Journal of Chromatography, 331 (1985) 265–272
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17 861

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC AND SPECTROSCOPIC CHARACTERIZATION OF STEREOISOMERIC RETINALOXIMES

IMPROVEMENTS IN RESOLUTION AND IMPLICATIONS OF THE METHOD

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(Received May 2nd, 1985)

SUMMARY

A study of mobile phases at different flow-rates for the improved high-performance liquid chromatographic resolution of retinaloxime stereoisomers is described. By using isopropanol as a phase modifier in diethyl ether–benzene (3:97, v/v) solution, the simultaneous separation of a mixture of ten retinaloxime isomers (all-*trans*-, 7-*cis*-, 9-*cis*-, 11-*cis*- and 13-*cis*-retinal *syn*- and *anti*-oximes) was satisfactorily achieved for the first time, leading to a sensitive method for the separation of retinal from biological materials. All peaks on the chromatogram were characterized by extensive ^{1}H NMR analysis. Limitations of the application of this method are discussed.

INTRODUCTION

The application of high-performance liquid chromatography (HPLC) to the simultaneous fractionation and determination of geometrically isomeric retinoids has been reported previously[1–4]. The usefulness of preparative HPLC for obtaining particular isomers has also been established, *e.g.*, for the rapid and convenient separation of 11-*cis*-retinal, the most important biological substance in the visual process[5].

For vision research, however, there is still an argent need to establish an analytical procedure applicable to biological materials. A serious problem is how to extract the intact isomers quantitatively without any change in their stereo-structures. The chloroform or dichloromethane method[6–8] can directly extract the isomers at low temperatures, but gives retinal recoveries (extraction yields) of only 15–30% or less. Groenendijk and co-workers[9–11] proposed the oxime method instead, with which the recoveries were excellent (95–100%) but the HPLC resolution was poor[9–15].

In this paper, we describe a method for the baseline separation by normal-phase HPLC of *syn*- and *anti*-oximes of *cis-trans* isomeric retinals (*syn*, the *cis*-position of the hydroxy group with reference to the hydrogen at C-15; *anti*, the *trans* position of the same groups). The simultaneous separation of all mono-*cis* isomers

0021-9673/85/$03.30

and the all-*trans* isomer was satisfactorily achieved. The limitations of the oxime method were briefly studied and several other trials to improve the technique were carried out.

EXPERIMENTAL

Materials

Mono-*cis*-retinals (7-, 9-, 11- and 13-*cis*) were prepared from all-*trans*-retinal ($\lambda_{max.}$ in ethanol, 380 nm; ε = 44 400) by irradiation with a fluorescent lamp (30 W), followed by HPLC separation[3,5]. The corresponding oxime was obtained quantitatively from each member of the retinal set as follows. A methanol solution of retinal was mixed with a 50- to 100-fold molar excess of aqueous hydroxylamine hydrogen carbonate (prepared by neutralization of the aqueous hydrochloride with saturated aqueous sodium hydrogen carbonate) and the final concentration of methanol was maintained at 70% by volume. The solution was stirred in the dark for 5 min at room temperature and then extracted twice with dichloromethane–methanol–water (1:1:1). The lower layer was washed twice with sodium chloride solution and dried over sodium sulphate. Evaporation of the solvent *in vacuo* left a mixture of *syn*- and *anti*-isomers in a ratio of about 3:1.

These isomers were separated by the following preparative thin-layer chromatographic (TLC) or HPLC technique: *syn*- and *anti*-isomers of all-*trans*-, 9-*cis*- or 11-*cis*-retinaloxime were separated on a TLC plate using cyclohexane–benzene–ethyl acetate (5:3:2), 3% diethyl ether in benzene or 5% diethyl ether in benzene, respectively, while those of 7-*cis*- or 13-*cis*-retinaloxime were separated by HPLC using 0.2% isopropanol in diethyl ether–benzene (3:97) as the eluent.

Chromatography

Preparative TLC. For the separation of the *syn*- and *anti*-isomers of each component, the sample was applied to a pre-coated plate [Kieselgel $60F_{254}$ (Merck), 0.5 and 0.25 mm thick], using the eluent described above.

