Journal of Chromatography, 361 (1986) 437–441
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 535

Note

Rapid high-performance liquid chromatographic analysis of retinal mixtures

REIMAR C. BRUENING*, FADILA DERGUINI and KOJI NAKANISHI*
Department of Chemistry, Columbia University, New York, NY 10027 (U.S.A.)
(Received January 9th, 1986)

An indispensable tool for the investigation of biochemical transformations involved in vision is the analysis of natural or synthetic retinal chromophores from visual pigments[1–4]. The instability and rapid isomerization of retinals, especially of 11-*cis*-retinal (see Fig. 1), the natural chromophore in animal rhodopsin[5], requires a fast analytical methodology in order to assess qualitatively and quantitatively the composition of isomeric mixtures. The lipophilic nature of retinal and its isomers makes normal-phase high-performance liquid chromatography (HPLC) on either silica- or cyano-supports the most commonly used technique for their separation and many systems have been described already[6,7]. However, two disadvantages seem to be shared by most of these methods. Common solvent systems are based on alkane–diethyl ether mixtures which not only present serious safety problems in large scale preparations, but also make it difficult to maintain the solvent composition constant unless an automatic on-line mixing device is employed. Fixed-wavelength detection on the other hand, the preferred technique for detection of eluting peaks, allows unambiguous assignments only if retention times stay constant, and calls in many cases for subsequent off-line measurements of UV spectra. In the following we report an HPLC set-up to overcome these drawbacks.

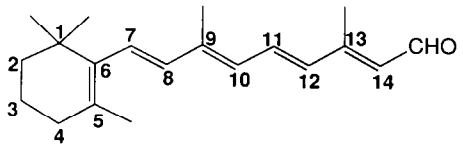

Fig. 1. Structure and numbering system of *trans*-retinal.

EXPERIMENTAL

Instrumentation and reagents

HPLC hardware consisted of a Perkin-Elmer chromatography station includ-

* Present address: Department of Chemistry, University of Hawaii at Manoa, 2545 The Mall, Honolulu, HI 96822, U.S.A.

0021-9673/86/$03.50

ing a Series 4 pump and an ISI 100 autosampler, both controlled by a PE 7000 laboratory computer. As detector we have used a combination of a Kratos Spectroflow 773 variable-wavelength UV detector and an LKB 2140 rapid spectral detector; the spectroflow was set on-line with an SP 4100 computing integrator from Spectra Physics and simultaneously looped via D/A interface back to the PE 7000. A 10-cm PTFE tubing 1/16 × 0.01 in. served to connect the detector to the LKB instrument. The LKB 2410 was controlled by an LKB WAVESCAN software through an IBM personal computer: isogram printouts were produced by a Canon inkjet colour printer on-line with the personal computer. Separations were achieved with a 250 × 4.5 mm I.D. column packed with 5 μm 100 Å spherical silica, manufactured by YMC.

A mixture of retinal isomers was prepared from *trans*-retinal by irradiation in acetonitrile according to Denny *et al.*[8]. 11-*cis*-Retinal was isolated from a crude sample by centrifugal partition chromatography[9,10]. The 13-*cis*, 9,13-di-*cis*-, 9-*cis*- and *trans*-retinal isomers used as reference were available in our laboratory.

The assignments of peaks were made by comparison of retention times with those of authentic samples if available, by comparison of the elution order with the literature[7,11], and also by comparison of UV data of the HPLC peaks as measured by the diode-array detector with published data[7,12,13].

RESULTS AND DISCUSSION

The commonly used solvent system for the separation of retinal isomers is a mixture of *n*-hexane–diethyl ether (95:5, v/v) at a flow-rate of 1 ml/min (ref. 7). However, we found that a mixture of 1,1,2-trichlorotrifluoroethane (freon 113)–methyl *tert.*-butyl ether (97:3, v/v), at a flow-rate of 0.7 ml/min, gives improved separation in a shorter period. As shown in Fig. 2, a 19 component mixture consisting

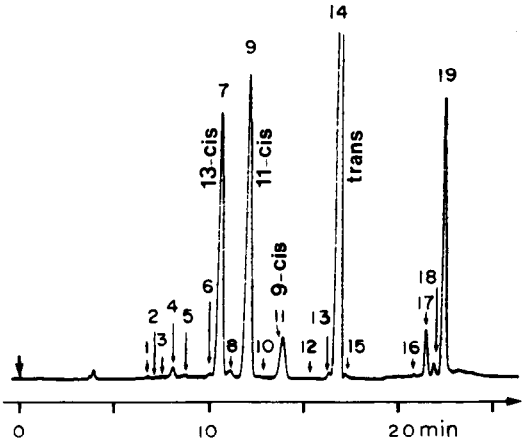

Fig. 2. Chromatogram [5 μm/100 Å spherical silica column, 250 × 4.5 mm I.D., freon 113–methyl *tert.*-butyl ether (97:3), 0.7 ml/min, 360 on detection] of an irradiated mixture of retinal (≥ 380 nm, in acetonitrile). The minor peaks represent the following retinals: peaks 1–4, tri-*cis* isomers; peak 5, 7,13-di-*cis*; peak 6, 11,13-di-*cis*; peak 8, 9,13-di-*cis*; peak 10, 9,11-di-*cis*; peak 12, 7,9-di-*cis*; peak 13, 7-*cis*. See text for basis of assignment.

of 14 retinal isomers and 5 more polar unknown contaminants could be satisfactorily separated in less than 25 min; all 14 retinals were eluted within 17 min. A 95:5 solvent ratio, at a flow-rate of 1 ml/min, led to elution of all isomers within 14 min with only slight reduction in resolution. This corresponds to *ca.* 70% of the retention time obtained with the conventional solvent system mentioned above.

