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SIMULTANEOUS DETERMINATION OF *cis–trans* ISOMERIC RETINALS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method for the simultaneous determination of geometric isomers of retinal in a mixture has been developed. The method can be applied to problems such as determination of optimal conditions for the preparation of a particular isomer, or the investigation of novel photoproducts from retinal. Aprotic solvent effects on *cis–trans* isomerization are discussed.

INTRODUCTION

Of vitamin A compounds, retinal (vitamin A aldehyde) is the sole example that has an important biological function in the *cis* form. Hence, the establishment of a method for the separation and determination of retinal isomers (Fig. 1) is essential in the study of problems concerned with vision[1]. Laborious adsorption chromatography on an alumina column was the first chromatographic technique to be applied for this purpose. Although thin-layer chromatography (TLC) has achieved some success[2,3], the complete separation of these isomers, especially of the 11-*cis* and 13-*cis* isomers, is not always feasible by TLC and the isomers are extremely labile when adsorbed on a TLC plate. A complete and accurate non-destructive method for the separation and determination of these isomers is therefore still desirable. Recent work[4–6] on the analysis of retinal isomers prompted us to report our results on the simultaneous determination of retinal isomers by high-performance liquid chromatography (HPLC).

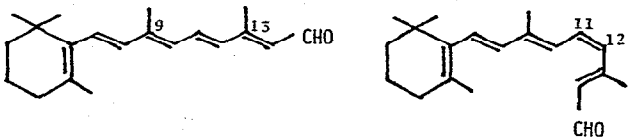

Fig. 1. Structures of retinal isomers.

MATERIALS AND METHODS

Materials

All-*trans*-retinal was prepared from crystalline all-*trans*-retinyl acetate by saponification, followed by oxidation with manganese dioxide. The 13-*cis* isomer was obtained from all-*trans*-retinal by a conventional isomerization technique. The 9-*cis* isomer was purchased from Sigma (St. Louis, Mo., U.S.A.) and the 11-*cis* isomer for identification purposes was a gift from Dr. T. Yoshizawa of Kyoto University, Japan. All specimens were well characterized spectroscopically and concentrations were determined spectrophotometrically. The solvents used in the mobile phase were of reagent-grade quality in order to minimize background absorbance in the ultraviolet (UV) detector of the HPLC apparatus, and usually no special purification was required.

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 (25 × 0.79 cm I.D.) packed with Zorbax SIL; pressure, 35 kg/cm^2 (flow-rate, *ca.* 2 ml/min); temperature, ambient; detector, UV at 254 or/and 380 nm; mobile phase, 12% diethyl ether in *n*-hexane for determination of the set of isomers; sample size, 1 μl (*ca.* 10 ng), injected with a 10-μl syringe by the stop–flow technique.

Cis–trans isomerization of retinal isomers

The solution of the all-*trans* isomer was stirred in a flask and exposed to the light from a 43-cm long fluorescent lamp (30 W, 680 lux) at a distance of 15 cm for a limited time (see Table II).

RESULTS

HPLC of cis–trans retinal isomers

Chromatograms and internal standards. A number of mobile phases, consisting of 10% chloroform, 12% diethyl ether, 1% ethyl acetate or 0.17% isopropanol in *n*-pentane or *n*-hexane, 2% diethyl ether + 0.15% isopropanol in *n*-pentane, and 10% diethyl ether + 0.07% acetonitrile in *n*-hexane were tested. Typical chromatograms of *cis–trans*-retinal isomers obtained under the preferred operating conditions are illustrated in Fig. 2. Baseline separation of the isomers was obtained and the relative retention data are given in Table I. Tetraphenylethylene or 2,6-di-*tert*.-butyl-*p*-cresol was selected from a number of compounds as a suitable internal standard to compensate for the column characteristics, instrumental variations and sample introduction techniques. In order to obtain approximately the same peak height as that of the all-*trans* isomer, a 1/9- or 3-fold weight of tetraphenylethylene or 2,6-di-*tert*.-butyl-*p*-cresol, respectively, was required.

Standard calibrations. From four 1% standard solutions of retinal isomers and a 0.1% (w/v) solution of tetraphenylethylene (internal standard) in *n*-hexane containing 10% of diethyl ether, 20 standard working solutions of retinal isomers were prepared in which the ratio of the isomer to the internal standard in each

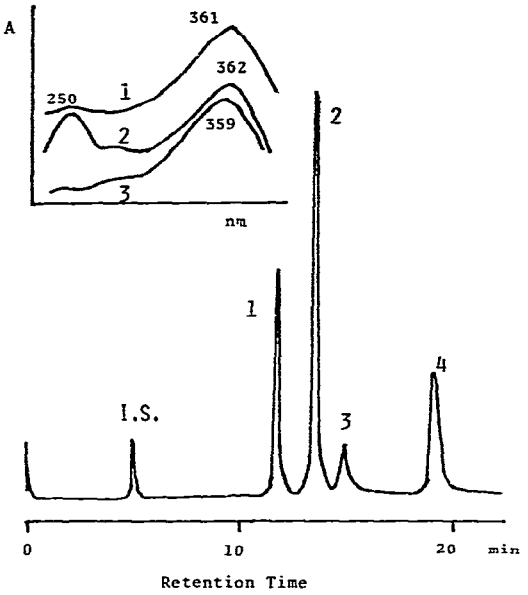

Fig. 2. HPLC behaviour of retinal isomers. Sample: an isomeric mixture of the irradiated all-*trans*-retinal (irradiated for 2 h in ethanol; iodine+). 1 = 13-*cis*; 2 = 11-*cis*; 3 = 9-*cis*; 4 = all-*trans*. Mobile phase: 12% diethyl ether in *n*-hexane. Internal standard (I.S.): 2,6-di-*tert*.-butyl-*p*-cresol.

