Assay of Retinoids in Biological Samples by Reverse-Phase High-Pressure Liquid Chromatography

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ABSTRACT

A separation procedure for retinoids based on reverse-phase high-pressure liquid chromatography with solvent mixtures of acetonitrile and water is described. The method may be applied to the screening of synthetic retinoids, which have potential for use in the prevention of cancer. It is easily adapted to a variety of biological samples and can be applied to other conventional retinoid assays in liver and plasma, detecting as little as 1 nmol retinyl acetate or of a synthetic vitamin A analog (21). For circumvention of these properties, synthetic retinoids are being developed. It was therefore desirable to develop a method that could quickly and accurately determine the amounts of retinoids in tissue samples and the degree to which synthetic retinoids might be converted to retinyl esters and stored in the liver. Furthermore, the limited amounts of the trial compounds required that the assay be more sensitive than were previous methods. Other applications of the assay include the estimation of plasma retinol and retinyl esters in vitamin A toxicity (17, 21) and the routine bioassay of the liver storage of retinoids (3), as well as the biochemical investigation of the metabolism of various retinoids (7, 10, 13, 15).

In the past the only simple and rapid procedures available for retinoid determination have been based on either the Carr-Price antimony trichloride colorimetric assay (3) or the trifluoroacetic acid modification of Dugan et al. (6), both of which confound retinol and retinyl esters. A separate estimation of retinol and its esters has required lengthy chromatographic procedures (10–13, 15, 19). The method proposed here is based on the reverse-phase HPLC system recently described by Frolik et al. (9), which can rapidly and reproducibly separate and simultaneously quantitate a variety of retinoids with little or no degradation. The use of a 2-step elution system results in a clean separation of retinol from many closely related synthetic derivatives and from other physiological forms of the vitamin such as retinoic acid, retinal, and retinyl esters. In addition, the retinyl esters are separated into their major fatty acid components, which by previous methods required 2 extra chromatographic steps (12).

With this method it has been shown that 2 synthetic retinoids, N-acetyl retinyl amine (14) and retinal acetylhydrazone (18) (Chart 1), each undergo conversion to retinol and retinyl esters in the vitamin A-deficient hamster. The splitting of these derivatives to a physiological form of the vitamin could account for the reported vitamin A activity of the compounds in the reversal of keratinization in tracheal organ cultures (23).

MATERIALS AND METHODS

Chemicals. Retinol was obtained from Eastman Organic Chemicals, Rochester, N. Y.; retinyl palmitate was from Sigma Chemical Corporation, St. Louis, Mo.; and axerophthenol was from BASF/AG, Ludwigshafen am Rhein, Germany. All other retinoids were a generous gift of Hoffmann-La Roche Inc., Nutley, N. J. Solvents used for HPLC were obtained from Burdick & Jackson Laboratories, Muskegon, Mich., and other reagents used were analytical grade. The water used for HPLC was redistilled over glass.

Dosing of Animals. Weanling male and female Syrian golden hamsters raised on a vitamin A-deficient regimen (4, 9) were used at 30 to 33 days of age. At this time the livers contained no detectable retinol and very small amounts of retinyl esters (average concentration of 1 nmol/g).

Hamsters were dosed daily with either retinoids or vehicle for 1 or 2 days by stomach intubation of 0.2 ml of a solution of ethanol:triocotanoin (1:3) containing 0.025% butylated hydroxytoluene.

1 To whom requests for reprints should be addressed.
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Retinyl acetate was added to each tissue with a solvent flow of 1.2 ml/min and a solvent flow of 1.2 ml/min and a solvent flow of 1.2 ml/min. The esters appeared to be stable for at least 5 weeks.

For determination of the efficiency of various solvents in extracting the retinoids, the lyophilization procedure of Ito ef al. (13) or that of Ito ef al. (13). Ether containing 0.1% butylated hydroxytoluene was used for extraction. An aliquot of the extract was evaporated to dryness under a stream of nitrogen, and the residue was redissolved in 100% chloroform, followed by the addition of 0.5 volume of methanol to give a final composition of 1:1. This solution was centrifuged to clarity and analyzed directly by HPLC. All procedures were carried out in minimal light or under red lights, and sample solutions were stored in the dark at -20° prior to chromatography. For the trifluoroacetic acid determinations, aliquots of the same HPLC solution as above were analyzed by a modification (8) of the procedure of Dugan ef al. (6).

