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Note

Effect of end-capping of reversed-phase high-performance liquid chromatographic matrices on the analysis of vitamin A and its metabolites

ROBERT W. CURLEY, Jr.* and DEAN L. CARSON
Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, The Ohio State University, 500 West 12th Avenue, Columbus, OH 43210 (U.S.A.)
and
CASIMIR N. RYZEWSKI
Bioanalytical Systems Group, Beckman Instruments, Inc., Arlington Heights, IL 60004 (U.S.A.)
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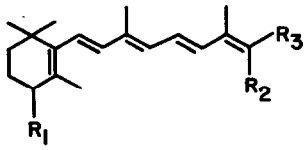
All-*trans*-retinoic acid (see Fig. 1) is a metabolite of retinol (vitamin A) capable of supporting the functions of retinol in the maintenance of normal growth and epithelial cell differentiation[1]. Retinoic acid and some of its analogues (retinoids) have generated much interest as agents useful for the treatment of skin disorders[2] and as potential cancer chemopreventive or chemotherapeutic compounds[3,4]. At present, the mechanism of action of these materials remains obscure although a number of possibilities have been proposed[5].

Recently, we have been engaged in the synthesis and biological evaluation of analogues of all-*trans*-retinoic acid and its natural isomer 13-*cis*-retinoic acid[6,7]. Our current efforts in the area involve the synthesis of electrophilic affinity label analogues derived from the metabolite 4-hydroxyretinoic acid in order to probe the cellular



1 R_1 = H, R_2 = H, R_3 = CH_2OH
2 R_1 = H, R_2 = H, R_3 = CHO
3 R_1 = H, R_2 = H, R_3 = COOH
4 R_1 = H, R_2 = COOH, R_3 = H
5 R_1 = OH, R_2 = H, R_3 = COOH

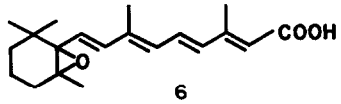


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Fig. 1. Structures of retinol and metabolites. 1 = Retinol; 2 = retinal; 3 = all-*trans*-retinoic acid; 4 = 13-*cis*-retinoic acid; 5 = 4-hydroxyretinoic acid; 6 = 5,6-epoxyretinoic acid.

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retinoic acid-binding protein potentially mediating the actions of all-*trans*-retinoic acid[5,7,8]. Successful isolation of these analogues will require the separation of retinoid isomers produced by our synthetic procedures[9].

Reversed-phase high-performance liquid chromatography (HPLC) has been extensively used to resolve retinol and its metabolites. Many of the most useful methods employ aqueous methanol-based mobile phases[10–13]. The nucleophilicity of methanol[14], however, makes its use as a mobile phase component incompatible with the planned electrophilic analogues of 13-*cis*-retinoic acid. Alternative approaches utilizing aqueous acetonitrile-based mobile phases seemed more likely to suit our needs[15,16].

In the present communication, we report a comparison of the effect of end-capping of the reversed-phase stationary phase on the chromatographic resolution of a number of retinoids of varying polarity. In addition, the ability of the two previously outlined mobile phases to resolve the representative group of retinol metabolites already mentioned, as well as retinal and 5,6-epoxyretinoic acid, has been evaluated.

EXPERIMENTAL

Materials and methods

The retinol, retinal, and all-*trans*-retinoic acid employed in this study were purchased from Aldrich (Milwaukee, WI, U.S.A.). 4-Hydroxyretinoic acid, 13-*cis*-retinoic acid, and 5,6-epoxyretinoic acid were generously provided by Dr. P. F. Sorter, Hoffmann La-Roche (Nutley, NJ, U.S.A.). Retinyl palmitate was obtained from Sigma (St. Louis, MO, U.S.A.). Methyl retinoate was prepared by treatment of retinoic acid with diazomethane according to standard procedures. HPLC grade water, methanol, acetonitrile, tetrahydrofuran, and ammonium acetate were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). Retinoid solutions at a concentration of 1 mg/ml were prepared in HPLC grade acetonitrile and stored under an argon atmosphere at −35°C until use. All manipulations were performed under yellow light (Sylvania F40 gold fluorescent lamps).

