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EFFECT OF 2-ALKANOLS ON THE SEPARATION OF GEOMETRIC ISOMERS OF RETINOL IN NON-AQUEOUS HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The effects of 2-alkanols as mobile phase modifiers on the resolution of 11-*cis*-, 13-*cis*-, 9-*cis*- and all-*trans*-retinol in high-performance liquid chromatography were studied. Partisil-10-ODS and Zorbax CN columns were used in series. The retentions of 9-*cis*-and all-*trans*-retinol were significantly influenced by the presence of long-chain 2-alkanols in the mobile phase. Baseline separation of 9-*cis*-and all-*trans*-retinol was achieved when 1% 2-heptanol in hexane was used as the eluent. On the other hand, the separation of 11-*cis*-from 13-*cis*-retinol required 5% dioxane in hexane. The method is applicable to the separation of retinol isomers present in biological samples. Analysis of normal rat liver lipids showed the presence of a small amount of 13-*cis*-retinol in addition to all-*trans*-retinol.

INTRODUCTION

The utilization of high-performance liquid chromatography (HPLC) for the separation of retinoids and their metabolites present in various tissue extracts has been reported[1–4]. In biological systems, vitamin A occurs in the form of *cis–trans* isomers of the alcohol (retinol)[5,6], aldehyde (retinal)[7,8], acid (retinoic acid)[9] and esters (retinyl fatty acyl esters)[10]. Several laboratories have reported the HPLC separation of several isomers of retinol[11], retinal[12,13] and retinoic acid[9,14]. Stancher and Zonta[15] recently compared normal- and reversed-phase HPLC systems for the separation of retinol isomers and reported that the reversed-phase systems are not suitable for the separation of 9-*cis*- from all-*trans*-retinol or of 13-*cis*-from 11-*cis*-retinol. The baseline resolution of 9-*cis*-and all-*trans*-retinol has been found to be difficult by normal-phase HPLC[16]. Here we report the use of Partisil-ODS and Zorbax CN columns connected in series for the separation of retinol isomers and their application to the separation of retinol isomers present in normal rat liver tissue.

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EXPERIMENTAL

Apparatus

The apparatus consisted of a Beckman Model 322 MP programmable liquid chromatograph with two Model 100A pumps. The UV spectrophotometer was a Hitachi Model 100-40 equipped with a variable wavelength between 195 and 850 nm. Pre-packed Partisil-10-ODS, Partisil-10-ODS2 and Partisil-5-ODS HPLC columns were obtained from Whatman (Clifton, NJ, U.S.A.). The 5-μm Zorbax CN and 5-μm Ultrasphere columns were purchased from Dupont Canada (Ontario, Canada) and Beckman Instruments (Ontario, Canada), respectively.

Materials and methods

Standard pure 11-*cis*-, 13-*cis*- and 9-*cis*-retinal were gifts from Dr. W. E. Scott (Hoffmann-La Roche, Nutley, NJ, U.S.A.) and all-*trans*-retinal was purchased from Sigma. Retinol isomers were obtained from the corresponding retinals by reduction with sodium borohydride as described by Bridges[17]. All operations were carried out under golden yellow light.

HPLC-grade dioxane, hexane and 2-propanol were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). 2-Butanol, 2-pentanol, 2-hexanol, 2-heptanol, 2-octanol, 2-nonanol and 2-decanol were purchased from Aldrich (Milwaukee, WI, U.S.A.). [11,12-^{3}H]Retinol (specific activity 51 Ci/mmol) was obtained from Amersham Searley.

The capacity ratios (k') were calculated from the retention times[18]: $K' = (t_r - t_0)/t_0$, where t_r is the retention time of a retained peak and t_0 is the retention time of an unretained peak, which is retinyl palmitate in this system.

Sprague–Dawley male rats (Canadian Breeding Farm, Quebec, Canada) maintained on normal laboratory feed were used for the study of endogenous retinol isomers. The retinol isomers were extracted from the rat liver tissue as described earlier[1].