The integrated peak areas were converted to nmol of retinoid by comparing the integrator number for the peak area at 325 nm of the retinoid of interest with an average standard integrator number/nmol of that retinoid, obtained from the chromatography and peak integration of several reference samples in the range of 0.1 to 2 μg.

**RESULTS**

The separation of retinol, retinal acetylhydrazone, N-acetyl retinyl amine, retinyl acetate, axerophthene (the vitamin A hydrocarbon), and retinyl palmitate by reverse-phase HPLC is shown in Chart 2. Depending on which retinoids are present in a particular tissue sample, the percentage of acetonitrile in the initial eluting solvent can be modified to give separation of the more polar retinoids in the minimal time. For example, retinol and retinyl acetate are eluted at 3.3 and 4.6 min, respectively, when the initial solvent is 84% acetonitrile in water.

With anhydrous ether as the solvent, the sodium sulfate powder procedure of Ames ef al. (2) and the lyophilization method of Ito ef al. (13) were shown to be equivalent for the determination of tissue retinoids, although the lyophilization procedure has distinct advantages when water-miscible solvents are used for extraction. The relative efficiency of ether, ethyl acetate, methanol, or chloroform:methanol approximately 10 min following the change of the eluant composition to 98% acetonitrile.

The identification of the various peaks was established by chromatography of standard compounds as well as by cochromatography of standard retinoid solutions with the tissue extracts.

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(1:1) in extracting retinol and retinyl esters from livers was compared. Differences among solvents were not significant. The ease of evaporation of the ether made it the solvent of choice for this analysis. However, the effective extraction of certain retinoids may require the use of a different solvent. For example, retinoic acid, which is more polar than retinol, was extracted by ether only to 50% as well as by methanol.

With the sodium sulfate powder method with ether as the extracting solvent, the recovery of 261 nmol retinol and 378 nmol retinyl palmitate was determined. A 100-µl aliquot of a chloroform solution of each retinoid was either applied directly onto the liver or added to the ether in the first extraction step. Drying of the chloroform solutions onto a 1-g sample of liver from a control hamster prior to grinding the liver with sodium sulfate resulted in 98.5 and 94.5% recovery of the retinol and retinyl palmitate, respectively, compared to the recovery of the same amount of standard added to the ether at the time of the first extraction step. Absolute recoveries in the latter procedure were 109 and 111%, respectively.

Unexpectedly, chloroform solutions (100 µl of a 1-mg/ml solution) of retinyl esters underwent a tissue-dependent hydrolysis to retinol in both the sodium sulfate powder method and the lyophilization procedure. Aliquots of a retinyl acetate solution dried onto the liver prior to grinding with sodium sulfate were hydrolyzed 55 to 92%, whereas an aliquot added to the medium prior to homogenization in the lyophilization method resulted in a lesser extent of hydrolysis (17 to 24%). Further investigation of the hydrolysis of retinyl esters by the sodium sulfate powder method showed that aliquots of a retinyl acetate solution that had been added either to a liver sample that had been boiled in 0.9% NaCl solution for 15 min or directly to sodium sulfate with no liver tissue present were recovered unchanged. Retinyl palmitate solutions, on the other hand, were hydrolyzed to retinol to only a minor degree by either procedure (grinding, 3.1%; lyophilization, 0.5%), and the synthetic retinoid, retinal acetylhydrazone, was not hydrolyzed at all by the same procedures. Retinyl acetate and retinyl palmitate solutions were hydrolyzed to retinol approximately 5 and 2%, respectively, when dried onto intestinal samples and ground with sodium sulfate. These findings are not inconsistent with the data of Ames et al. (2), as their reported 99% recovery for retinyl acetate was quantitated by the Carr-Price reaction, which cannot distinguish between retinol and retinyl esters. It follows, however, that the lyophilization procedure, which minimizes these effects, is the method of choice for this analysis.