Reversed-phase HPLC

HPLC was carried out on a Beckman Model 332 gradient liquid chromatograph system equipped with a Beckman Model 164 ultraviolet detector (Beckman Instruments, San Ramon, CA, U.S.A.) monitoring eluent at 340 nm and a Kipp and Zonen (Delft, The Netherlands) Model BD41 dual-pen recorder.

Retinoids were analyzed by isocratic reversed-phase chromatography on two columns: a 10-μm, irregular particle, non-end-capped octadecylsilane column (Ultrasil-ODS, 250 mm × 4.6 mm I.D.; Beckman Instruments) and a 5-μm, spherical particle, fully end-capped octadecylsilane column (Ultrasphere-ODS, 250 mm × 4.6 mm I.D.; Beckman Instruments). Mobile phases employed in specific separations are indicated in the legend of the appropriate figure. All columns were eluted at ambient temperature and a flow-rate of 1.5 ml/min.

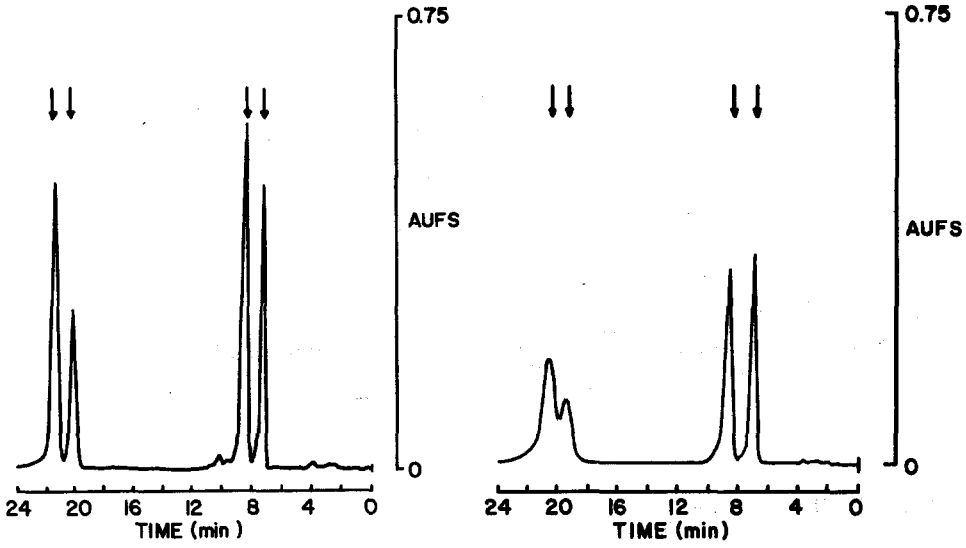

Fig. 2. Reversed-phase HPLC of retinoids on Ultrasphere-ODS with 0.01 *M* ammonium acetate in methanol–0.01 *M* ammonium acetate in water, pH 7 (88:12) as the mobile phase. Arrows indicate elution positions of (from right to left), 13-*cis*-retinoic acid, retinoic acid, retinol, and retinal.

Fig. 3. Reversed-phase HPLC of retinoids on Ultrasil-ODS with 0.01 *M* ammonium acetate in methanol–0.01 *M* ammonium acetate in water, pH 7 (83:17) as the mobile phase. Arrows indicate elution positions of (from right to left), 13-*cis*-retinoic acid, retinoic acid, retinol, and retinal.

