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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF *cis–trans* STEREOISOMERIC 3-DEHYDRORETINALS IN THE PRESENCE OF RETINAL ISOMERS

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SUMMARY

A high-performance liquid chromatographic method for the separation of geometric isomers of 3-dehydroretinal in the presence of retinal isomers has been devised. Simultaneous determination of 11-*cis*-retinal and 11-*cis*-3-dehydroretinal was also achieved. *Cis–trans* photoisomerization of 3-dehydroretinal and retinal under the same conditions was examined and the possible importance of the 9-*cis* isomer in the former polyenal series is considered.

INTRODUCTION

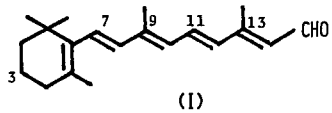
Visual pigments so far found in nature involve an 11-*cis*-retinal or 11-*cis*-3-dehydroretinal chromophore, covalently bound to an apoprotein opsin[1]. For studying the visual process including the former polyenal and its related retinoids, high-performance liquid chromatography (HPLC) has been demonstrated to be the most efficient method as both an analytical[2] and a preparative[3,4] tool. In contrast, there has been no report of the HPLC analysis of the latter polyenal and its related retinoids, mainly because of their unusual instability. Recently, Liu *et al.*[5] were able to separate the 7-*cis* isomer by HPLC from an irradiated mixture of all-*trans*-3-dehydroretinal, whereas chromatographic resolution between the common geometric isomers was poor and the important 11-*cis* isomer was obscured and remained unidentified.

As an extension of our previous studies on the simultaneous HPLC analysis of *cis–trans* isomeric retinals[6], retinols and retinals[7], or 5,6-epoxyretinals[8], we describe here the HPLC analysis of *cis–trans* isomers of retinal (vitamin A_1 aldehyde) (Fig. 1, I) and 3-dehydroretinal (vitamin A_2 aldehyde) (Fig. 1, II) in the presence of each other.

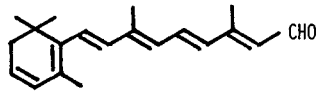
EXPERIMENTAL

Materials

Retinal isomers (all-*trans*, 13-*cis*, 11-*cis* and 9-*cis*) were obtained as previously described[3,6], and the 7-*cis* isomer was separated from an irradiated mixture of all-



(I)



(II)

Fig. 1. The all-*trans* forms of retinal (I) and 3-dehydroretinal (II).

trans-I by HPLC employing a μPorasil column (see Fig. 3A) in place of the Zorbax SIL previously used[6]. All-*trans*-II was prepared from all-*trans*-I according to the literature[9] and was purified by chromatography through a 5% water-deactivated alumina column (developed with 2–17% diethyl ether in *n*-hexane), followed by preparative thin-layer chromatography (TLC) on a pre-coated silica gel plate (60F-254, Merck, Darmstadt, G.F.R.; developed with 20% diethyl ether in *n*-hexane). Four mono-*cis*-II isomers (13-*cis*, 11-*cis*, 9-*cis* and 7-*cis*) were obtained from an irradiated mixture of all-*trans*-II (acetonitrile solution) by preparative TLC (as above), followed by HPLC separation (developed with 12% diethyl ether in *n*-hexane under 20 kg/cm² pressure). Attempted direct conversion of 11-*cis*-I into the corresponding 11-*cis*-II by treatment with N-bromosuccinimide and 1,5-diazabicyclo[5.4.0]undec-5-ene was unsuccessful.

All specimens were well characterized spectroscopically (Table I and Fig. 2), and concentrations were determined spectrophotometrically. The solvents used in the mobile phase were of reagent grade (for HPLC) quality in order to minimize background absorbance in the ultraviolet (UV) detector of the HPLC apparatus, and usually no special purification was required.

TABLE I

UV SPECTRAL DATA FOR 3-DEHYDRORETINALS

Values in parentheses denote inflection.

3-Dehydroretinal isomer	λ_{max} *(nm)*		
	In ethanol	*In n-hexane*	*+$NaBH_4$ in ethanol*
All-*trans*	400, (308)	387, (305)	352, 287, 276
7-*cis*	378		329, 288, 278
9-*cis**	390, 313		347, 286, 277
11-*cis*	393, 314, 250		346, 286, 277
13-*cis*	394, (307)		352, 287, 276

* See Fig. 2.

