Separation of the natural retinoids by high-pressure liquid chromatography

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Abstract A reverse phase high-pressure liquid chromatography system for rapid separation of various retinoids (vitamin A and its analogs) with little or no degradation is described. This method permits detection of as little as 22 pmol of retinoic acid. The procedure has been applied to the study of retinoic acid metabolism in vitamin A-deficient hamsters.

Supplementary key words retinol · retinoic acid · reverse phase

The study of vitamin A metabolism has been hampered by the need for a method by which it is possible to separate closely related labile compounds. Adsorption chromatography has, in the past, been the most widely used procedure for isolation and purification of the various natural retinoids (1). Recently, a liquid–gel partition chromatography system has been developed that utilizes Sephadex LH-20 to successfully detect several metabolites of retinoic acid (2, 3). The main disadvantage of this method is the time required for each chromatographic determination. In order to overcome this drawback, the use of high-pressure liquid chromatography (HPLC) for the rapid separation of retinoids was examined.

Recently, HPLC on silica columns has been used by a number of investigators in various areas of retinoid research (4–8). Silica, however, is very sensitive to the presence of water and to the use of gradients employing solvents of widely varying polarity (9). Therefore, these systems have been used in retinoid studies only for isocratic separations of closely related compounds. In order to develop a method that could readily be adapted to a wide range of retinoids, it was decided to use the technique of reverse phase chromatography. It is the purpose of this report to illustrate the use of high-pressure reverse phase liquid chromatography for the rapid separation of retinol, retinal, retinoic acid, and retinyl acetate. The direct application of this technique to the study of the metabolism of retinoic acid in the plasma and urine of retinoid-deficient hamsters will also be demonstrated.

EXPERIMENTAL METHODS

High-pressure liquid chromatography

All HPLC work was performed on a Spectra Physics Model 3500 B (Spectra Physics, Santa Clara, CA) apparatus fitted with a Valco sample injection valve (Valco Inst. Co., Houston, TX). Detection was by a variable wavelength UV monitor (Model 770, Schoeffel Instrument Corp., Westwood, NJ) with a maximum sensitivity of 0.01 AUFS. The two columns used were a 3.0 mm ID × 25 cm long, 5 μm Spherisorb ODS column (Spectra Physics) and a 4.6 mm ID × 25 cm long, 10 μm Partisil-10-ODS-2 column (Whatman Inc., Clifton, NJ). All chromatography was done at ambient temperature with a flow rate of approximately 1.1 ml/min.

Animals

Pregnant Syrian golden hamsters (Sprague Dawley, Madison, WI) were obtained 4–5 days before delivery and maintained on a diet containing a 1:1 ratio of Wayne Lab Blox (Allied Mills Inc., Chicago, IL) and pelleted vitamin A-deficient food modified for hamsters with 5% nonnutritive fiber added (10) (TekLad Mills, Madison, WI). Immediately after delivery the mothers were placed entirely on the vitamin A-deficient diet. The litter size was reduced to eight per mother. The hamsters were weaned at 21 days and then maintained on the vitamin A-deficient diet until ready for use (30–33 days old).

Preparation of plasma and urine

Hamsters were placed under ether anesthetic and injected intrajugularly with 50 μl of ethanol–0.9% NaCl 1:1 containing 0.17 μg of [11,12-^{3}H]-

Abbreviations: HPLC, high-pressure liquid chromatography.

