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STATISTICAL SOLVENT OPTIMIZATION FOR THE SEPARATION OF GEOMETRIC ISOMERS OF RETINOL BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A systematic approach to the optimization of solvents for separation of isomers of retinol by straight-phase high-performance liquid chromatography is described. The optimum resolution of the 11-*cis* isomer from the 13-*cis* isomer required solvent characteristics different from those for the optimum resolution of the 9-*cis* isomer from the all-*trans* isomer. A compromise solvent composition produced good resolution of all four isomers in less than 12 min when used with an Excalibar column packed with 5-μm spherical silica.

INTRODUCTION

In the eye, 11-*cis* retinaldehyde combines with opsin to form the active visual pigment rhodopsin, and 9-*cis* retinaldehyde can combine with opsin to form isorhodopsin, a similar (but nonphysiological) pigment. The various isomers of retinaldehyde are readily converted in the eye to the corresponding retinol isomers by retinol dehydrogenase. Thus, in studying the nature and specificity of regeneration of rhodopsin after bleaching, it is necessary to have a suitable technique for the separation and quantitation of retinol isomers.

The complete separation of the major isomers of retinol by high-performance liquid chromatography (HPLC) has proven difficult. The 11-*cis* and 13-*cis* isomers tend to coelute in one peak, followed by the 9-*cis* and all-*trans* isomers in the second peak. Stancher and Zonta[1] concluded that straight-phase HPLC produced better resolution of retinol isomers than reversed-phase HPLC, but were unable to achieve more than slight resolution of 9-*cis* from all-*trans* retinol. No data were given for the separation of 11-*cis* from 13-*cis* retinol. Bhat *et al.*[2] achieved good resolution of 11-*cis* from 13-*cis* retinol on Zorbax CN with 2-propanol in hexane as eluent; they also achieved good resolution of 9-*cis* from all-*trans* retinol by using 2-octanol in hexane as solvent and a Zorbax CN column and a Partisil 10-ODS column in series. Nevertheless, they were unable to achieve good resolution of both isomers simultaneously. Tsukida *et al.*[3] studied the resolution of isomers of retinol on silica in four different solvents but were unable to achieve more than partial separation of all the isomers

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simultaneously. Bridges, Fong and Alverez[4] were also able to achieve usable resolution of the 11-*cis* and 13-*cis* retinol isomers in a gradient of dioxane in hexane on a Lichrosorb column, although no resolution of 9-*cis* from all-*trans* was obtained. Paanakker and Groenendijk[5] reported baseline separation of 9-*cis* and all-*trans* retinol, as well as usable resolution of 11-*cis* and 13-*cis* retinol, on 5-μm Si-60 packing with dioxane in hexane as eluent. Although this separation would be sufficient for our purposes, we have been unable to duplicate it with the Excalibar column (5-μm spherical silica packing). In our hands, the 9-*cis* isomer elutes close to all-*trans* retinol instead of being completely resolved as reported by Paanakker and Groenendijk. Because of the problems associated with these separation methods, which to the best of our knowledge were determined by empirical methods, we used a systematic approach to the optimization of elution solvent for the separation of retinol isomers in a straight-phase chromatographic system.

A systematic approach to solvent optimization for the reversed-phase separation of substituted naphthalenes was developed by Glajch *et al*[6] on the basis of the solvent classification scheme of Snyder[7]. Snyder classified solvents according to their action as proton donors, proton acceptors and strong dipoles, and proposed a quaternary solvent system of methylene chloride, ethyl ether, chlorform and hexane in which varying the proportions of the individual solvent components should produce the full range of solvent strengths and selectivity possible for straight-phase chromatography.

In the present paper this solvent optimization approach has been applied to the separation of isomers of retinol on straight-phase HPLC columns.