RESULTS

Fig. 1 illustrates the separation of some isomers of retinol on a Zorbax CN column with 1% of 2-propanol in hexane as the mobile phase. Baseline separation of 13-*cis*- from 11-*cis*-retinol was achieved, but all-*trans*- and 9-*cis*-retinol were eluted in a single peak. The flow-rate and solvent ratios of 2-propanol and hexane had no effect on the resolution of all-*trans*- and 9-*cis*-retinol.

An attempt was made to resolve 9-*cis*- and all-*trans*-retinol in addition to 11-*cis*- and 13-*cis*-retinol by connecting a Partisil-10-ODS column in series with the Zorbax CN column. Fig. 2A shows that a partial resolution of 9-*cis*- from all-*trans*-retinol was achieved, but that the baseline separation of 11-*cis*- and 13-*cis*-retinol was lost when the same mobile phase system was used. Again, alterations in the flow-rate or in the percentage of 2-propanol in hexane did not affect the separation of the isomers of retinol.

An increase in the hydrophobicity of the column (*i.e.*, Partisil-10-ODS 2 or Ultrasphere-ODS instead of Partisil-10-ODS) resulted in the complete loss of the resolution of 9-*cis*- from all-*trans*- and of 11-*cis*- from 13-*cis*-retinol (data not shown).

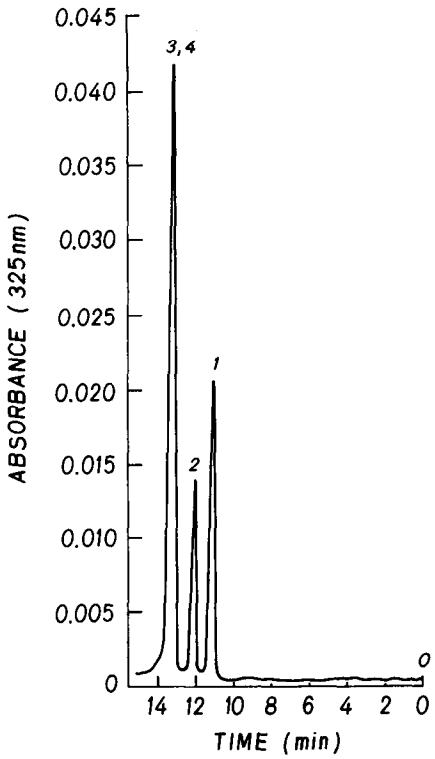

Fig. 1. Chromatogram of a test mixture of *cis–trans* isomers of retinol. Zorbax CN 5-μm column (25 cm × 4.6 mm I.D.); mobile phase, 1% of 2-propanol in hexane; flow-rate, 1.0 ml/min; pressure, 200 p.s.i.; wavelength of detection, 325 nm; sample size, 400–500 ng in 20 μl of methanol. Peaks: 1 = 11-*cis*-retinol; 2 = 13-*cis*-retinol; 3, 4 = 9-*cis*- and all-*trans*-retinol.

The effect of an increase in the hydrophobicity of the mobile phase on the separation of isomers of retinol was investigated. The hydrophobicity of the mobile phase was increrased by adding 1% of 2-alcohols with longer carbon chains. Inclusion of 2-butanol or 2-pentanol in the mobile phase improved the resolution of 9-*cis*- and all-*trans*-retinol (Fig. 2B and C). Further, baseline separation of 9-*cis*- and all-*trans*-retinol was obtained when 1% of 2-heptanol was used in the mobile phase (Fig. 2D). Fig. 2E, F and G show the effects of 1% of 2-octanol, 2-nonanol and 2-decanol, respectively, in hexane on the resolution of 9-*cis*- and all-*trans*-retinol. The retention times of geometrical isomers of retinol increased as the higher 2-alkanols were added to the mobile phase. The capacity ratios (k') of isomers of retinol in the presence of 2-alkanols in the mobile phase are presented in Table I. The higher 2-alkanols had very little effect on the resolution of 11-*cis*- and 13-*cis*-retinol even though there was an increase in the retention (Table I). In all of these analyses the flow-rate and the amount of 2-alkanols in hexane were 2.0 ml/min and 1%, respectively.