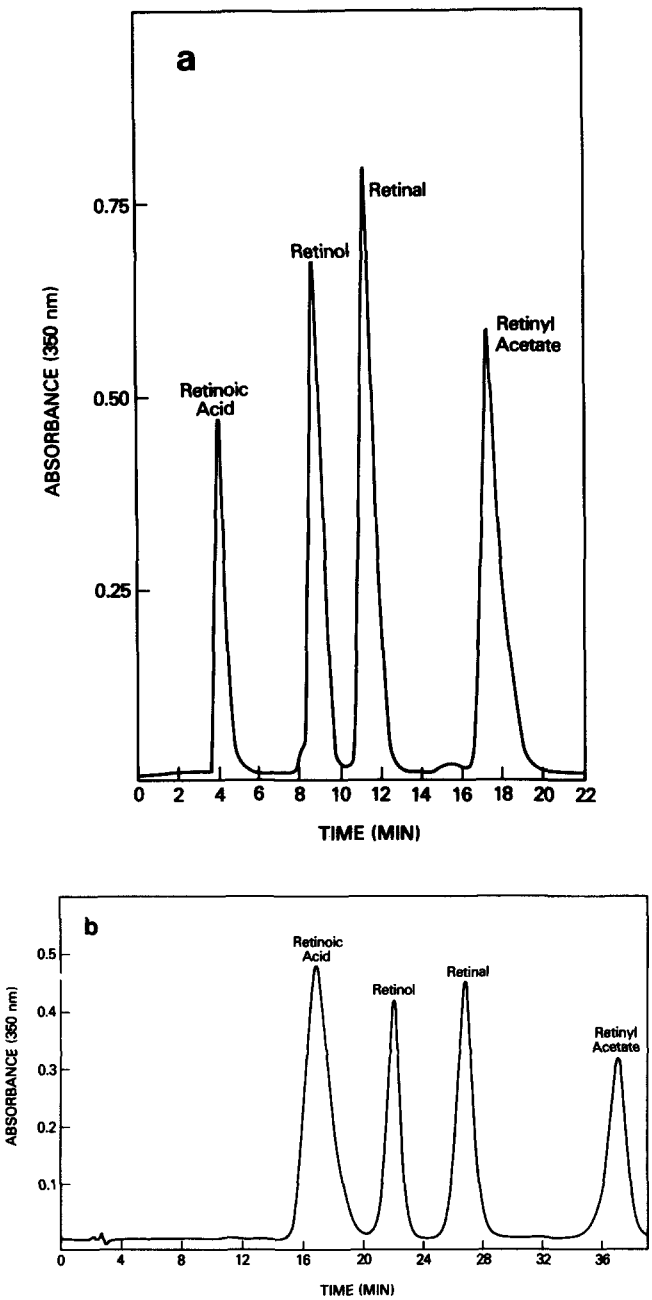

Fig. 1. High-pressure liquid chromatography of four retinoids. A mixture of retinoic acid, retinol, retinal, and retinyl acetate (total 350 mm absorbing material equaled 0.91 absorbance units) was injected: (*a*) in 10 μl of 100% methanol onto a Spherisorb ODS column (3.0 mm ID × 25 cm). Acetonitrile–1% ammonium acetate 60:40 was used as the developing solvent. At a pressure of 2620 psi, a flow rate of 1.1 ml/min was achieved; (*b*) in 50 μl of 100% methanol onto a Partisil ODS-2 column (4.6 mm ID × 25 cm). Acetonitrile–1% ammonium acetate 80:20 was used as the developing solvent. At a pressure of 700 psi a flow rate of 1.1 ml/min was achieved.

retinoic acid (6.0×10^6 dpm/animal). Six hr after injection, plasma was obtained by heart puncture. Urine was collected directly from the bladder and therefore represented only the material present in the bladder at the time of death. The samples were lyophilized to dryness and the residue was extracted with chloroform–methanol and methanol as described by Ito et al. (2) with the following changes. Unlabeled carrier retinoic acid (25 μg) was added to the sample prior to lyophilization which was carried out in 2–3 hr. The final extract was dissolved in 0.25–2 ml of methanol and centrifuged, and an aliquot was applied directly to the HPLC column.

Determination of radioactivity

Samples were collected from the column at 1-min intervals. The solvent was evaporated using a Vortex–evaporator (Buchler Inst., Fort Lee, NJ). To the dried sample, 0.2 ml of NCS (Amersham/Searle, Arlington Heights, IL)–H_2O 9:1 and 5 ml of Econofluor (New England Nuclear, Boston, MA) was added. The final sample was counted in a Packard TriCarb Model 3385 scintillation counter equipped with an external standard system. Aliquots of plasma and urine and their extracts were counted in a similar manner.