HPLC. HPLC analysis was performed on a Shimadzu LC-3A instrument equipped with a LiChrosorb column (25 × 0.8 cm I.D.). Several eluents were examined at different flow-rates (see Results and Discussion). The absorbance at 360 nm was recorded (sensitivity 0.08), and the percentage of each *syn*-isomer was calculated from the peak area or peak height and ε_{360} (molar absorptivity at 360 nm) (Table I).

Spectra

Ultraviolet (UV) spectra were recorded on a Shimadzu-UV 200S instrument. Nuclear magnetic resonance (NMR) spectra were obtained at 200 MHz on a Varian XL200 instrument in C^2HCl_3 or in C^2HCl_3 containing ^2H_2O, with tetramethylsilane as the internal reference.

Photoisomerization

A solution of the all-*trans*-*syn*-isomer in an appropriate solvent was irradiated with a fluorescent lamp (30 W) from a distance of 15 cm for 3 h.

TABLE I

UV ABSORPTION PROPERTIES OF RETINALOXIMES

Solvent: isopropanol–diethyl ether–benzene (0.2:3:97).

Retinaloxime	$\lambda_{max.}$ *(nm)*	$\varepsilon_{max.}$	ε_{360}	$\lambda'_{max.}$* *(nm)*
Syn:				
All-*trans*	362	52 400	52 200	357
7-*cis*	348	46 000	43 000	342
9-*cis*	358	44 000	43 600	352
11-*cis*	360	37 000	37 000	355
13-*cis*	358	48 000	47 800	354
Anti:				
All-*trans*				361

* In ethanol.

RESULTS AND DISCUSSION

Geometrical isomers of retinal are important biological substances as sources of prosthetic groups in a variety of light-sensitive chromoproteins. All-*trans*-, 11-*cis*- and 13-*cis*-retinal have been well recognized in visual rhodopsin[16], squid retinochrome[17] and *Halobacterium* rhodopsins (bacteriorhodopsin, halorhodopsin and slow-rhodopsin)[18,19]. However, the roles of the other isomers, 9-*cis*- and 7-*cis*-retinal, also need to be considered[8] in order to obtain a full understanding of the particular biochemical phenomena in which natural retinoids participate.

TABLE II

RETENTION TIMES (min) OF RETINALOXIMES

HPLC: LiChrosorb column (25 × 0.8 cm I.D.), UV detection (360 nm).

Retinaloxime	*Mobile phase (flow-rate, ml/min)*								
	Dioxane–n-hexane, 5:95 (2)	*Acetone–benzene, 1:99 (1.2)*	*Isopropanol–diethyl ether–benzene*						
			0:3:97			*0.2:3:97*			*0.5:3:97 (2.5)*
			(1.5)	*(2.5)*	*(3)*	*(1.5)*	*(2.5)*	*(3)*	
Syn:									
All-*trans*	12.4	9.4	7.8	12.4	10.8	5.6	9.4	8.4	6.2
7-*cis*			8.1				9.8	8.7	
9-*cis*	13.2	10.9	9.3	14.2	12.2	6.4	10.7	9.6	6.7
11-*cis*	10.7	8.3	7.0	10.6	9.1	5.0	8.5	7.6	5.7
13-*cis*	13.2	11.6	9.8	14.8	12.8	6.8	11.7	10.4	7.1
Anti:									
All-*trans*	23.5	28.3	19.9			12.4	21.1	19.5	11.7
7-*cis*			19.2				20.3	18.7	
9-*cis*	22.1	20.7	18.1			11.3	19.2	17.7	10.8
11-*cis*	19.3	16.6	14.0			9.0	15.7	14.2	9.1
13-*cis*	17.3	14.6	12.7			8.1	13.7	12.5	8.1

TABLE III

^{1}H NMR DATA OF *cis–trans* ISOMERIC RETINALOXIMES

J_{7-8} = 16 (*trans*), 12.5 (*cis*); J_{10-11} = 11–12; J_{11-12} = 15-16 (*trans*), 11 (*cis*); J_{14-15} = 10–11 Hz.