The choice of a solvent for redissolving the sample following evaporation of an aliquot of the extracting solvent was particularly critical, as shown in Table 1. Both 100% methanol and 100% acetonitrile, while adequately dissolving the retinol in the sample, were poor solvent choices for the solution of the very nonpolar retinyl esters. Increasing amounts of chloroform in mixture with methanol resulted in maximal solubilization of both retinol and retinyl esters. The limited solubility of chloroform mixtures in acetonitrile:water solutions, however, necessitated a compromise between the percentage of acetonitrile in the initial eluant (and therefore the degree of resolution desired) and the percentage of chloroform in the sample solution. With 90% acetonitrile as eluant, 10-µl samples could be injected in 100% chloroform, but if 70% acetonitrile was used (as is necessary to resolve closely related retinoids) the high degree of immiscibility of the 100% chloroform sample solution and the eluant caused a loss of resolution of the early peaks. The ester peaks, however, were unaffected. Selection of the percentage of acetonitrile in the initial eluant and of the sample solvent composition must therefore depend on the particular array of retinoids present and the degree of resolution necessary to quantitate them accurately.

A linear relationship was found between the total dose administered to the hamster and the liver retinoid concentration in the dose range tested of 0.2 to 1 mg retinyl acetate per day for 1 or 2 days (Chart 3). As can be seen, liver storage of retinoids was increased by a multiple dosing schedule. For example, the liver storage of retinyl esters was increased approximately 60% by the administration of 0.5 mg retinyl acetate for each of 2 successive days, as compared to a single 1-mg dose. Whether this phenomenon

<table>
<thead>
<tr>
<th>Sample solvent composition</th>
<th>Relative solubility</th>
<th>Miscibility in parts/100 in acetonitrile:water mixtures at</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Retinol</td>
<td>Retinyl esters</td>
</tr>
<tr>
<td>100% methanol</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>100% CH₃CN</td>
<td>0.96</td>
<td>0.91</td>
</tr>
<tr>
<td>CHCl₃:methanol (1:1)</td>
<td>0.94</td>
<td>1.95</td>
</tr>
<tr>
<td>CHCl₃:methanol (2:1)</td>
<td>0.97</td>
<td>1.99</td>
</tr>
<tr>
<td>100% CHCl₃</td>
<td>0.98</td>
<td>2.05</td>
</tr>
</tbody>
</table>

* Results are the average of duplicate determinations on aliquots of an ether extract of a liver containing 60 nmol retinol and 815 nmol retinyl esters per g. An aliquot of the ether extract was taken to dryness under nitrogen and redissolved in 100 µl of the solvent indicated. Sample volumes for HPLC were 10 µl, and the elution was as described in Chart 2.

† This is an approximate determination done by adding 25-µl portions of the sample solvent to 500 µl of the acetonitrile:water mixture and noting the point at which turbidity or a distinct 2-phase system was produced.
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Chart 3. Total retinoid (nmol) per g liver versus cumulative dose of retinyl acetate. Vitamin A-deficient hamsters (3 in each group) were given daily p.o. doses of either 0.2, 0.5, or 1.0 mg retinyl acetate in 0.2 ml ethanol:trioctanoin (1:3) containing 0.025% butylated hydroxytoluene for either 1 (----) or 2 (---) days prior to sacrifice 24 hr after administration of the last dose. Tissues were extracted with ether by the method of Ames et al. (2). The total nmol retinoid is the sum of the values for retinol and all retinyl esters as determined by HPLC (•) or the value derived from the trifluoroacetic acid method (△) of Dugan et al. (6) determined on an aliquot of the same sample.

results from a decreased ability of the deficient animal to store the initial dose of retinyl acetate or from an increase in liver storage with time, as reported by Ames and Harris (1), is unknown. The supplementation schedule chosen for synthetic retinoids was a p.o. dose of 0.5 mg/day for 2 days followed by sacrifice 24 hr after administration of the final dose.