Chemicals

All retinoids were a gift from Hoffmann-La Roche Inc., Nutley, NJ. The [11,12-^{3}H]retinoic acid (sp act 4.85 mCi/mg and 37 mCi/mg) was purified on a Spherisorb ODS column. The initial solvent for purification was acetonitrile–0.2% ammonium acetate 2:98. After a 5 min delay, a linear gradient was started with a sweep time of 35 min and a final solvent concentration of acetonitrile–0.2% ammonium acetate 60:40. There was a 10-min hold 30% into the gradient. The retinoic acid from the Spherisorb ODS column was applied to a Partisil ODS-2 column which was developed isocratically with acetonitrile–1% ammonium acetate 60:40. Solvents for chromatography were obtained from Burdick and Jackson Laboratories, Inc., Muskegon MI. All other solvents were of analytical grade. The ammonium acetate was of reagent grade and was purchased from Eastman Kodak Co., Rochester, NY.

RESULTS

Fig. 1 illustrates the chromatographic profiles from two different reverse phase HPLC systems that successfully separated several of the natural retinoids. The Spherisorb ODS column (Fig. 1*a*), with its shorter retention times, is advantageous for isolation of compounds of low polarity. The more polar retinoids separate better on the Partisil ODS-2 column

(Fig. 1*b*). Inclusion of the ammonium acetate was found to be necessary to avoid broadening of the retinoic acid peak. As little as 0.2% ammonium acetate could be utilized for the Spherisorb ODS column. However, the percentage could not be decreased below the 1% level when chromatography was performed on Partisil ODS-2.

In addition to reverse phase chromatography, several other liquid–solid chromatographic adsorbents, including silica and alumina, were also tested for their ability to separate the various retinoids. As mentioned before, silica has been used in the past for isocratic HPLC chromatography of many closely related vitamin A compounds (4–8). However, for quick elution from silica of retinoids that have a marked difference in polarity (e.g., retinol vs. retinoic acid), a solvent gradient was found to be necessary. With the use of such a gradient, reproducible retention times were difficult to obtain. The more polar solvent appeared to strip water from the silica thereby continually changing its adsorption characteristics (9). For this reason, when samples of widely varying polarity are encountered, a reverse phase system with its ability to withstand large changes in solvent polarity would be the adsorbent of choice.

Alumina has also been widely used as an adsorbent for retinoid chromatography (11–13) and is available for use in HPLC. However, when the alumina adsorbent was tested, retinoic acid could not be eluted from the column with either 100% isopropyl alcohol, 100% chloroform, or 100% methanol. Because of this, alumina was not considered to be useful for the chromatography of retinoids, especially those compounds containing a carboxylic acid function.

Besides changing the adsorbent in liquid–solid HPLC, the eluting solvent can also be varied. Since methanol–water is a commonly used solvent system in reverse phase chromatography, its ability to separate retinoids was investigated. Although retinoic acid and retinyl acetate separated cleanly from retinol and retinal, the latter two compounds could not be separated from each other, even using the powerful separation technique of recycling. This solvent system, therefore, although perhaps useful for chromatography of certain retinoids, would not be useful for the separation of the natural retinoids.

Having selected the reverse phase adsorbent and the acetonitrile–ammonium acetate solvent system as being the most versatile chromatographic techniques of the methods examined, the degree of recovery and stability of retinoids applied to this system was examined. In order to determine recovery of the applied retinoid and its stability during chromatography, a known aliquot of purified [11,12-^{3}H]retinoic acid was applied to the Partisil ODS-2 column (**Fig. 2**). A solvent gradient was run that separated compounds more polar than the parent retinoid. Total recovery of applied radioactive material was 96–104%, while 91% of the applied radioactive material migrated in the retinoic acid region of the column effluent. As can be seen on the expanded portion of the chromatogram, several peaks more polar than the retinoic acid did appear. These peaks represented 4.8% of the applied radioactivity. Rechromatography of the material in the retinoic acid region of the column effluent again yielded approximately 4% of the applied material migrating in fractions 35–55. It is uncertain whether these polar peaks are due to degradation of the retinoic acid during chromatography or to the presence of an equilibrium mixture of retinoic acid and its isomers (as is known to occur for retinal [14]), perhaps catalyzed by the