Using the same columns connected in series, baseline separation of 11-*cis*- and 13-*cis*-retinol could be obtained by eluting the column with 5% dioxane in hexane (Fig. 3A). However, the resolution of all-*trans*- from 9-*cis*-retinol was not achieved by this system. We attempted to achieve a separation of the four isomers in a single

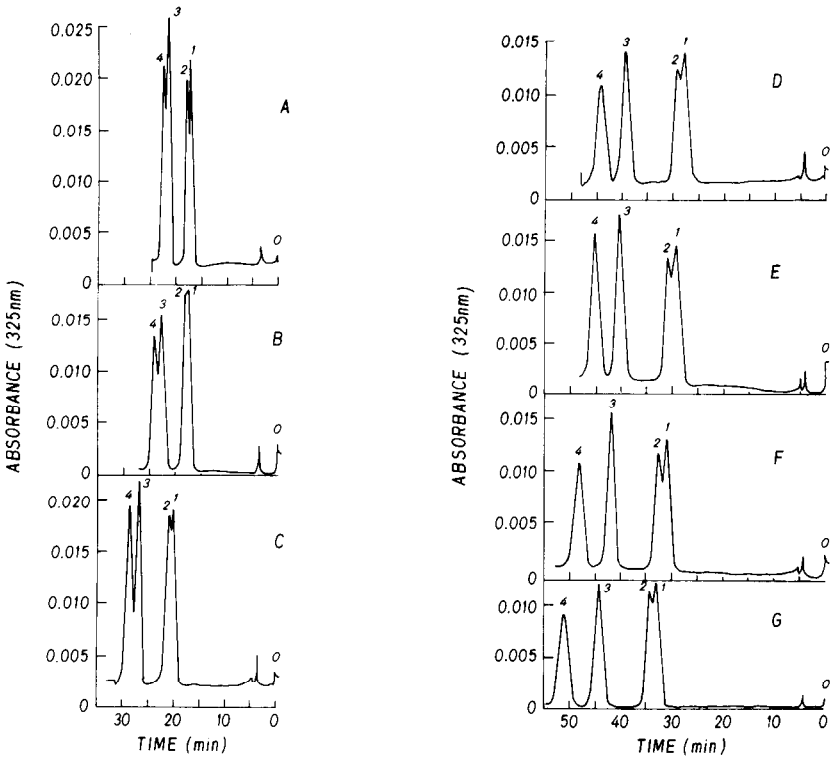

Fig. 2. Chromatogram of a text mixture of four isomers of retinol. Partisil-10-ODS (25 cm × 4.6 mm I.D.) and Zorbax CN 5-μm (25 cm × 4.6 mm I.D.) columns connected in series. Mobile phase: 1% of (A) 2-propanol; (B) 2-butanol; (C) 2-pentanol; (D) 2-heptanol; (E) 2-octanol; (F) 2-nonanol; and (G) 2-decanol in hexane. Flow-rate, 2.0 ml/min; pressure, 800 p.s.i.; sample size, wavelength and peak identification as in Fig. 1.

run by combining the two systems. The separation of 9-*cis*- from all-*trans*-retinol was not improved when a combination of dioxane and 2-octanol in hexane was used in the mobile phase (Fig. 3B).

Application to the purification of radioactive retinol

As all-*trans*-retinol is not stable, small amounts of *cis*-isomers are invariably formed when it is stored for long periods. It has been found that [11,12-^{3}H]retinol from Amersham Searly is relatively pure and further purification can be achieved by the HPLC method described here.