Proton	*Splitting**	*δ* (C^2HCl_3)									
		All-trans		*7-cis*		*9-cis*		*11-cis*		*13-cis*	
		Syn	*Anti*	*Syn*	*Anti*	*Syn*	*Anti*	*Syn*	*Anti*	*Syn*	*Anti*
1,1-CH_3	s	1.03	1.03	1.04	1.04	1.04	1.05	1.02	1.03	1.03	1.03
5-CH_3	s	1.72	1.72	1.52	1.52	1.75	1.76	1.71	1.72	1.72	1.72
9-CH_3	s	1.99	2.00	1.89	1.90	1.99	2.00	1.95	1.96	1.99	2.00
13-CH_3	s	2.01	2.06	1.99	2.03	1.99	2.04	2.07	2.12	2.04	2.08
7-H	d	6.25	6.27	5.88	5.90	6.24	6.26	6.26	6.26	6.26	6.29
8-H	d	6.12	6.14	6.10	6.11	6.66	6.66	6.11	6.12	6.13	6.14
10-H	d	6.14	6.16	6.19	6.23	6.04	6.06	6.57	6.62	6.15	6.18
11-H	dd	6.80	6.88	6.72	6.80	6.90	6.97	6.46**	6.51**	6.75	6.82
12-H	d	6.36	6.42	6.32	6.36	6.29	6.34	5.93	5.97	6.81	6.90
14-H	d	6.14	6.73	6.14	6.68	6.13	6.68	6.19	6.75	5.99	6.55
15-H	d	8.19	7.58br***	8.18	7.52	8.18	7.53	8.16	7.47	8.32	7.64
OH				7.88br***							

* s, Singlet; d, doublet; dd, doublet of doublets.
** Triplet-like.
*** br, Broad.

In spite of extensive HPLC studies of these isomeric retinals[1–5,11], there is still an urgent need for a procedure for their analysis in biological materials, because the following two problems have not yet been solved concurrently: first, the method should give a high extraction yield of the chromophoric retinal from retinal-bearing pigments without any change in its stereo-structure, and second, each isomer should be resolved rapidly and completely.

Two methods have previously been proposed, *viz.*, solvent extraction–HPLC and oxime derivatization–HPLC. Each method has its advantages and disadvantages: the former is straightforward, but it gives poor recoveries (15–30% or less); the latter leads to a quantitative extraction, but HPLC fails to separate the isomeric retinaloximes.

Because of its excellent recoveries (95–100%), we decided to adopt the latter method and examined solvent systems to establish the optimal eluent. Benzene or *n*-hexane containing dioxane, tetrahydrofuran, diethyl ether, acetone, ethyl acetate

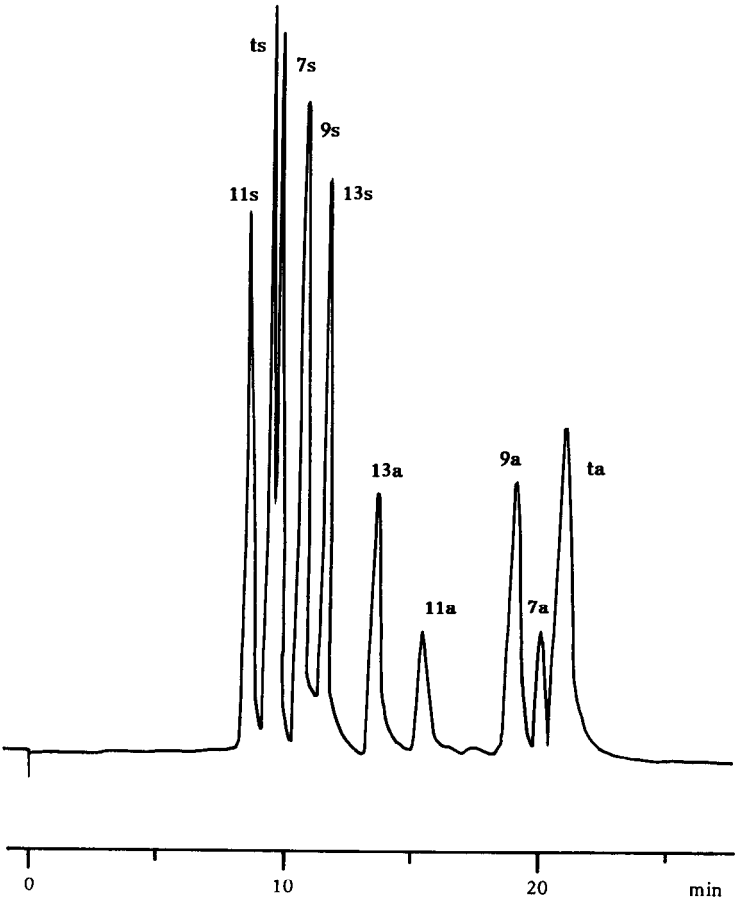

Fig. 1. HPLC elution pattern of a mixture of ten geometrical isomers of retinaloxime. Column, LiChrosorb (25 × 0.8 cm I.D.); eluent, 0.2% isopropanol in diethyl ether–benzene (3:97); flow-rate, 2.5 ml/min; UV detection, 360 nm; sensitivity, 0.08; sample, 1 μl. t = all-*trans*; s = *syn*; a = *anti* (*e.g.*, 7s = 7-*cis*-*syn*; 9a = 9-*cis*-*anti*; ts = all-*trans*-*syn*).