MATERIALS AND METHODS

The chromatographic equipment consisted of a Constametric IIIG pump, Spectromonitor III variable-wavelength UV–VIS detector (both from Laboratory Data Control, Riviera Beach, FL, U.S.A.), and a Rheodyne (Berkeley, CA, U.S.A.) 7125 sample injection valve. Columns used were a μPorasil silica 300 × 3.9 mm column (Waters Assoc., Milford, MA, U.S.A.) and an Excalibar 5-μm silica 250 × 4.5 mm column (Applied Science, State College, PA, U.S.A.). Solvents obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.) were filtered through a Millipore (Bedford, MA, U.S.A.) 0.45-μm Fluoropore filter before use. Data analysis and plotting were performed with SAS and SAS/GRAPH software (SAS Institute, Cary, NC, U.S.A.). All procedures were carried out under Westinghouse (Minneapolis, MN, U.S.A.) F40 Gold fluorescent lamps, which emit little light of wavelength less than 500 nm.

All-*trans* retinaldehyde obtained from Sigma (St. Louis, MO, U.S.A.) was dissolved in acetone and photoisomerized in an ice bath by exposure to a 100 W incandescent lamp for 2 h at a distance of 1 ft. Pure isomers of retinaldehyde were prepared by preparative chromatography of the resulting photoisomer mixture on an M9 Partisil-10 500 × 9.4 mm column (Whatman Chemical Separation, Clifton, NJ, U.S.A.). Published straight-phase HPLC separations, such as the one in ref. 5, agree on the elution order of isomers of retinaldehyde (13-*cis* first, followed by 11-*cis*, 9-*cis* and all-*trans*), and this sequence has been used as the primary means of isomer identification. To obtain retinol isomers, solutions containing approximately 0.1 mg of the photoisomer mixture or of purified retinaldehyde isomers were evaporated under

vacuum at 30°C in a Buchler (Fort Lee, NJ, U.S.A.) rotary evaporator, redissolved in 1 ml methanol, and reduced with 1 mg sodium borohydride. After a few minutes, 1 ml of water was added, and the retinol isomers were extracted with 1 ml methylene chloride. Retinal oxime isomers were prepared according to Groenendijk *et al.*[8] from photoisomerized retinaldehyde. Briefly, 1 *M* aqueous hydroxylamine (pH 6.5) was added to retinaldehyde isomers in methanol to produce a 70% methanol solution. This was then extracted with methylene chloride to obtain the retinal oxime isomers. Peak identification was based on the elution position of retinal oximes derived from purified individual retinaldehyde isomers.

Pure samples of each retinol isomer were injected to determine retention times and peak widths. Identification of the retinol isomers was based on the identity of the retinaldehyde isomers from which they were derived. Retention times were determined automatically by using a Hewlett Packard (Avondale, PA, U.S.A.) 3390A integrator, whereas peak widths at base were measured manually from the integrator trace. The resolution of adjacent peaks was calculated as

$$R = (t_2 - t_1)/2(w_1 + w_2) \quad (1)$$

where t_1, t_2 are the average retention times and w_1, w_2 are the average base peak widths of the two peaks.

Methylene chloride, isopropyl ether (in place of ethyl ether) and chloroform were chosen as the polar modifiers of the hexane mobile phase, in keeping with Snyder's suggestion[7]. The concentration of polar modifying solvent in hexanes necessary to elute all-*trans* retinol in approximately 10 min was determined empirically. Retention times and peak widths for 9-*cis*, 11-*cis*, 13-*cis* and all-*trans* retinol were obtained in each of the three primary solvents. Additional solvents were obtained by mixing the three primary solvents in various proportions, as shown in Table I. The choice of solvent mixtures to test was based on the experimental design suggested by Snee[9] and employed by Glajch *et al.*[6]. Eqn. 1 was used to calculate resolution values for the 9-*cis*/all-*trans* and 11-*cis*/13-*cis* peak pairs in each of these solvents.