An aliquot of the radioactive retinol was mixed with cold standard retinol isomers and separated by HPLC using 1% of 2-octanol in hexane as the eluent. Fig. 4A shows the UV detection of standard retinol isomers. The trace due to the radioactive retinol is presented in Fig. 4B. Small amounts of radioactivity co-eluting with standard 11-*cis*-, 13-*cis*- and 9-*cis*-retinol were detected as impurities. The radioactive peak corresponding to all-*trans*-retinol was further collected and an aliquot was re-injected into the HPLC system to check whether isomers detected in Fig. 4B were formed during handling and chromatography. Fig. 4C illustrates the chromatographic profile of purified radioactive retinol, which is 100% pure.

TABLE I

INFLUENCE OF 1% OF 2-ALKANOLS IN HEXANE ON CAPACITY RATIO (k') OF GEOMETRICAL ISOMERS OF RETINOL ON PARTISIL-10-ODS AND ZORBAX CN COLUMNS CONNECTED IN SERIES

Chromatographic conditions as in Fig. 2.

Alkanol	*Retinol isomer*			
	11-cis	*13-cis*	*9-cis*	*All-trans*
2-Propanol	3.00	3.25	4.13	4.38
2-Butanol	3.13	3.38	4.38	4.88
2-Pentanol	3.75	4.13	5.50	6.00
2-Heptanol	5.38	6.00	8.25	9.35
2-Octanol	5.75	6.50	8.50	9.63
2-Nonanol	6.25	7.00	9.00	10.50
2-Decanol	6.75	7.13	9.50	11.00

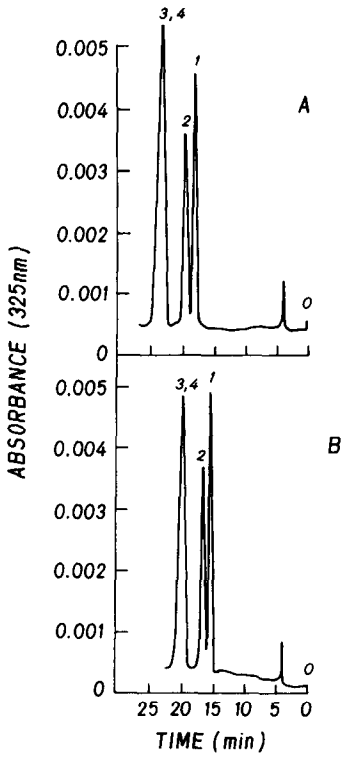

Fig. 3. Separation of a test mixture of *cis–trans* isomers of retinol. Conditions as in Fig. 2, except the composition of mobile phase: (A) 5% of dioxane in hexane; and (B) 4% of dioxane and 1% of 2-octanol in hexane.

Application to tissue extracts

It was important to examine whether the HPLC system developed here is applicable to biological samples. Fig. 5 illustrates the HPLC profile of an aliquot of lipid extract from normal rat liver. A UV peak in the area of 13-*cis*-retinol was detected. The UV-absorbing peak corresponding to 13-*cis*-retinol was further shown to be 13-*cis*-retinol using the dioxane–hexane system (data not shown).

DISCUSSION

Normal-phase HPLC systems have long been considered suitable for the separation of isomers of retinal[10,16] and retinol[11,19]. Bridges *et al.*[16] reported the use of programmed-gradient HPLC for the separation of mixtures of isomers of retinal,