RESULTS

Separations with aqueous methanol-based mobile phases

The separation of retinol and its prominent metabolites retinal, all-*trans*- and 13-*cis*-retinoic acid by isocratic reversed-phase HPLC with aqueous methanol-based mobile phases is shown in Figs. 2 and 3. Fig. 2 demonstrates their resolution on the Ultrasphere-ODS column. Fig. 3 shows the chromatography of the same four retinoids on the Ultrasil-ODS column with an aqueous methanol mobile phase designed to give retention times similar to those in Fig. 2.

Separations with aqueous acetonitrile-based mobile phases

Fig. 4 represents the most favorable separation of the four retinoids obtained with the Ultrasphere-ODS column and an aqueous acetonitrile-based mobile phase. The chromatogram shown in Fig. 5 displays the results obtained for the resolution of the same four retinoids on the Ultrasil-ODS column with an aqueous acetonitrile mobile phase designed to give retention times similar to those in Figs. 2 and 3. The effects of addition of a small amount of relatively non-polar organic modifier (in this case 5% tetrahydrofuran) on the resolution observed in Fig. 5 is shown in Fig. 6. This figure also demonstrates that the chromatographic system will resolve some of the more polar metabolites of retinoic acid, *i.e.* 4-hydroxyretinoic acid and 5,6-epoxyretinoic acid.

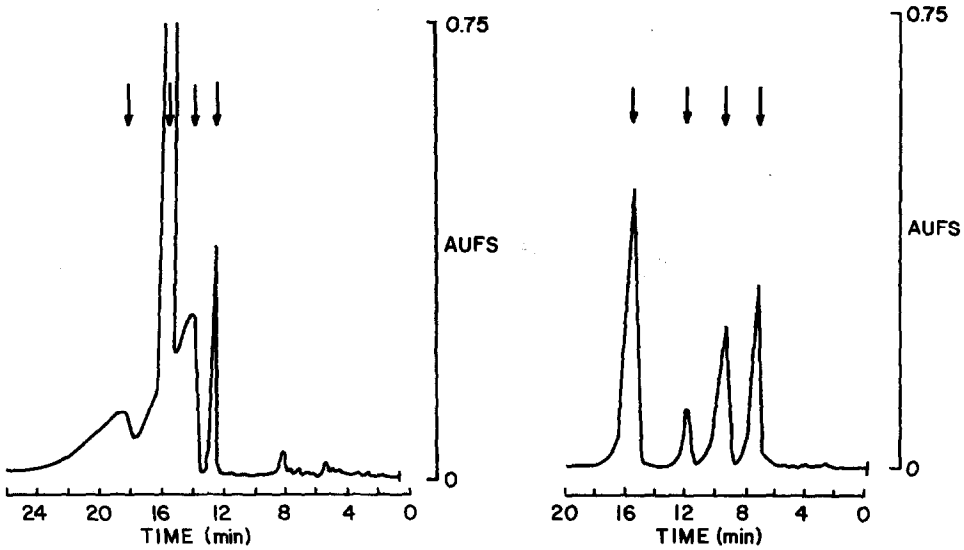

Fig. 4. Reversed-phase HPLC of retinoids on Ultrasphere-ODS with acetonitrile–0.05 *M* ammonium acetate in water, pH 7 (90:10) as the mobile phase. Arrows indicate elution positions of (from right to left), retinol, 13-*cis*-retinoic acid, retinal, and retinoic acid.

Fig. 5. Reversed-phase HPLC of retinoids on Ultrasil-ODS with acetonitrile–0.05 *M* ammonium acetate in water, pH 7 (80:20) as the mobile phase. Arrows indicate elution positions of (from right to left), 13-*cis*-retinoic acid, retinoic acid, retinol, and retinal.

DISCUSSION

For separations of retinol and its oxidation metabolites in this aqueous methanol-based mobile phase the fully end-capped, 5-μm octadecylsilane column is preferable to the non-end-capped, 10-μm octadecylsilane stationary phase (*cf.* Figs. 2 and 3). Although adequate for resolving all-*trans*-retinoic acid and 13-*cis*-retinoic acid, the 10 μm non-end-capped column does not resolve the later eluting retinol and retinal as well as the 5-μm end-capped matrix.