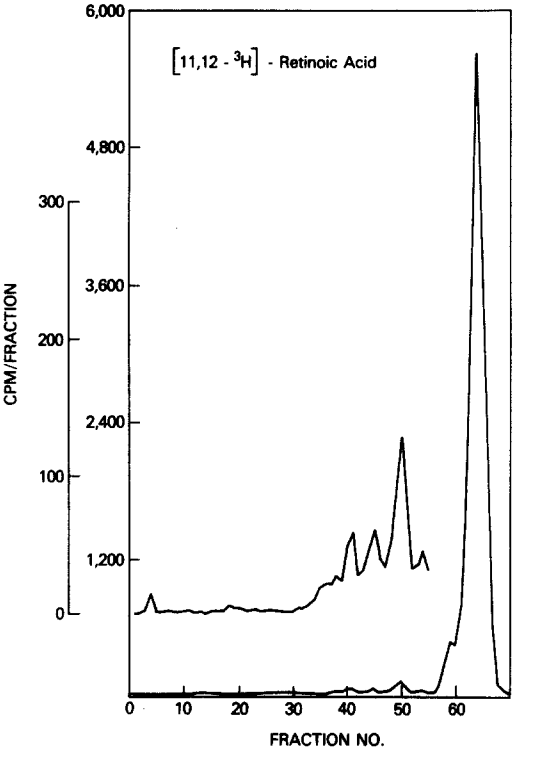

Fig. 2. High-pressure liquid chromatography of [11,12-^{3}H]-retinoic acid. [11,12-^{3}H]Retinoic acid (17,500 cpm) was injected in 80 μl of 100% methanol onto a Partisil ODS-2 column. After developing the column for 5 min with acetonitrile–1% ammonium acetate 2:98, a 35-min linear gradient was run with a 10-min hold 8 min into the gradient. The final solvent concentration was acetonitrile–1% ammonium acetate 75:25. A flow rate of 1.1 ml/min was maintained during the entire chromatographic run. One-min fractions were collected, evaporated, and counted as described in Methods.

presence of tritium in the molecule. The sensitivity of the technique was demonstrated by the ability to detect as little as 6.7 ng (22 pmol) of nonradioactive retinoic acid by absorbance at 350 nm.

Minor modifications of the eluting solvent allowed this system to be used with a wide variety of retinoids. For example, the use of acetonitrile–1% ammonium acetate 80:20 on the Partisil ODS-2 column gave a good separation of the natural retinoids (Fig. 1*b*). A slight change of the solvent to acetonitrile–1% ammonium acetate 60:40 now allowed an excellent separation of 13-*cis* from all-*trans*-retinoic acid (**Fig. 3**). Retinyl palmitate did not elute from the Spherisorb ODS column until the solvent composition reached acetonitrile–0.2% ammonium acetate 98:2 (data not shown). The procedure could therefore be readily modified to obtain the degree of separation needed for the particular retinoids being studied.

No separation technique is useful unless it can be easily applied to actual experimental conditions. Therefore, purified [11,12-^{3}H]retinoic acid (0.17 μg) was injected intrajugularly into five vitamin A-deficient hamsters. Six hours later the animals were killed and the plasma and urine were collected and extracted as described in Methods. The results of the extraction are shown in **Table 1**. It can be seen that the majority of the radioactive material present in the plasma and urine at this time was chloroform–methanol soluble.

In order to determine the usefulness of the HPLC system for examining the nature of this radioactive material, the chloroform–methanol extracts were applied directly to the Partisil ODS-2 column. The column was developed with a solvent gradient that separated the polar metabolites of retinoic acid. The unchanged parent compound appeared in fractions 60–62. As can be seen in **Fig. 4**, the chromatographic system separated the radioactive material in the chloroform–methanol extract into several more polar peaks (**Table 2**). To make certain that these peaks were due to in vivo metabolism and not to in vitro degradation that might be occurring during extraction and chromatography, [11,12-^{3}H]-retinoic acid was added directly to retinoid-deficient hamster plasma and urine. The samples were then extracted and chromatographed as before. The chromatographic profiles (Fig. 4) looked very similar to the initial starting material (Fig. 2) with only 2.4–3.6% of the applied radioactive material occurring in the more polar regions of interest (Table 2). It therefore appeared that the observed metabolites found in the plasma and urine were indeed formed in vivo and were not artifacts of the extraction and chromatographic procedure. Identical results were obtained in a second experiment.