TABLE IV

DIAGNOSTIC ^{1}H NMR DATA OF *cis*-RETINALOXIMES

All the changes larger than ±0.2 ppm are given. Positive values mean downfield shifts in the *cis* compounds.

Proton	*Δδ**							
	Syn				*Anti*			
	7-cis	*9-cis*	*11-cis*	*13-cis*	*7-cis*	*9-cis*	*11-cis*	*13-cis*
5-CH_3	−0.20				−0.20			
7-H	−0.37				−0.37			
8-H		+0.54				+0.52		
10-H			+0.43				+0.46	
11-H			−0.34				−0.37	
12-H			−0.43	+0.45			−0.45	+0.48
J_{7-8}	12.5				12.5			
J_{11-12}			11				11	
	δ							
14-H			5.99–6.15				6.56–6.76	
15-H			8.18–8.32				7.48–7.65	

* Isomerization shifts: $\Delta\delta = \delta_{cis} - \delta_{trans}$.

TABLE V

DIRECT PHOTOISOMERIZATION OF ALL-*trans*-RETINAL-*syn*-OXIME

Comparative data with those of all-*trans*-retinal in different solvents.

Isomer	*Isomer distribution (%)*			
	Acetonitrile	*Methanol*	*n-Hexane*	*Tetrahydrofuran*
Retinal*:				
All-*trans* (recovered)	21	60.5***	54	24.5
7-*cis*	3	0.5***	0	Trace
9-*cis*	12.5	3***	5	9.5
11-*cis*	44	18***	0	24
13-*cis*	19.5	18***	41	42
Retinaloxime**:				
Syn:				
All-*trans* (recovered)	77	70	87.5	94
7-*cis*	Trace	Trace	Trace	Trace
9-*cis*	14	14	5.5	3.5
11-*cis*	3	5	0	0
13-*cis*	6	11	7	2.5
Anti isomers	Trace	Trace	Trace	Trace

* Irradiated with a fluorescent lamp (30 W) for 30 min in tetrahydrofuran or for 1 h in other solvents. Estimated from calibration graphs prepared from peak-height ratio *vs.* weight ratio[2,3].

** Irradiated with a fluorescent lamp (30 W) for 3 h. Estimated from peak height and ε_{360}.

*** Solvent: ethanol.

or ispropanol in various ratios were tested at different flow-rates. A good separation of a complex mixture of ten retinaloximes was achieved by employing 0.2% isopropanol in diethyl ether–benzene (3:97, v/v) at a flow-rate of 2.5–3 ml/min (Table II and Fig. 1).

All HPLC peaks appeared were identified by ^{1}H NMR analysis on an isolated pure form (Tables III and IV and Fig. 2). Thus the simultaneous separation of the all-*trans*- and all of the mono-*cis*-isomers (7-, 9-, 11- and 13-*cis*) was satisfactorily achieved for the first time. The detection limit (sensitivity 0.005) was 0.2 ng for all-*trans*-retinal-*syn*-oxime and 1 ng for 11-*cis*-retinal-*syn*-oxime. The percentage distribution or amount of each isomer can easily be calculated from its peak area or peak height and the ε_{360} values (Table I). A typical result for the direct photoisomerization of all-*trans*-retinal-*syn*-oxime is shown in Table V. The oxime was irradiated in an appropriate solvent by a fluorescent lamp (30 W) as described under Experimental. Under these experimental conditions, conversion of the *syn* to the *anti* form took place only to a slight extent, while employment of a more intense light source