Equipment and operating conditions

The HPLC analysis was carried out on a Shimadzu–DuPont 830 liquid chromatograph, equipped with a UV-202 spectrophotometer. From preliminary trials, the preferred operating conditions were established as follows: column, a stainless-steel tube (30 × 0.4 cm I.D.) packed with μPorasil; mobile phase and pressure, 6 or

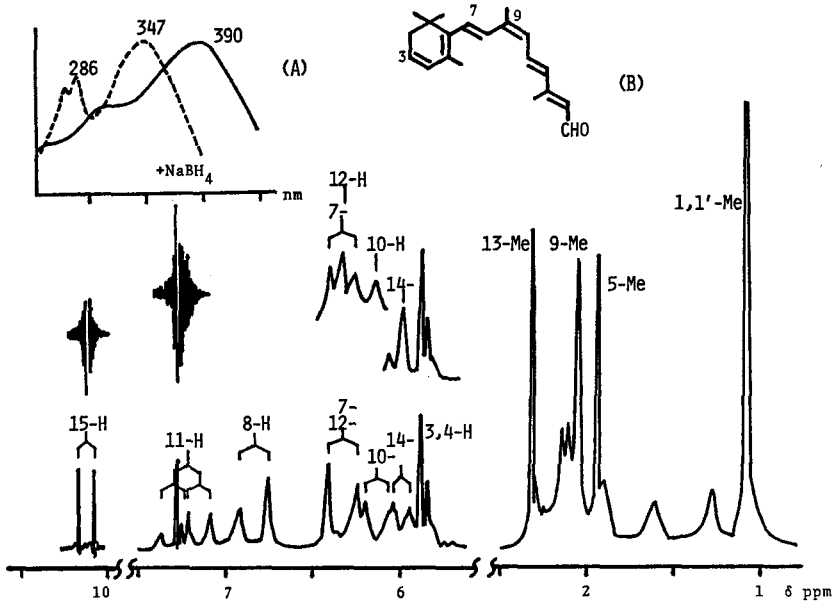


Fig. 2. Spectroscopic properties of 9-*cis*-3-dehydroretinal: (A) UV in ethanol; (B) ^{1}H NMR in C^2HCl_3 (90 MHz).

12% diethyl ether in *n*-hexane under 20 or 35 kg/cm^2 (see Table II); temperature, ambient; detection, UV at 254 nm; sample size, 1–5 μl (*ca.* 100 ng), injected with a 10-μl syringe by the stop-flow technique.

Stereoisomerization of all-trans-3-dehydroretinal and all-trans-retinal

The solution of the all-*trans* isomer in an appropriate solvent was stirred in a flask and was irradiated with a desk fluorescent lamp (20 W) at a distance of 15 cm for 60 min (see Table III).

RESULTS AND DISCUSSION

Identification of the samples

Specimens of *cis–trans* isomeric II were completely characterized as pure substances based on their spectral properties. Of the spectral data, UV data are most convenient and useful, especially in connection with those of the reduced form 3-dehydroretinols (Table I and previous results[10]). On the other hand, ^{1}H nuclear magnetic resonance (NMR) signals are less diagnostic once a specimen is contaminated with any other isomer, although ^{1}H NMR spectra give the most decisive information on their stereochemistry. As the 9-*cis* isomer seems to be a representative member of the II series (see Table III), UV and ^{1}H NMR spectra of this isomer are specially depicted in Fig. 2.

HPLC separation of the isomers

It is well known that HPLC is uniquely suited to the analysis of labile compounds such as *cis*-retinoids. In our laboratory[6], *cis–trans*-I isomers were analysed

initially by HPLC employing a column packed with Zorbax SIL. However, their resolution was much improved when this stationary phase was replaced with μPorasil (Fig. 3A). For analysing *cis–trans*-II isomers, a number of mobile phases consisting of 6–18% diethyl ether-containing *n*-hexane were tested under various pressures (10–50 kg/cm²). *Cis*-II isomers were extremely unstable and deteriorated on passing through columns with large inner diameters, *e.g.*, a 0.79-cm I.D. column filled with Zorbax SIL. Other conditions being equal, the extent of deterioration of the isomers appeared to depend on the amount of silica that the sample encountered. Columns with narrower bores, *e.g.*, 0.21-cm I.D. for Zorbax SIL or 0.4-cm I.D. for