TABLE I

SOLVENT COMPOSITIONS USED AND RESOLUTION VALUES OBTAINED

Solvent	*Fractional composition*			*Composition (%)*			*Average R values*	
	1	*2*	*3*	*Isopropyl ether*	*Methylene chloride*	*Chloroform*	*11-cis/ 13-cis*	*9-cis/ all-trans*
1	1	0	0	21.3	0	0	0.096	0.67
2	0	1	0	0	75	0	0.43	0.051
3	0	0	1	0	0	25	0.084	0.35
4	1/2	1/2	0	10.6	37.5	0	0.43	0.26
5	1/2	0	1/2	10.6	0	12.5	0.13	0.36
6	0	1/2	1/2	0	37.5	12.5	0.68	0.20
7	1/3	1/3	1/3	7.1	25	8.3	0.47	0.37
8	2/3	1/6	1/6	14.2	12.5	4.2	0.43	0.40
9	1/6	2/3	1/6	3.6	50	4.2	0.44	0.19
10	1/6	1/6	2/3	3.6	12.5	16.7	0.29	0.49

RESULTS

The resolution values obtained for each of the isomers in a given solvent system (Table I) were fitted to a quadratic model of resolution as a function of solvent composition. This model was then used to produce the contour plots shown in Fig. 1. The best resolution of the 9-*cis*/all-*trans* isomer pair is predicted to be obtained with 21.3% isopropyl ether in mixed hexanes, and the best resolution of the 11-*cis*/13-*cis* pair is predicted to occur with 45% methylene chloride plus 10% chloroform in hexanes.

The optimum solvent composition for simultaneous resolution of all four retinol isomers on the μPorasil column was determined by manually overlaying the 9-*cis*/all-*trans* and 11-*cis*/13-*cis* resolution contour plots. The solvent composition chosen was 26% methylene chloride plus 14% isopropyl ether in hexanes, which yields a methylene chloride:isopropyl ether ratio of 1.9. This solvent ratio was employed on the more efficient Excalibar column (5-μm spherical packing) with a more favorable flow-rate of 1 ml/min. The overall solvent strength was increased to elute all the retinol isomers in approximately 10 min. The chromatogram resulting from the use of 35% methylene chloride plus 18.5% isopropyl ether in mixed hexanes is shown in Fig. 2.

Retention times for isomers of retinal oxime were also determined in each solvent combination tested. These are compared with the retention times obtained

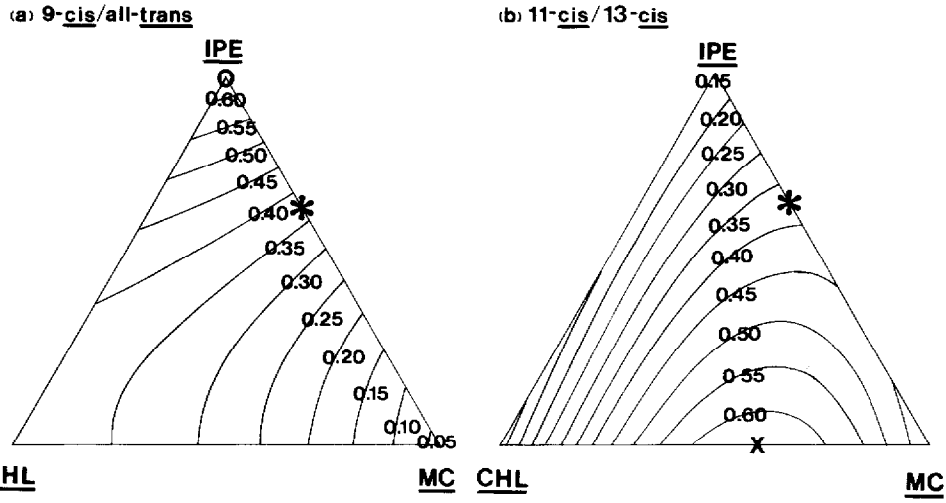

Fig. 1. Trilinear contour plot of resolution of adjacent retinol isomer peaks on the μPorasil column as a function of solvent composition. Numbers on the contour lines represent the value of R (eqn. 1) along the line. Solvent composition at vertices: CHL = 25% chloroform in mixed hexanes; MC = 75% methylene chloride in mixed hexanes; IPE = 21.3% isopropyl ether in mixed hexanes. * represents the position of the predicted optimum solvent for simultaneous resolution of all four isomers (26% methylene chloride plus 14% isopropyl ether in mixed hexanes). (a) Resolution of 9-*cis* from all-*trans* retinol. **O** represents the predicted optimum solvent composition for resolution of the 9-*cis*/all-*trans* isomer pair (21.3% isopropyl ether in mixed hexanes). (b) Resolution of 11-*cis* from 13-*cis* retinol. **X** represents the predicted optimum solvent composition for resolution of 11-*cis*/13-*cis* isomer pair (45% methylene chloride plus 10% chloroform in mixed hexanes).