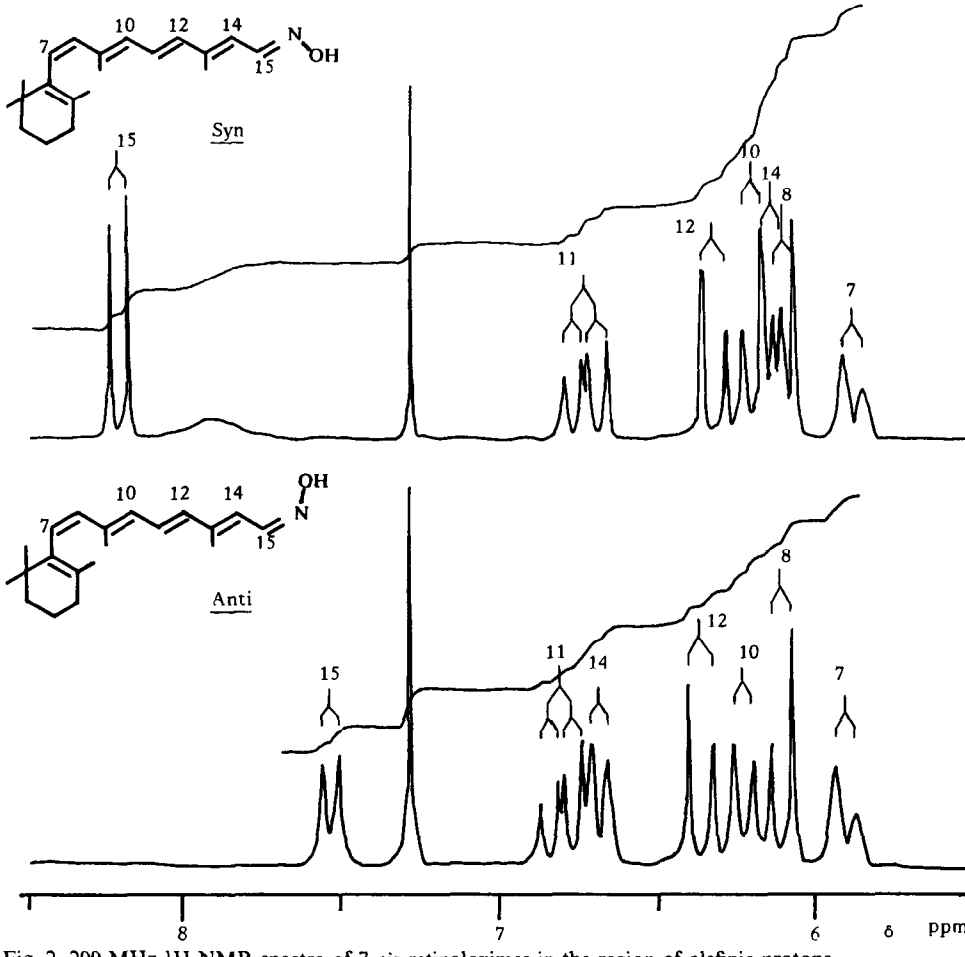

Fig. 2. 200 MHz ^{1}H NMR spectra of 7-*cis*-retinaloximes in the region of olefinic protons.

(300 W high-pressure Hg lamp) or prolonged irradiation increased the formation of *anti* components. Compared with the results on all-*trans*-retinal[1,3], it is noteworthy that retinaloxime is fairly photo-stable and little isomerization of the all-*trans*- to the 11-*cis*-isomer was observed.

A standard procedure for the extraction of the intact retinal from retinal-containing bio-pigments has already been developed[10,13], and is now complemented by the HPLC separation of the derivatized retinals, permitting complete resolution. Hence the two prerequisites indicated above have been fulfilled and a sensitive method for the analysis of retinal isomers in biological materials has been established. However, this method has some disadvantages, *viz.*, oxime derivatization affords an additional pair of stereo-isomers, *syn*- and *anti*-oximes, and the *syn*/*anti* formation ratio depends on the kind of isomer and the experimental conditions (*e.g.*, 3–4[10] or *ca.* 2.4[13] in solution, 1.9 in a membrane[10]). Further, *anti* peaks are usually broad and are sometimes overlapped by those of retinol isomers[13]. To overcome these problems, several methods such as reversed-phase TLC or HPLC of the oximes, derivatization of retinal using NH_2OCH_3, symmetric 1,3-diketones, cyclic secondary amines or cysteine methyl ester or conversion of the oxime to the nitrile were tried. Some of these trials are still in progress, but so far the oxime method described here appears to be the most promising.

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