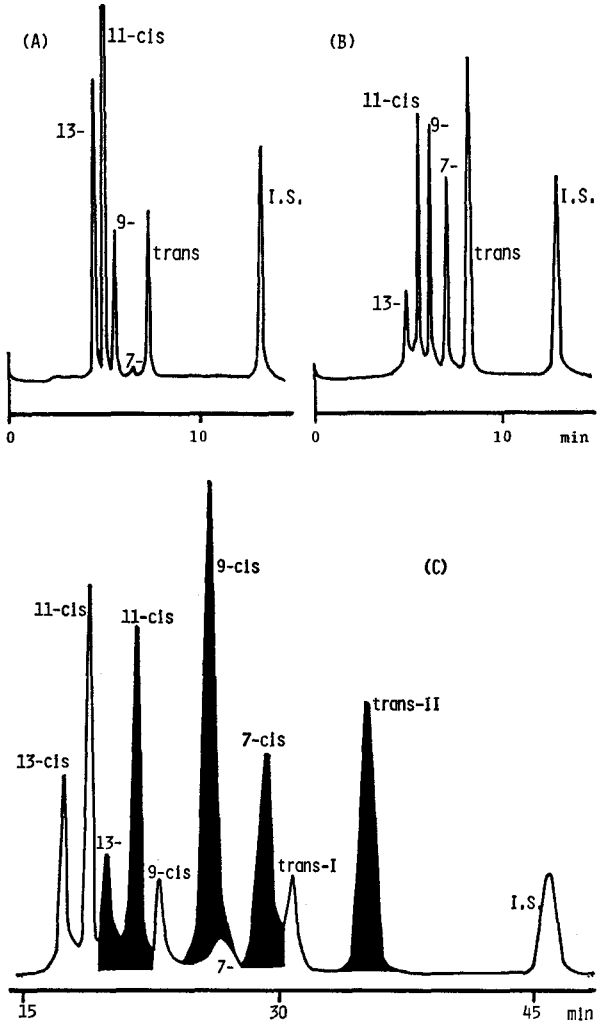


Fig. 3. HPLC behaviour of retinal (I) and 3-dehydroretinal (II) isomers. (A) Isomeric mixture of irradiated all-*trans*-retinal (μPorasil, 12% diethyl ether in *n*-hexane, 35 kg/cm², *m*-nitrobenzaldehyde as I.S.); (B) isomeric mixture of irradiated all-*trans*-3-dehydroretinal (conditions as in A); (C) isomeric mixture of irradiated all-*trans*-retinal and 3-dehydroretinal (μPorasil, 6% diethyl ether in *n*-hexane, 20 kg/cm², *m*-nitrobenzaldehyde as I.S.).

μPorasil, are therefore recommended for the chromatography of these isomers. Further, *m*-nitrobenzaldehyde was carefully selected as a suitable internal standard (I.S.) from several candidates to compensate for the column characteristics, instrumental variations and sample introduction techniques. As shown in typical chromatograms (Fig. 3B and C), clear baseline separation of isomeric II has been achieved. This separation can be performed in the absence or presence of isomeric I within 10 and 40 min, respectively, and their relative retention data are given in Table II. A recent paper[5] on the direct irradiation of all-*trans*-II reported the results of HPLC analysis of an irradiated mixture of all-*trans*-II and emphasized the formation of the novel 7-*cis*-isomer. However, compared with our results (see Fig. 3B and C), their HPLC provided poor resolution of the isomers and the most important 11-*cis* isomer was not separated and remained unidentified.

TABLE II

RELATIVE RETENTION TIMES (RRT) IN HPLC ANALYSIS OF ISOMERIC RETINALS AND 3-DEHYDRORETINALS

Column, μPorasil (30 × 0.4 cm I.D.). RRT values are given relative to the internal standard (*m*-nitrobenzaldehyde) = 1.00 in each instance (absolute retention times: 12.7 min in eluent I and 46.0 min in eluent II).