Practically, a base-line separation is achieved for all isomers in Fig. 2, including the tri- and di-*cis* isomers which usually are difficult to resolve. Thus under normal conditions, the 9,11,13-tri-*cis* (peak 3), 7,9,13-tri-*cis* (peak 4), 7,13-di-*cis* (peak 5) and 11,13-di-*cis* (peak 6) isomers elute together with the 13-*cis* isomer (peak 7); the 9,13-di-*cis* isomer (peak 8) also partially overlaps with peak 7. However, all these isomers are well-separated in Fig. 2. Similarly, 9,11-di-*cis* (peak 10) and 7,9-di-*cis*-retinals (peak 12) are usually poorly resolved from the 9-*cis*-isomer (peak 11), but this is not the case here.

Fig. 3 shows the isogram of retinal peaks 1–14 in Fig. 2 as obtained by diode-array detection. The spectral window was set from 240 to 370 nm and spectra were taken at 400-ms intervals. After data collection the sensitivity of each peak was readjusted according to the sample amount; in this manner it was possible to visualize even the minor components. The respective peaks in Fig. 3 were identified on the following basis:

Peaks 1–4. These early eluting peaks were tentatively identified as the tri-*cis* isomers based on their UV absorption patterns and retention times by comparison with reported values[7,11,12]. Peak 2 could be assigned to the 7,9,11-tri-*cis*-retinal as shown by comparison of its UV isogram with the reported absorption maximum (346 nm) for this isomer[7,11]. Peaks 3 in particular can be attributed to the very blue shifted 9,11,13-tri-*cis*-retinal, the maximum of which is reported to be 302 nm in hexane and ethanol[12]. Peak 4 is probably the 7,9,13-tri-*cis* isomer which has been reported to elute with the 7,13-di-*cis*-retinal under various HPLC conditions[11].

Peak 5. The absorption pattern of peak 5 is in agreement with the reported value of 357 nm for 7,13-di-*cis*-retinal[7,11].

Peak 6. We believe that this blue-shifted peak represents the unstable 11,13-di-*cis* isomer, the UV spectrum of which shows an intense broad band at 302 nm and a shoulder at about 355 nm[13].

Peaks 7–9, 11 and 14. Retinal isomers were ascertained by co-injection with authentic samples.

Peaks 10, 12 and 13. Assignment of these to 9,11-di-*cis*-, 7,9-di-*cis*- and 7-*cis*-retinals respectively, follow the elution pattern reported for *n*-hexane[7]. Peak 10 attributed to 9,11-di-*cis*-retinal appears to be contaminated as judged from the shape of the isogram. The contaminating congenor is presumably the 7,11-di-*cis* isomer which normally elutes close to 11-*cis*-retinal[11]. The isogram shows another 340 nm absorbing minor component between peaks 12 and 13. In the regular HPLC diagram, Fig. 2, these two minor components are not detectable due to the insufficient amount.

Since isograms are produced on-line during the chromatographic run, this technique is well-suited for the instantaneous characterization of peaks due to unstable compounds. Another advantage of the isographic display lies in the possibility to assess directly the purity of an eluting peak.

1,1,2-Trichlorotrifluoroethane[14] and methyl-*tert.*-butyl ether[15] are both solvents less polar than *n*-hexane and diethyl ether[16], the conventionally employed sol-