TABLE 1. Distribution of radioactive material in plasma and urine 6 hr after injection of 0.17 μg of [11,12-^{3}H]retinoic acid

	Plasma	Urine
% Dose/ml	1.8 ± 0.2[a]	5.2
% $CHCl_3$–MeOH soluble	89	104
% MeOH-soluble	17	8
Residue, %	1.8	0.3
Total recovery, %	107.8	112.3

[a] Standard deviation (average of five separate animals).

Five retinoid-deficient hamsters were dosed intrajugularly with 0.17 μg of [11,12-^{3}H]retinoic acid. Six hr later the animals were killed and the plasma and urine were collected and pooled. They were then extracted as described in the text.

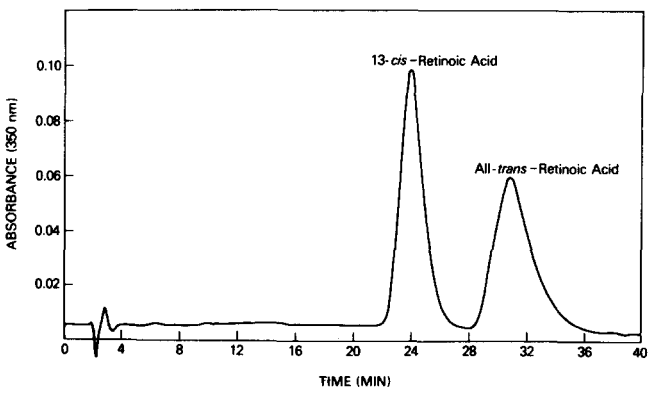

Fig. 3. Separation of 13-*cis*-retinoic acid from all-*trans*-retinoic acid. A mixture of 40 ng of 13-*cis*-retinoic acid and 40 ng of all-*trans*-retinoic acid was injected in 50 μl of 100% methanol onto a Partisil ODS-2 column. The column was developed with acetonitrile–1% ammonium acetate 60:40 at a flow rate of 1.1 ml/min.

DISCUSSION

The main concern of these studies has been the development of a simple, rapid, reliable separation procedure for the natural retinoids. The method developed utilizing reverse phase HPLC offers all the advantages innate in HPLC. These include high resolution, short elution times, low solvent volumes, and high sensitivity. In addition, the system gives quantitative recoveries with little or no production of artifacts, a necessary attribute for any study of retinoid metabolism.

The use of reverse phase adsorption chromatography over normal adsorption chromatography offers several additional advantages. Since the system is not sensitive to the presence of water in the sample or in the solvents, the tissue organic solvent extract can