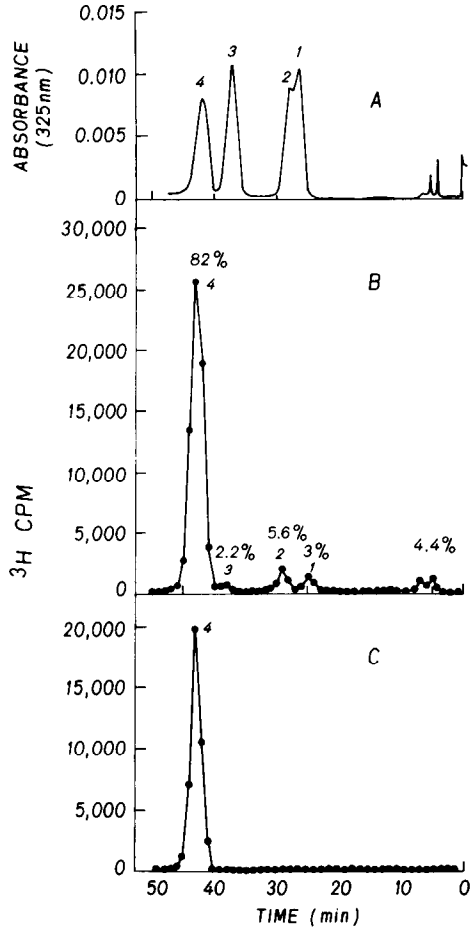

Fig. 4. Purification of labelled retinol by HPLC. Approximately 50,000 cpm of [11,12-^{3}H]retinol was mixed with a standard mixture of retinol isomers in methanol and 50 μl were injected. (A) UV-absorbing peaks of standard retinol isomers; (B) radioactive trace of the fractions collected from the column; and (C) chromatogram of purified all-*trans*-[11,12-^{3}H]retinol. Mobile phase: 1% of 2-octanol in hexane. Other chromatographic conditions and identification of peaks as in Fig. 2.

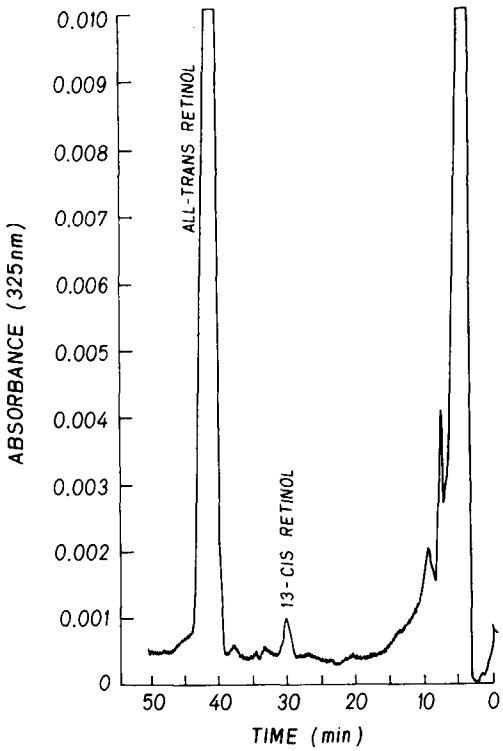

Fig. 5. HPLC separation of endogenous retinol isomers present in the liver tissue of a normal rat. An aliquot (50 μl) of methanolic extract was injected on to the column. Chromatographic conditions as in Fig. 4.

retinol and retinyl esters. They were partially successful in resolving 9-*cis*- from all-*trans*-retinol. Initially we attempted to separate the retinol isomers by reversed-phase HPLC using available ODS columns (5-μm Ultrasphere ODS, 10-μm Partisil ODS-2, Zorbax-ODS, 5-μm Partisil-ODS) and methanol–water as the eluent; we were unable to resolve all-*trans*- from 9-*cis*- and 13-*cis*- from 11-*cis*-retinol (data not shown). This confirmed the report of Stancher and Zonta[15], who also could not separate all the retinol isomers by reversed-phase systems. Normal-phase systems have their own disadvantages and the loss of resolution of retinol isomers due to the presence of polar lipids in the tissue extract has been reported[11]. The advantage of our system is that the HPLC column becomes equilibrated rapidly in the mobile phase and can be easily washed with methanol and water after 25–30 injections of tissue extract.

With the Partisil-10-ODS column we observed the partial resolution of all-*trans*- from 9-*cis*- and 13-*cis*- from 11-*cis*-retinol, when 1% of 2-propanol in hexane was used as the mobile phase. However, the peaks were very broad (data not shown). Zorbax CN is a polar bonded-phase packing that can be used in both normal- and reversed-phase techniques. However, neither the Partisil-10-ODS nor the Zorbax CN column used alone yielded satisfactory separations of all the isomers of retinol. We found that by combining Partisil-10-ODS and Zorbax CN columns, the four isomers of retinol can be separated in two steps using two different solvent systems.