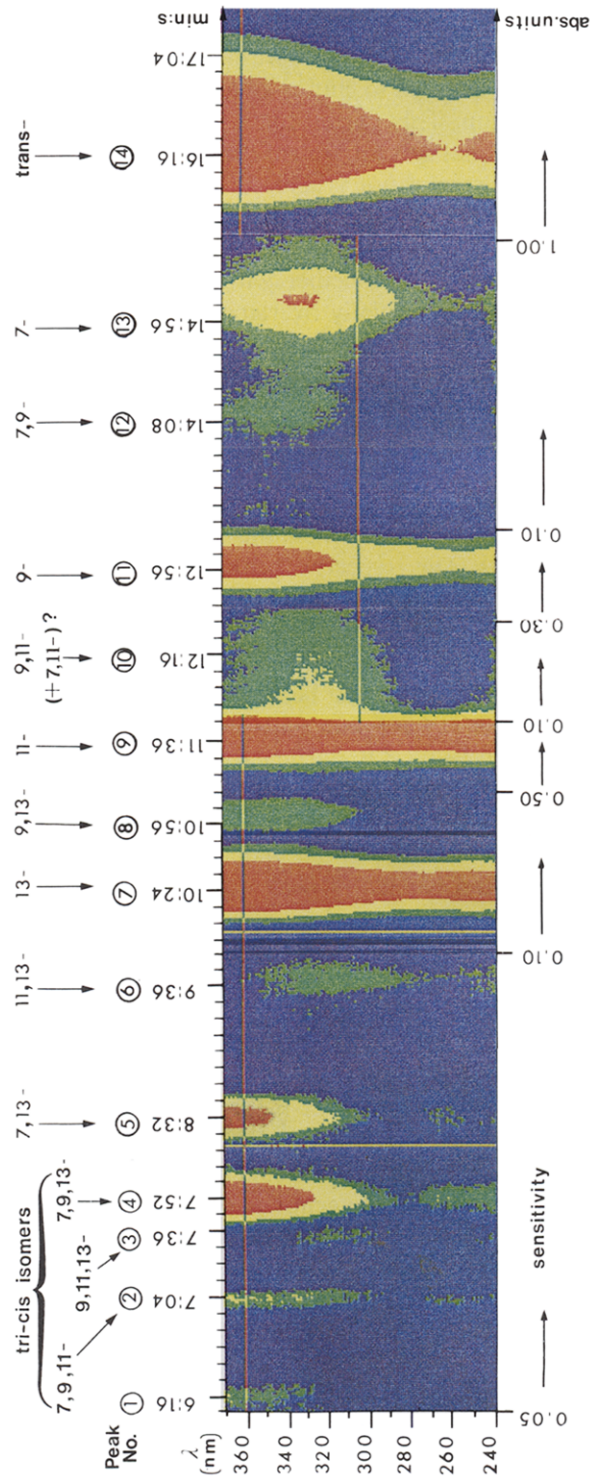

Fig. 3. Isogram of retinal peaks 1–14 shown in Fig. 2. Spectral window 240–370 nm; sensitivities indicated at the bottom of the figure were adjusted according to relative peak intensities of respective peaks. Shades of the isogram approximate the absorption maxima.

vents in retinal separations. It is therefore surprising that the retention times in our separation are actually shorter and yet result in improved resolution. Since a separation of the same retinal mixture with *n*-hexane–diethyl ether (95:5) gave on our column similar results to those published[7], it becomes obvious that the new mobile phase exhibits enhanced resolution with polyenes. It is possible that it is also suited for polyene antibiotics and related compounds.

ACKNOWLEDGEMENTS

We are grateful to Professor R. S. H. Liu for discussion. The studies were supported by NSF CHE 84-12513.

REFERENCES

1 H. Sichi, *Biochemistry of Vision*, Academic Press, New York, 1981.
2 *Methods Enzymol.*, 81 and 88 (1982).
3 K. Nakanishi, in S. Natori, N. Ikekawa and M. Suzuki (Editor), *Advances in Natural Products Chemistry*, Kodansha/Wiley, New York, 1981, p. 552.
4 V. Balogh-Nair and K. Nakanishi, *Methods Enzymol.*, 88 (1982) 496.
5 G. Wald and P. K. Brown, *Proc. Natl. Acad. Sci. U.S.A.*, 36 (1950) 84.
6 M. A. Adams and K. Nakanishi, *J. Liq. Chromatogr.*, 2 (1979) 1094; and references cited therein.
7 R. S. H. Liu and A. E. Asato, *Tetrahedron*, 40 (1984) 1931; and references cited therein.
8 M. Denny, M. Chun and R. S. H. Liu, *Photochem. Photobiol.*, 33 (1981) 267.
9 W. Murayama, T. Kobayashi, Y. Kosuge, H. Yano, Y. Nunogaki and K. Nunogaki, *J. Chromatogr.*, 239 (1982) 643.
10 R. C. Bruening, F. Derguini and K. Nakanishi, *J. Chromatogr.*, 357 (1986) 340.
11 R. S. H. Liu, H. Matsumoto, A. Kini, A. E. Asato, M. Denny, A. Kropf and W. J. DeGrip, *Tetrahedron*, 40 (1984) 473.
12 C. G. Knudsen, S. C. Carey and W. H. Okamura, *J. Am. Chem. Soc.*, 102 (1980) 6355.
13 C. G. Knudsen, R. A. S. Chandraratna, L. P. Walkeapaa, Y. S. Chauhan, S. C. Carey, T. M. Cooper, R. R. Birge and W. H. Okamura, *J. Am. Chem. Soc.*, 105 (1983) 1626.
14 J. L. Glajch, J. J. Kirkland and W. G. Schindel, *Anal. Chem.*, 54 (1982) 1276.
15 C. J. Little, A. D. Dale, J. A. Whatley and J. A. Wickings, *J. Chromatogr.*, 169 (1979) 381.
16 J. T. Przybytek (Editor), *High Purity Solvent Guide*, Burdick and Jackson Laboratories, Inc., Publ., Muskegon, MI, 1982, p. 133.