Since the colorimetric determination of vitamin A has formed the basis of many assays (1, 2, 17), it was desirable to compare that method with the present HPLC method. The amount of sample used for the trifluoroacetic acid determination was on the average 20 times the amount used for the HPLC. As seen in Chart 3, the values were quite consistent with those for the HPLC, averaging about 91% of the HPLC value. Although the trifluoroacetic acid method is faster, the HPLC method provides significantly more information about the nature of the retinoids involved.

The chromatographic profile of the ether extract of the liver and small intestine of a vitamin A-deficient hamster fed 2 p.o. doses of 0.5 mg retinal acetylhydrazone per day is shown in Chart 4. The parent compound (λmax 380 nm) is easily distinguished from retinol (λmax 325 nm) by the relative peak heights of the 365- and the 325-nm detector. In the retinyl ester portion of the chromatogram, only Ester 3 was positively identified by cochromatography as retinyl palmitate. The other peaks in the retinyl palmitate portion of the chromatogram were determined to be retinyl esters by several criteria. Chromatography of larger samples permitted the collection of samples in the regions identified as Ester 1, Ester 2 + 3, and Ester 4. Each of these samples had a UV absorption spectrum characteristic of retinyl esters (λmax 325 nm). Furthermore, hydrolysis of each of these fractions yielded retinol as the only 325-nm-absorbing ma-
terial on HPLC. Comparison of the elution order of the ester peaks with known elution patterns from reverse-phase paper chromatographic systems (8, 10, 12) permits tentative identification of Esters 1, 2, and 4 as retinyl linoleate, oleate, and stearate, respectively. Whereas the parent retinoid represents over 40% of the total in the intestine, it represents only about 2% of the total retinoid in the liver, possibly implying that hydrolysis occurs prior to absorption.

The retinoid concentrations, in nmol/g liver or small intestine, of hamsters given either retinyl acetate, retinal, or the synthetic retinoids, retinyl acetylated hydrazone or N-acetyl retinyl amine, on a dose schedule of 0.5 mg/day for 2 days are tabulated in Table 2. The 2 synthetic retinoids are converted to retinol and retinyl esters, although considerably less efficiently than is retinyl acetate or retinal. Neither retinal nor retinyl acetate was detected in the tissues of hamsters dosed with those retinoids. This presumably results from their rapid conversion to other retinoid species.

In the case of retinyl acetate, hydrolysis during the work-up procedure could account for this, in part. However, the partial hydrolysis in liver extracted by the lyophilization procedure (17 to 24%), coupled with the fact that no retinyl acetate was detected by either extraction procedure, makes it unlikely that this artifactual hydrolysis has been taken into consideration in the interpretation of the data. Only for the synthetic analogs, which seem to have a longer half-life in vivo, is the parent compound still detected 1 day after the last dose. Regardless of the retinoid dosed, the major retinoid species in the liver are retinyl esters with varying smaller amounts of retinol. The slightly different retinyl ester distribution in the intestine as compared to the liver is consistent with that reported previously (10).

**DISCUSSION**

HPLC is increasingly becoming the method of choice for various biochemical assays because of both the high resolution attainable and the short elution times as compared to conventional chromatographic systems. This work represents an application of the reverse-phase HPLC system of Frolik et al. (9) to the bioassay of tissue retinoids. The reverse-phase system was chosen because of the excellent recoveries attainable, the reproducibility, and the mild nature of the chromatographic process, which minimizes the production of artifacts. The method as described in this paper is capable of quantitating retinoids at less than 1 nmol/g tissue, which represents an approximately 20-fold increase in sensitivity over previous methods (1).

The use of vitamin A-deficient animals is important when working within the limits of the sensitivity of this assay, a feature that was also included in the liver storage assay procedure of Ames and Harris (11). The vitamin A-deficient hamsters have on the average less than 1 nmol retinyl esters per g liver and therefore make the detection of ester levels greater than 2 nmol/g significant compared to controls. This permits the conservative use of synthetic retinoids in the assay.