TABLE I

RELATIVE RETENTION TIMES IN HPLC ANALYSIS OF ISOMERIC RETINALS

Column, Zorbax SIL (25 × 0.79 cm I.D.); eluent, 12% diethyl ether in *n*-hexane; pressure, 35 kg/cm². Retention times are given relative to the internal standard = 1.00 in each instance.

Retinal	*Internal standard*	
	*Tetraphenyl-ethylene**	*2,6-Di-tert.-butyl-p-cresol***
13-*cis*-	2.19	2.37
11-*cis*-	2.50	2.70
9-*cis*-	2.75	2.98
All-*trans*-	3.53	3.82

* Retention time = 5.4 min.
** Retention time = 5.0 min.

solution was 2, 4, 6, 8 or 10. These standard working solutions were injected alternately into the instrument under the above operating conditions and the HPLC results were calibrated as the peak-height ratio *versus* the weight ratio of the isomer to internal standard (Fig. 3).

Isomerization of all-trans retinal. Typical results obtained on the irradiated retinal are given in Tables II and III. It is significant that the isomerization pattern depends markedly on the solvent employed. As can be seen in Table II, formation of the 13-*cis* isomer clearly occurred when an *n*-hexane solution containing all-*trans* retinal and a catalytic amount of iodine was allowed to stand in the dark, although

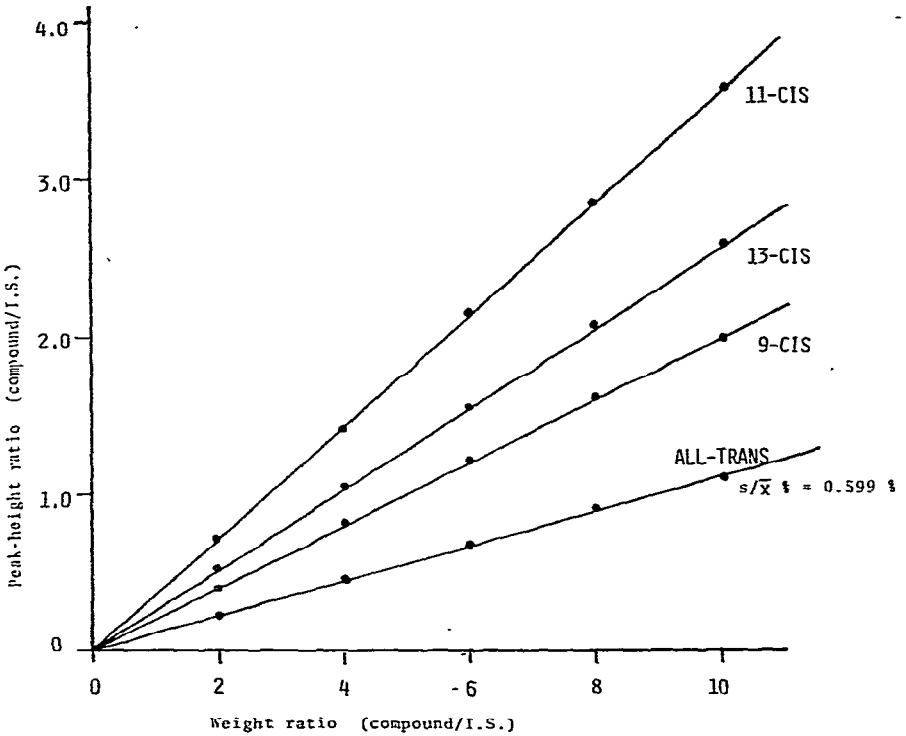

Fig. 3. Calibration graphs for retinal isomers. Mobile phase: 12% diethyl ether in *n*-hexane. Internal standard (I.S.): tetraphenylethylene.

