di-*cis* conformation. Although the separation is not fully convincing, in biological samples 11-*cis*- and all-*trans*-retinal are by far the most frequent isomers and the chromatogram in Fig. 3 shows that the separation obtained from pure standards of these two isomers was well-resolved.

To quantify the extracted retinal, an internal standard was needed. As [^{14}C]-all-*trans*-retinal could not be used as an internal standard, because of its low specific activity (9 mCi mmol^{-1}), several compounds were tested. First we selected molecules with a conformation similar to retinal and therefore with similar behavior during the extraction. Then we eliminated compounds whose retention times were too close to those of the photoisomerized retinal isomers. Thus, β-ionone, acitretin, 3-methyl-7-(1,1,3,3-tetramethyl-5-indanyl)2,4,6-octatrienoic acid (TIMOTA), retinoic acid and TMMP were selected and tested. Solutions containing a mixture of [^{14}C]-all-*trans*-retinal and one of each of the compounds studied were extracted and injected under the conditions described above. The recovery of each compound was measured and compared with that obtained with [^{14}C]-all-*trans*-retinal. The recoveries for β-ionone, acitretin, TIMOTA, retinoic acid and TMMP were 9±8, 47±5, 60±16, 61±4 and 95±11% (mean±SD, n=3) respectively. The best result was obtained with TMMP (Fig. 1), a synthetic analog of retinoids, which was therefore selected as a suitable internal standard.

We determined the detection limit, defined as the amount of analyte giving a peak surface area three times the maximum noise peak of a blank biological sample observed at the retention times of each analyte. The values were about 5 ng (18 pmol) for 11-*cis*-retinal and about 2 ng (7 pmol) for all-*trans*-retinal.

The precision of the method was assessed by the degree of repeatability of the retention times (RT) in a series of several injections during a single session. A good repeatability for the retention times of 11-*cis*-retinal, RT=26.19±0.07 min (n=5); all-*trans*-retinal, RT=29.8±0.1 min (n=5) and TMMP, RT=14.9±0.1 min (n=4) was observed (mean±SD). The coefficients of variation for the retention times were 0.29% for 11-*cis*-retinal, 0.35% for all-*trans*-retinal and 0.97% for TMMP.

In future investigations on retina, we need to be able to assay the 11-*cis*-retinal isomer. It is well known that dark-adapted retina contains almost exclusively the 11-*cis*-retinal chromophore of rhodopsin [27,28]. We therefore tested this new reversed-phase HPLC method on suspensions of

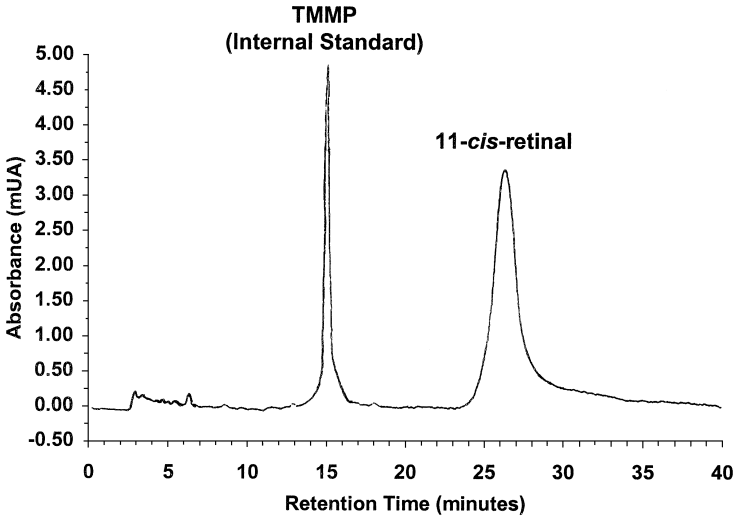

Fig. 4. Chromatogram obtained from HPLC analysis of 11-*cis*-retinal in rat retina. Columns, Vydac C_{18} (5 μm, 150×4.6 mm) in series with Vydac C_{18} (5 μm, 250×4.6 mm); detection absorption at 380 nm; mobile phase, 0.1 *M* ammonium acetate–acetonitrile (40:60, v/v); flow-rate, 1.6 ml min^{-1}.

retinas obtained from rats after dark adaptation, and prepared as indicated in Section 2.3. As expected, we obtained for each assay a peak that we identified as 11-*cis*-retinal (Fig. 4) from its retention time, $RT = 26.0 \pm 0.3$ min ($n = 5$), and its UV absorption spectra. The amount of 11-*cis*-retinal found (1.0 ± 0.3 nmol per whole retina, $n = 5$) was consistent with the values reported in the literature [29,30].

In conclusion, an isocratic reversed-phase HPLC method was developed for the separation of 13-*cis*-, 11-*cis*-, 9-*cis*- and all-*trans*-retinal isomers. The method provided a satisfactory resolution for the separation of 11-*cis*- and all-*trans*-retinal, the most important isomers in retina, and showed good sensitivity, recovery and reproducibility. For these reasons, this method is suitable for the assay of 11-*cis*- and all-*trans*-retinal isomers in retina.

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