Isomer	*RRT × 100*	
	*Eluent I**	*Eluent II***
13-*cis*-I	34	38
11-*cis*-I	37	41
13-*cis*-II	39	43
11-*cis*-II	43	47
9-*cis*-I	43	50
9-*cis*-II	48	57
7-*cis*-I	49	59
7-*cis*-II	55	64
All-*trans*-I	55	67
All-*trans*-II	64	77

* Eluent I: 12% diethyl ether in *n*-hexane (pressure, 35 kg/cm²).
** Eluent II: 6% diethyl ether in *n*-hexane (pressure, 20 kg/cm²).

Simultaneous determination of 11-cis-retinal and 11-cis-3-dehydroretinal

In order to obtain calibration graphs for determining these isomers, different standard working solutions containing the isomer and internal standard were prepared from a 0.1% standard solution of 11-*cis*-I or 11-*cis*-II [solvent, diethyl ether–*n*-hexane (6:94)] and a 0.1% (w/v) solution of I.S. (same solvent). These standard working solutions were injected alternately into the instrument under the operating conditions and the HPLC results were calibrated as the peak-height ratio *versus* the weight ratio of the isomer to I.S. As shown in Fig. 4, linear calibration graphs were obtained and the simultaneous determination of these 11-*cis*- isomers by HPLC in the presence of each other has been established. This method provides nanogram sensitivity and adequate reproducibility and would be a useful technique in the future for studying vision chemistry in detail, *e.g.*, the dynamic behaviour of visual pigments.

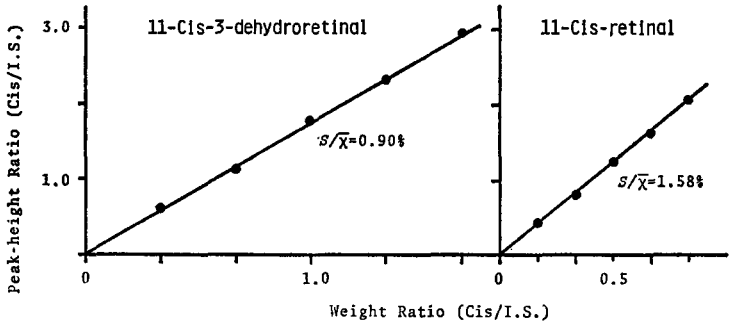


Fig. 4. Calibration graphs for 11-*cis*-retinal and 11-*cis*-3-dehydroretinal (μPorasil, 6% diethyl ether in *n*-hexane, 20 kg/cm², *m*-nitrobenzaldehyde as I.S.).

Cis–trans photoisomerization

Unlike the I series, few photochemical studies on the II series of isomers have been reported. Typical HPLC results on the *cis–trans* stereoisomerization of I and II are given in Table III. It is significant that the photoisomerization pattern is different between these two types of polyenals. The 13-*cis* is the most common *cis* type formed in *n*-hexane solution by direct irradiation. In the aprotic polar solvent acetonitrile, however, the formation of 11-*cis*-I or 9-*cis*-II predominates and the isomer distributions decrease in the following order:

11-*cis* ≫ 13- and 9-*cis* ≫ 7-*cis* (in the I series)

or

9-*cis* ≫ 11- and 7-*cis* ≫ 13-*cis* (in the II series)

All visual pigments found in nature have been recognized to involve the 11-*cis*-I or 11-*cis*-II chromophore bound to lysine in the apoprotein via a protonated Schiff base linkage. Our results on the photochemically induced *cis–trans* isomerization strongly suggest that the 9-*cis* form may be a representative *cis*-member in the II series and may have important biological significance as a visual chromophore as the 11-*cis* isomer in the I series.

TABLE III

DIRECT PHOTOISOMERIZATION OF ALL-*trans*-RETINAL AND 3-DEHYDRORETINAL

Irradiation with a fluorescent lamp (20 W) at a distance of 15 cm for 60 min.

All-trans-aldehyde	*Solvent*	*Isomer distribution (%)*				
		All-trans	*13-cis*	*11-cis*	*9-cis*	*7-cis*
I	*n*-Hexane	54	41	0	5	0
	Ethanol	60.5	18	18	3	0.5
	Acetonitrile	21	19.5	44	12.5	3
II	*n*-Hexane	82	17	0	1	0
	Ethanol	52	8	17	19	4
	Acetonitrile	44	5	13	26	12

ACKNOWLEDGEMENTS

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