TABLE II

cis–trans PHOTOISOMERIZATION OF ALL-*trans* RETINAL* IN *n*-HEXANE OR ETHANOL SOLUTION

Solvent	*Iodine as catalyst*	*Isomeric ratio in a mixture (all-trans:13-cis:9-cis:11-cis:others***)*	
		*Irradiated****	*In the dark overnight*
n-Hexane	Added	51.6:44.8:3.6:0:traces	61.6:38.4:traces:0:0
n-Hexane	None	54.1:41.1:4.8:0:traces	97.3:2.7:traces:0:0
Ethanol	Added	51.1:27.9:3.3:17.7:traces	95.5:3.8:0:0.7:0
Ethanol	None	60.8:18.2:2.9:18.1:traces	96.5:2.0:0:1.5:0

* $\lambda_{max.}$ = 381 nm (ethanol) or 368 nm (*n*-hexane).
** Di-*cis* (?).
*** Irradiated with a fluorescent lamp (30 W) for 1 h in *n*-hexane or for 30 min in ethanol.

this formation did not occur when an ethanolic solution was used. However, all-*trans*-retinal did not afford the 11-*cis* isomer in an irradiated *n*-hexane solution, but it did form this isomer in an irradiated ethanolic solution, irrespective of the presence of iodine. In aprotic solvents, the formation of the 11-*cis* isomer is believed to be affected strongly by the properties of the solvent employed. For example, it is a

TABLE III

cis–trans PHOTO-ISOMERIZATION OF ALL-*trans* RETINAL* IN APROTIC SOLVENTS

Aprotic solvent				*Irradiation time (min)***	*Percentage composition*				
*Type***	*Solvent*	*Dielectric constant at 25°*	*Viscosity at 25° (cP)*		*All-trans-*	*13-cis-*	*9-cis-*	*11-cis-*	*Others*§
5c	*n*-Hexane	1.9§§	0.31	60	54.1	41.1	4.8	0	
				120	40.7	52.4	5.2	0	1.7
4b	Diglyme	5.5	0.98	15	59.4	25.6	2.9	12.1	
				45	28.1	40.9	6.9	24.1	
4b	Tetrahydrofuran	7.6	0.55§§	30	24.6	41.7	9.5	24.2	
4b	Pyridine	12.3	0.95§§	10	34.8	27.9	6.6	30.7	
				60	19.3	31.4	13.0	34.9	1.4
5a	Acetone	20.7	0.32	30	23.1	26.7	11.9	38.3	
5a	Acetonitrile	36.0	0.32§§§	20	20.4	18.7	11.7	43.4	5.8
4a	Dimethyl sulphoxide	46.6	1.99	5	46.5	18.1	5.9	28.2	1.3
				60	19.7	24.5	16.9	36.8	2.1

* $\lambda_{max.}$ = 385 nm (pyridine, dimethylsulphoxide), 378 nm (diglyme, tetrahydrofuran, acetonitrile), 375 nm (acetone) or 368 nm (*n*-hexane).

** Kolthoff's classification of organic solvents[7]: 4a, 4b = dipolar protophilic; 5a = dipolar protophobic; 5c = inert.

*** Irradiated with a fluorescent lamp (30 W); no iodine catalyst was added.

§ Di-*cis* (?).

§§ At 20°.

§§§ At 30°.

general tendency that the amount of 11-*cis* isomer formed increases rapidly with an increase in the dielectric constant of the solvent (Table III; it reached 43% of the total in an irradiated acetonitrile solution), although it might be different in a viscous solvent such as dimethyl sulphoxide.

Experiments in which benzophenone, acetophenone or 2-acetonaphthone was used as a photosensitizer failed to provide any unusual isomerization products. However, when retinal was irradiated with a much more powerful light source (a 300-W high-pressure mercury lamp), HPLC clearly indicated the occurrence of a novel electro-cyclized retinal, the structure of which will be described elsewhere.

DISCUSSION

As all visual pigments found in nature involve an 11-*cis*-retinal or 11-*cis*-3-dehydroretinal chromophore bound to lysine in the apoprotein via a protonated Schiff base linkage and *cis–trans* isomerization of this polyene is essential for the visual process, the accurate determination of these isomers is an important task in the evaluation of various problems concerned with vision. However, retinal isomers are readily oxidized by air and are sensitive to light, and isomerize rapidly in a solution upon exposure to light, heat or chemical reagents. Therefore, one of the main problems in retinal analysis is the rapid and accurate determination of labile retinal isomers in a mixture.

Modern HPLC is uniquely suited to the analysis of a wide range of labile materials[8]. After the operating conditions, including the internal standard, had been carefully examined, an HPLC method for the simultaneous determination of retinal isomers was established. The operating conditions selected represent the most favourable compromise between speed of determination and maximum separation of the isomers in a mixture. Both clear resolution and accurate quantitation were achieved in less than 20 min using 12% diethyl ether in *n*-hexane as the eluent and tetraphenylethylene as the internal standard. The linearity of the response of the UV spectrophotometer to increasing concentrations of the isomers is good, and excellent linearity of the calibration graphs of the weight ratio *versus* peak-height ratio of sample to internal standard is obtained (Fig. 3).

The HPLC method is non-destructive and provides nanogram sensitivity and adequate reproducibility. The practical applicability of the method for the unequivocal identification and simultaneous determination of closely related isomers in a photochemically induced isomeric mixture of retinal was confirmed (Tables II and III). The method has also been applied successfully to further problems such as the determination of optimal conditions for the preparation of 11-*cis*-retinal and the investigation of novel photoproducts from retinal. The high speed, excellent resolution, precise and accurate results in trace analysis, flexibility and mild operating conditions are advantages of the method, which is believed to be superior to analytical procedures previously proposed for such applications.

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