There is a reversal of apparent matrix superiority upon changing to aqueous acetonitrile-based mobile phases. In this case, even at relatively high concentrations

TABLE I

STATIONARY PHASE PROPERTIES

Property	*Ultrasphere-ODS*	*Ultrasil-ODS*
Average particle size (μm)	5	10
Particle shape	Spherical	Irregular
Particle surface area (m^2/g)	200	350
Carbon loading (%)	12	20
End-capping	Exhaustive	None

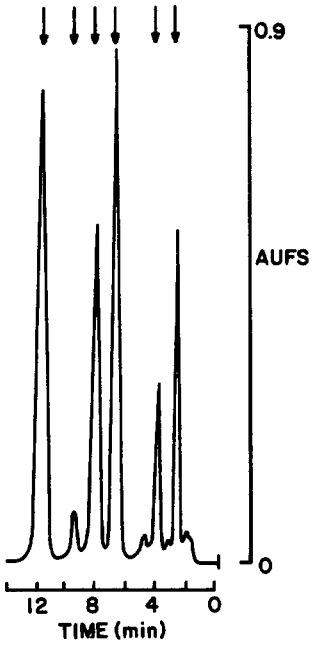

Fig. 6. Reversed-phase HPLC of retinoids on Ultrasil-ODS with acetonitrile–0.05 *M* ammonium acetate in water, pH 7–tetrahydrofuran (76:19:5) as the mobile phase. Arrows indicate the elution positions of (from right to left), 4-hydroxyretinoic acid, 5,6-epoxyretinoic acid, 13-*cis*-retinoic acid, retinoic acid, retinol, and retinal.

of acetonitrile, there is an undesired increase in the retention times of the retinoids on the Ultrasphere-ODS column. In addition, an unanticipated change in elution order of the four retinoids occurs as well as an excessive amount of peak tailing for the ionizable retinoic acids. These undesirable chromatographic effects were not moderated by an increase in ionic strength (0.1 *M* ammonium acetate), a change to the more hydrophobic salt triethylamine acetate, or addition of tetrahydrofuran (data not shown).

Alternatively, chromatography on Ultrasil-ODS is very acceptable in the aqueous acetonitrile mobile phase. Although there is a minimal amount of peak tailing in this system, this isocratic method should prove ideal in situations where methanol must be avoided as a component in the mobile phase. In addition, the resolution in this system can be improved along with a reduction in retention time by addition of a small proportion of tetrahydrofuran. Even with this added less polar organic modifier, relatively polar metabolites of retinol can still be determined (Fig. 6).

Addition of 5% tetrahydrofuran to the mobile phase employed with the Ultrasil-ODS column will also permit analysis of relatively non-polar esters of retinol and all-*trans*-retinoic acid. For example, the methyl ester of all-*trans*-retinoic acid is eluted with a retention time of 20 min with the mobile phase employed in Fig. 6 (data not shown). With a change in the acetonitrile–water–tetrahydrofuran proportion to 85:10:5, this stationary–mobile phase combination will even permit ready analysis of

retinyl palmitate, a very non-polar metabolite of retinol (t_R = 34 min; data not shown).

The apparent superiority of Ultrasil-ODS for chromatography of retinoids in aqueous acetonitrile is most likely related to the chemistry of the stationary phases. The general properties of the two modified silicas are listed in Table I. Although the Ultrasil particles are larger than those of the Ultrasphere matrix, the former material has a greater surface area due to a higher particle porosity. This property, along with the absence of end-capping of the matrix, may account for the favorable utility of the Ultrasil matrix in the present application.

In summary, we have developed an efficient, isocratic, reversed-phase chromatographic procedure for the resolution of a wide range of retinol metabolites. This procedure may prove to be generally useful as well as being particularly suited to use in separation problems which require the avoidance of nucleophilic solvents such as methanol.

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