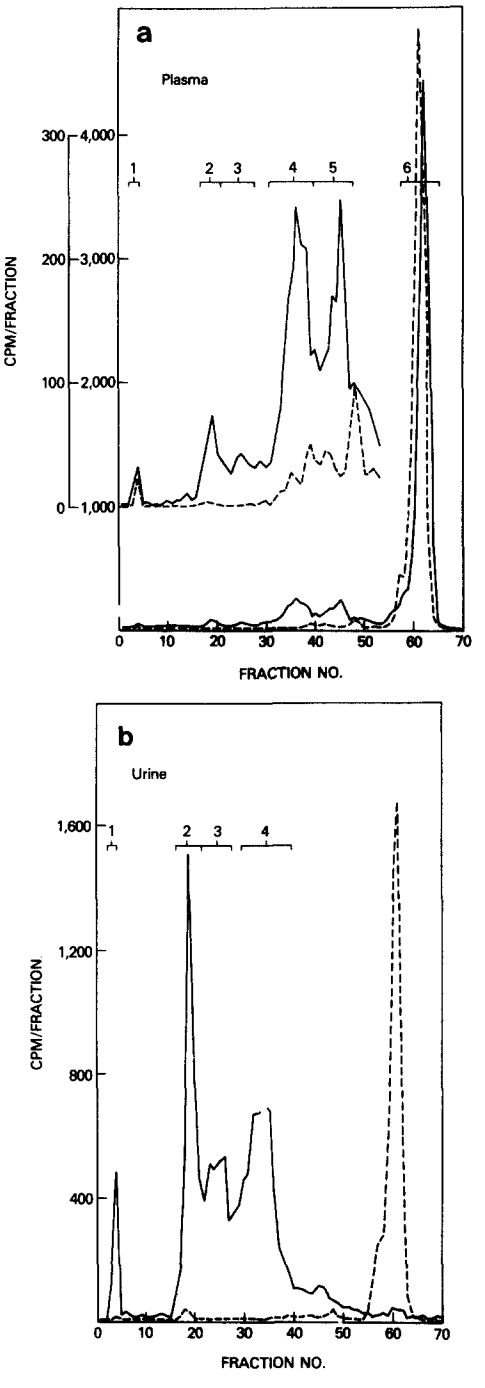

Fig. 4. High-pressure liquid chromatography of metabolites formed in vivo from [11,12-^{3}H]retinoic acid. Retinoid-deficient hamsters were injected intrajugularly with 0.17 μg of [11,12-^{3}H]retinoic acid 6 hr prior to being killed. The plasma and urine were extracted as described in Methods. Aliquots (0.08–0.10 ml in 100% MeOH) were injected onto a Partisil ODS-2 HPLC column which was developed as described in Fig. 2. The dashed line represents control samples in which [11,12-^{3}H]retinoic acid was added directly to the tissue which was then extracted and chromatographed as above. The brackets denote the regions of the chromatogram combined to obtain the data for Table 2. The upper tracing (*a*) represents a portion of the chromatogram on an expanded scale.

be applied directly to the column without concern for its water content. Furthermore, reverse phase HPLC is particularly advantageous to the study of retinoic acid metabolism since it appears from past work (3, 15) that most metabolites of retinoic acid are more polar than the parent compound. In a reverse phase system, these polar compounds rapidly elute from the column prior to retinoic acid, whereas in normal adsorption chromatography, a strongly polar metabolite would bind tightly to the support requiring perhaps extreme solvent changes for elution. A final advantage to this technique is its wide applicability. Minor changes in solvent composition will give separation of a wide range of retinoids (see, for example, Fig. 1*b* and Fig. 3). Therefore, the need for a large variety of solvents is avoided.

Although it was not the purpose of these initial experiments to conduct an extensive study of the metabolism of retinoic acid, it was felt necessary to test the system with actual experimental samples. Direct application to the HPLC column of tissue extracts from retinoid-deficient hamsters injected 6 hr earlier with [11,12-^{3}H]retinoic acid confirmed data obtained from the rat (3, 15). As expected, several polar metabolites of retinoic acid appeared in the tissues studied. Also in agreement with these previous reports, no free retinoic acid was detected in the urine. However, one major difference can be noted. The percentage of free, unchanged retinoic acid appearing in the plasma chloroform–methanol extract is higher in the results presented in this paper than in earlier work (3). Whether this is due to the experimental animal used (hamster vs. rat) and/or to the level of retinoic acid administered (0.17 μg vs. 10–17 μg) will have to be determined in future work.

In conclusion, the results obtained in this study

TABLE 2. Distribution of [11,12-^{3}H]retinoic acid and its polar metabolites in plasma and urine

Metabolite Region	Plasma	Urine
	% applied radioactivity	
1	0.2 (0.1)[a]	3.7 (0.2)
2	1.2 (0.06)	23 (1.2)
3	1.1 (0.03)	15 (0.04)
4	6.7 (1.2)	30 (1.2)
5	5.5 (1.0)	— (1.0)
6	62 (76)	1 (74)
Total	82 (82)	83 (80)

[a] The numbers in parentheses represent the distribution of radioactive material in control [11,12-^{3}H]retinoic acid plasma and urine extracts.

Animals were treated as described in Table 1. Chloroform–methanol extracts were analyzed using the HPLC system described in Fig. 2. Metabolites less polar than retinoic acid were not examined.

clearly indicate the potential usefulness of reverse phase HPLC in the study of retinoid metabolism.

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