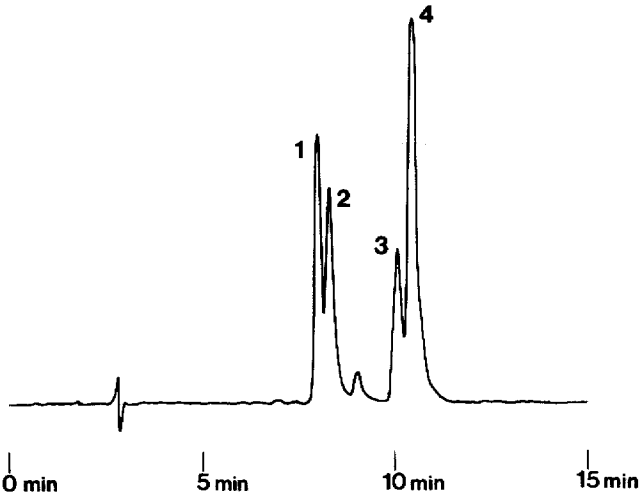

Fig. 2. Separation of isomers of retinol on the Excalibar column (5-μm spherical silica packing) using solvent conditions extrapolated from the overall optimum determined for the μPorasil column. Solvent = 35% methylene chloride plus 18.5% isopropyl ether in mixed hexanes at a flow-rate of 1 ml/min. Peak identification: 1 = 11-*cis*; 2 = 13-*cis*; 3 = 9-*cis*; 4 = all-*trans*. The small peak between peaks 2 and 3 is a contaminant in the standard mixture, possibly 9,13-di*cis* retinol.

