Journal of Chromatography, 357 (1986) 340–343
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 534

Note

Preparative scale isolation of 11-*cis*-retinal from isomeric retinal mixture by centrifugal partition chromatography

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(Received January 9th, 1986)

Retinals perform crucial functions in nature because they constitute the chromophore of various rhodopsins. This is exemplified by the visual pigment rhodopsin and the proton-translocating bacteriorohodopsin which contain 11-*cis*-retinal and *trans*-retinal as the chromophore, respectively[1–3]. A common method used in recent years to clarify the properties of these pigments is to study regenerated rhodopsins; these are prepared by detachment of the retinal from the apoprotein and reincubation with various retinal analogues[4] including isotopically labeled compounds. A preparative method for the purification of these light- and heat-sensitive retinals, natural and synthetic, thus becomes highly desirable. Being the most polar and stablest isomer, *trans*-retinal can be obtained rather readily by high-performance liquid chromatography (HPLC)[5,6] or flash chromatography; however, this does not apply to other isomers which are harder to purify. The same is true for synthetic retinal analogues[4] since the elution order generally follows that of natural series. Retinal purification is thus mostly confined to analytical or semi-preparative HPLC which, however, produces only sub-mg quantities per injection. Obtaining the crucial 11-*cis*-isomer in a pure state is particularly difficult because it usually elutes in the middle of the various isomers[6,7]. However, in one case using a μBondapak CN column, 1% ether in hexane[8], the 11-*cis*-isomer could be made to elute first in the four-component mixture, 11-*cis* → 13-*cis* → 9-*cis* and all-*trans* (the normal phase elution sequence is 13-*cis*, 11-*cis*, 9-*cis* and all-*trans*). The μBondapak CN column thus facilitates collection of the 11-*cis* isomer but on an analytical scale.

We recently observed that the elution sequence of the four retinals resulting from the above mentioned support is also obtained in the liquid–liquid distribution pattern with certain solvent systems (see below). This then allowed us to purify relatively large quantities of 11-*cis*-retinal using centrifugal counter-current (or centrifugal partition) chromatography (CPC)[9–12], a technique which has recently been applied to extremely labile natural products[13,14].

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0021-9673/86/$03.50

EXPERIMENTAL

Instrumentation and reagents

A centrifugal partition chromatography apparatus (Model CPC-B92-N) manufactured by Sanki Engineering (Nagaokakyo, Kyoto, Japan) was used. It consisted of a continuous flow centrifuge (B92-N) containing twelve cartridges (total volume 180 ml) made of monochlorotrifluoroethylene resin, a constant flow pump (LBP II type triple plungers), a valve connection unit (FCU-II) linked to a 2-ml PTFE sample loop injector, an electric power control unit (PCB II), a recorder, and a fraction collector. Detection of isomeric retinals were carried out by a flow-cell UV spectrophotometer (Jasco UVIDEC-100) and monitored at 420 nm (sensitivity 2.0 a.u.f.s.); this wavelength was used because the intensity at the absorption maxima around 360 nm was too high.

A mixture of cyclohexane–pentane–acetonitrile (5:2:5, v/v) containing 0.1% methanol (MCB solvents, HPLC-grade) served as the biphasic solvent system. The upper phase (mobile phase) was pumped through the lower stationary phase at 2.5 ml/min, thus adopting the "ascending" mode. The rotation speed was adjusted to 1500 rpm which stabilized the pump discharge pressure at 700 p.s.i.

Thin-layer chromatographic (TLC) analysis of the CPC fractions was performed on Whatman HPTLC plates using the upper phase of the solvent system as developing solvent and 3% (w/v) vanillin in absolute ethanol containing 1% (v/v) sulfuric acid as detector. A Perkin-Elmer chromatography station including a Series 4 pump and an ISI 100 auto-sampler both controlled by a PE 7000 laboratory computer was used to monitor the purity of isolated retinals and to identify each isomer by comparison of HPLC retention times with those of authentic samples. The column, 250 × 4.5 mm I.D., was packed with 5 μm/100 Å spherical silica, (YMC, Mt. Freedom, NJ, U.S.A.); the solvent system was 5% ether in *n*-hexane, flow-rate 1 ml/min, or 1,1,2-trichlorotrifluoroethane–methyl *tert.*-butyl ether mixture (97:3)[7], flow-rate 0.7 ml/min. A Kratos spectroflow 773 variable-wavelength detector, monitoring wavelength 360 nm, was employed.

Sample

The retinal mixture (160 mg) was a crude "11-*cis*-" retinal sample stored at −20°C for several years. This was dissolved in 2 ml of the lower solvent phase and injected into the continuous flow centrifuge. The authentic retinals used as reference were available in our laboratory. All experiments were carried out at room temperature and under dim red light.

RESULTS AND DISCUSSION

The centrifugal partition chromatogram obtained under these experimental conditions is shown in Fig. 1. The separation was complete after 2.5 h. The elution sequence of the 11-*cis* and 13-*cis* isomers under these CPC conditions is the reverse of that resulting from silica gel adsorption chromatography with an alkane–diethyl ether solvent system. The shaded portion of the first peak shown in Fig. 1 gave 50 mg of HPLC-pure 11-*cis*-retinal. The slowest eluting peak yielded 40 mg of HPLC-pure *trans*-retinal, whereas the middle fraction gave a total of 40 mg of a mixture of

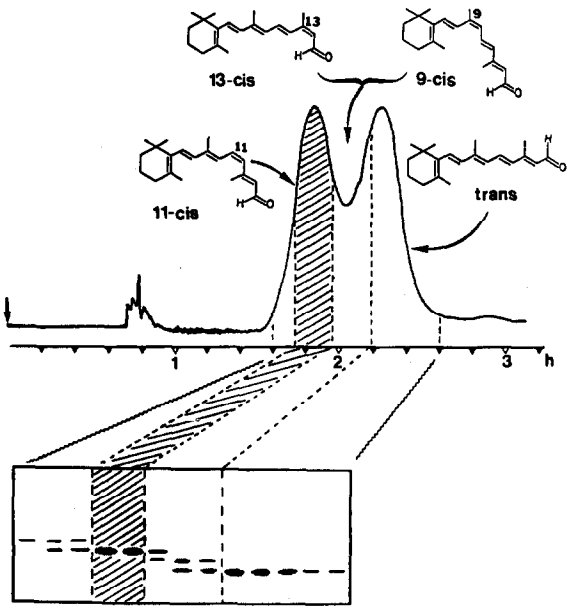

Fig. 1. Upper trace: CPC of 160 mg of crude isomeric retinal mixture consisting of four major retinals. Lower trace: TLC trace of CPC fractions. The solvent system and other experimental conditions are described in the text. Note that the TLC solvent system employed here results in the same retention time sequence as that of CPC.

the four isomers. The volume of mobile phase pumped was *ca.* 240 ml before elution of the solutes and an additional 150 ml for completion of analysis. The rest of the sample which remained in the stationary phase was a polar tar which did not move on TLC. It weighed 30 mg, thus accounting for recovery of all material and showing that pre-purification of crude sample is not necessary.

The centrifugal partition (or counter-current) chromatography technique[9–14] combines the advantages of classical counter-current chromatography and preparative flow-through centrifuge and allows efficient separation of sensitive material on a gram scale. Moreover, in the present application on retinals, the non-aqueous solvent system permitted recovery of solutes under non-isomerizing low temperature conditions.

ACKNOWLEDGEMENT

The studies were supported by NIH grant EY 01253.

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