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 [11,12-3H]-...
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.

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)–H₂O 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 [11,12-³H]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. 1a), 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.

retinoic acid (6.0 × 10⁶ 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.
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 continuously 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 [11,12-3H]retinoic acid. [11,12-3H]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.

![Fig. 2. High-pressure liquid chromatography of [11,12-3H]-retinoic acid.](Image)
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. 1b). 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 acetonit-
trile–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 par-
ticular retinoids being studied.

No separation technique is useful unless it can be
easily applied to actual experimental conditions.
Therefore, purified [11,12-3H]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 cer-
tain that these peaks were due to in vivo metabolism
and not to in vitro degradation that might be occurring
during extraction and chromatography, [11,12-3H]-
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.

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 reti-
noid metabolism.

The use of reverse phase adsorption chromatog-
raphy 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. 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 acetonit-
trile–1% ammonium acetate 60:40 at a flow rate of 1.1 ml/min.

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

<table>
<thead>
<tr>
<th></th>
<th>Plasma</th>
<th>Urine</th>
</tr>
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<tbody>
<tr>
<td>% Dose/ml</td>
<td>1.8 ± 0.2*</td>
<td>5.2</td>
</tr>
<tr>
<td>% CHCl3–MeOH soluble</td>
<td>89</td>
<td>104</td>
</tr>
<tr>
<td>% MeOH-soluble</td>
<td>17</td>
<td>8</td>
</tr>
<tr>
<td>Residue, %</td>
<td>1.8</td>
<td>0.3</td>
</tr>
<tr>
<td>Total recovery, %</td>
<td>107.8</td>
<td>112.3</td>
</tr>
</tbody>
</table>

* Standard deviation (average of five separate animals).

Five retinoid-deficient hamsters were dosed intrajugularly with
0.17 µg of [11,12-3H]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.
High-pressure liquid chromatography of metabolites formed in vivo from \([11,12-^3H]\)retinoic acid. Retinoid-deficient hamsters were injected intrajugularly with 0.17 \(\mu g\) of \([11,12-^3H]\)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 \([11,12-^3H]\)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.

Fig. 4. High-pressure liquid chromatography of metabolites formed in vivo from \([11,12-^3H]\)retinoic acid. Retinoid-deficient hamsters were injected intrajugularly with 0.17 \(\mu g\) of \([11,12-^3H]\)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 \([11,12-^3H]\)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.

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

<table>
<thead>
<tr>
<th>Metabolite Region</th>
<th>Plasma</th>
<th>Urine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% applied radioactivity</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.2 (0.1)*</td>
<td>3.7 (0.2)</td>
</tr>
<tr>
<td>2</td>
<td>1.2 (0.06)</td>
<td>23 (1.2)</td>
</tr>
<tr>
<td>3</td>
<td>1.1 (0.03)</td>
<td>15 (0.04)</td>
</tr>
<tr>
<td>4</td>
<td>6.7 (1.2)</td>
<td>30 (1.2)</td>
</tr>
<tr>
<td>5</td>
<td>5.5 (1.0)</td>
<td>— (1.0)</td>
</tr>
<tr>
<td>6</td>
<td>62 (76)</td>
<td>1 (74)</td>
</tr>
<tr>
<td>Total</td>
<td>82 (82)</td>
<td>83 (80)</td>
</tr>
</tbody>
</table>

* The numbers in parentheses represent the distribution of radioactive material in control \([11,12-^3H]\)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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REFERENCES