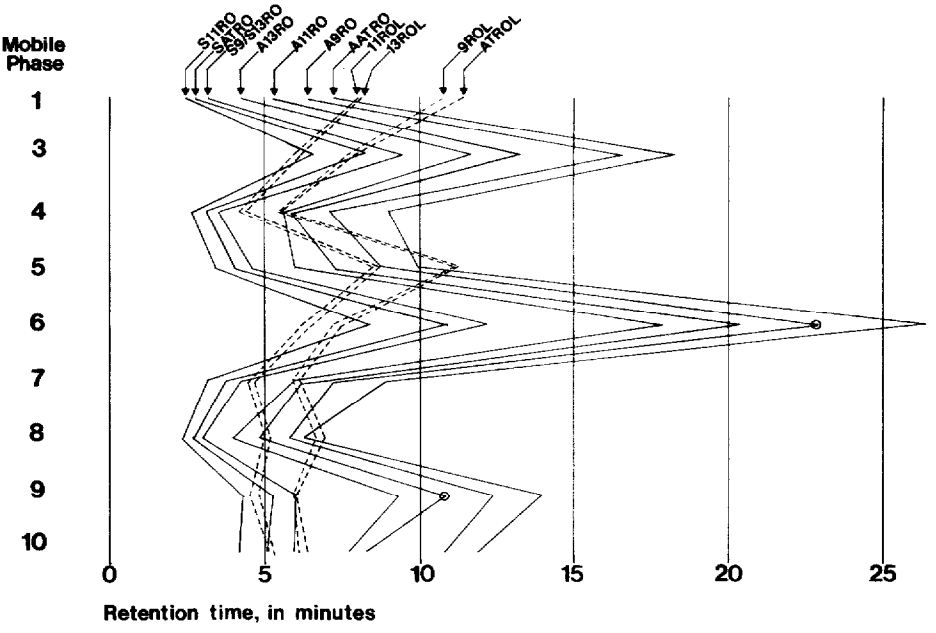

Fig. 3. Retention time maps for retinal oxime isomers and retinol isomers in different mobile phases. Flow-rate was 3.0 ml/min on the μPorasil column. Mobile phase identification is as in Table I. The two circled points represent estimated retention times for retinal oxime isomers not identifiable in the isomer mixture in that particular mobile phase. Maps for retinal oxime isomers are shown with solid lines, while retinol isomers are mapped with dotted lines. Isomer abbreviations, in order of elution with solvent 1: S11RO = *syn* 11-*cis* retinal oxime; SATRO = *syn* all-*trans* retinal oxime; S13/S9RO = *syn* 13-*cis* and *syn* 9-*cis* retinal oxime (eluting in a single peak); A13RO = *anti* 13-*cis* retinal oxime; A11RO = *anti* 11-*cis* retinal oxime; A9RO = *anti* 9-*cis* retinal oxime; AATRO = *anti* all-*trans* retinal oxime; 11ROL = 11-*cis* retinol; 13ROL = 13-*cis* retinol; 9ROL = 9-*cis* retinol; ATROL = all-*trans* retinol. There are no reversals in elution order between isomers within a functional group.

for the retinol isomers in the same solvents in Fig. 3. Retinal oxime exists as *syn* and *anti* conformers, and the four main isomers of retinaldehyde thus become eight retinal oxime isomers. The *syn* 9-*cis* and *syn* 13-*cis* isomers did not resolve under any of the conditions used in this study, so that the standard retinal oxime mixture was resolved into seven peaks. The retention times for mobile phase 2 (75% methylene chloride) are not presented in Fig. 3 because the retinal oxime isomers were so poorly resolved in this solvent that peaks for the individual isomers in the standard mixture could not be distinguished.

DISCUSSION

Separation of the 9-*cis*/all-*trans* pair requires almost opposite conditions from those required for separation of the 11-*cis*/13-*cis* isomer pair (see Table I and Fig. 1). This observation agrees with results reported by Bhat *et al.*[2]. In their study, which was conducted with a Partisil-10-ODS and a Zorbax CN column in series, separation of 9-*cis* from all-*trans* retinol was favored by long-chain alcohols, whereas resolution of 11-*cis* from 13-*cis* retinol was favored by more polar solvent modifiers, such as dioxane. Methylene chloride was the most effective polar modifier for separating the 11-*cis*/13-*cis* pair in the present study, and we also have found that dioxane favors the resolution of the 11-*cis*/13-*cis* pair of isomers on silica columns. Similarly, we have found isopropyl ether, as well as ethyl acetate, to be effective in separating the 9-*cis*/all-*trans* pair. Oddly, both ethyl acetate and dioxane, which exert opposite effects on the retinol isomer resolution, are in Snyder's solvent group VIa and have very similar proton donor and dipole interaction parameters[7]. Thus, other factors, such as solvent–solute localization effects or hydrogen bonding interactions, must be affecting separation in this situation. Snyder has since modified both his analysis of the factors important in straight-phase chromatography and his choice of solvents for an optimization study[10,11].

The same series of solvents used in the present study produced large changes in the retention times of retinol isomers relative to the retention times of retinal oxime isomers (Fig. 3). In contrast, the retention time for each isomer relative to other isomers of the same compound remained fairly constant. This type of optimization scheme thus seems more effective for separations of compounds with different functional groups than for separations of geometric isomers of the same compound, where the nature of the packing may play a more crucial role than the solvent.

The overall resolution of the μPorasil (10-μm packing) column at a flow-rate of 3 ml/min was not quite adequate for this difficult separation, such that resolution of a truly useful degree was not obtained for any of the solvents tested. Consequently, resolution data were most accurately obtained from injections of individual isomers rather than from direct analysis of an isomer mixture. Small variations in retention times between runs were troublesome, but fairly accurate data were obtained by making multiple injections of the pure isomers and using averages in the analysis. Despite these problems, the trace obtained with the Excalibar column (Fig. 2) demonstrates the potential of this approach. It is particularly encouraging that the results obtained on the μPorasil column (10-μm irregular silica packing) at a flow-rate of 3 ml/min were transferable with considerable success to the Excalibar column (5-μm spherical silica packing) at a flow-rate of 1 ml/min. Although this may not be the

absolute optimum solvent for this separation on the Excalibar column, the resolution is as good as, or better than, any obtained by us or by others[2–5] by nonsystematic approaches.

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