The increase in the capacity ratios when the higher 2-alkanols are added as modifiers could be due to the partitioning of the solute in the mobile phase rather than the interaction on the hydrophobic region of the stationary phase. Alternatively, it is possible that the 2-alkanols may adsorb on the stationary phase, exposing the length of the carbon chain for interaction with the solute[20]. It is interesting that different isomers behave differently with the 2-alkanols in the mobile phase. It appears that 9-*cis*- and all-*trans*-retinol require increased hydrophobicity of the mobile phase for their baseline resolution, whereas 13-*cis*- and 11-*cis*-retinol need increased polarity of the mobile phase for their separation (5% dioxane in hexane) in this system.

The standard retinol isomers (400–500 ng) were injected in 20 μl of absolute methanol. An increase in the volume of methanol, as in Figs. 4A, B and C and Fig. 5 (50 μl of methanol) reduces the retention times of retinol isomers. This change in retention time could be overcome by dissolving the sample in hexane, or keeping the amount of methanol constant (10 μl) throughout the experiment.

The HPLC method described in this paper can be used for the study of *cis–trans* isomerization of retinol in tissues other than the eye. This initial study indicated the presence of 13-*cis*-retinol in the liver lipid extract from a normal rat. Further, the procedure can be applied to the purification of labelled retinol for metabolic studies.

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REFERENCES

1 P. V. Bhat, L. M. De Luca and M. L. Wind, *Anal. Biochem.*, 102 (1980) 243.
2 P. V. Bhat and L. M. De Luca, *Ann. N.Y. Acad. Sci.*, 359 (1981) 135.
3 A. B. Roberts, C. A. Frolik, M. D. Nichols and M. B. Sporn, *J. Biol. Chem.*, 254 (1979) 6303.
4 M. H. Zile, R. C. Inhorn and H. F. De Luca, *J. Biol. Chem.*, 257 (1982) 3544.
5 C. D. B. Bridges, *Exp. Eye Res.*, 22 (1976) 435.
6 D. W. Stainer, T. K. Murray and J. A. Campbell, *Can. J. Biochem. Physiol.*, 38 (1960) 1219.
7 R. Hubbard and G. Wald, *J. Gen. Physiol.*, 36 (1952) 269.
8 S. Futterman, *Exp. Eye Res.*, 18 (1974) 89.
9 P. R. Sundaresan and P. V. Bhat, *J. Lipid Res.*, 23 (1982) 448.
10 R. A. Alvarez, C. D. B. Bridges and S.-L. Fong, *Invest. Ophthalmol. Visual Sci.*, 20 (1981) 304.
11 J. E. Paanakker and G. W. T. Groenendijk, *J. Chromatogr.*, 168 (1979) 125.
12 J. P. Rotmans and A. Kropf, *Vision Res.*, 15 (1975) 1301.
13 F. G. Pilkiewicz, M. J. Pettei, A. P. Yudd and K. Nakanishi, *Exp. Eye Res.*, 24 (1977) 421.
14 R. M. McKenzie, D. M. Hellwege, M. L. McGregor, N. L. Rockley, P,. J. Riquetti and E. C. Nelson, *J. Chromatogr.*, 155 (1978) 379.
15 B. Stancher and F. Zonta, *J. Chromatogr.*, 234 (1982) 244.
16 C. D. B. Bridges, S.-L. Fong and R. A. Alvarez, *Vision Res.*., 20 (1980) 355.
17 C. D. B. Bridges, *Methods Enzymol.*, 81 (1982) 463.
18 A. P. Goldberg, *Anal. Chem.*, 54 (1982) 342.
19 K. Tsukida, A. Kodama, M. Ito, M. Kawamoto and K. Takahashi, *J. Nutr. Sci. Vitamininol.*, 23 (1977) 263.
20 K.-G. Wahlund and I. Beijersten, *Anal. Chem.*, 54 (1982) 128.