A major feature of this chromatographic system is the resolution of the long-chain fatty acid esters of retinol. Although absolute identification of the ester peaks other than retinyl palmitate would require cochromatography with standard compounds, comparison of the elution pattern with published R values from a reverse-phase paper chromatography system (16) allows for tentative identification as stated in the legend for Chart 4. Supporting this tentative identification is the close agreement of the relative proportions of the different ester components in the liver and intestine with that reported by Goodman et al. (10). Most notable is the decrease in the proportion of palmitate and the increase in the proportion of stearate in the intestine as compared to the liver. The "critical pair" (20) of the oleate (18:1) and palmitate (16:0) esters could not be separated completely even under conditions resulting in large separations of this peak from the other ester peaks.

A useful refinement of this assay would be the addition of an internal reference standard to the extracting solvent to eliminate sample variance due to evaporation and other losses. The choice of a standard should be limited to

**Table 2**

<table>
<thead>
<tr>
<th>Retinoid given</th>
<th>Tissue</th>
<th>Retinol</th>
<th>Parent retinoid</th>
<th>Σ esters</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retinyl acetate</td>
<td>liver</td>
<td>34.6</td>
<td>ND</td>
<td>441</td>
<td>5.5</td>
<td>12.6</td>
<td>70.5</td>
<td>11.5</td>
</tr>
<tr>
<td>Retinal</td>
<td>liver</td>
<td>61.7</td>
<td>ND</td>
<td>337</td>
<td>5.3</td>
<td>9.3</td>
<td>73.1</td>
<td>12.3</td>
</tr>
<tr>
<td>Retinyl acetate</td>
<td>intestine</td>
<td>11.5</td>
<td>ND</td>
<td>51.1</td>
<td>6.2</td>
<td>9.1</td>
<td>2.0</td>
<td>32.7</td>
</tr>
<tr>
<td>Retinal</td>
<td>intestine</td>
<td>11.6</td>
<td>1.4</td>
<td>60.7</td>
<td>3.4</td>
<td>7.1</td>
<td>77.9</td>
<td>11.7</td>
</tr>
<tr>
<td>N-Acetyl retinyl</td>
<td>liver</td>
<td>1.4</td>
<td>1.6</td>
<td>7.3</td>
<td>13.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.2</td>
<td>71.7</td>
<td>7.9</td>
</tr>
<tr>
<td>amine</td>
<td>intestine</td>
<td>ND</td>
<td>3.0</td>
<td>&lt;0.5&lt;sup&gt;d&lt;/sub&gt;</td>
<td></td>
<td></td>
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</tbody>
</table>

<sup>a</sup> Each value represents the average of separate determinations on tissues from 3 hamsters. The extraction was by the method of Ames (2) with ether as the extracting solvent. Values below the detection limits of the assay are indicated as ND. The livers and intestines from vehicle-dosed hamsters contained no detectable retinol and an average ester level of 1 nmol/g liver and <0.1 nmol/g intestinal mucosa.

<sup>b</sup> Ester 3 is retinyl palmitate, and Esters 1, 2, and 4 can be tentatively identified as retinyl linoleate, oleate, and stearate, respectively.

<sup>c</sup> Represents the sum of Ester 1 and a larger peak migrating between Ester 1 and Ester 2.

<sup>d</sup> Only retinyl palmitate was detected, and the level was too low to assess accurately.
compounds not found in biological samples and intermediate in polarity between retinol and the long-chain retinyl esters, so that its recovery would parallel the esters, which are the predominant retinoid species. Two compounds fulfilling these requirements are retinyl acetate and axerophthenol, the hydrocarbon analog of retinol. Retinyl acetate was used successfully in this capacity, and axerophthenol, which remains unaltered during work-up by either of the described procedures, offers the additional advantage of being able to be applied directly to tissue samples. Each of these compounds is easily separated from retinol and most synthetic end group retinoid derivatives (Chart 2).

The application of this method to the detection of tissue retinoids following dosing with synthetic retinoids such as N-acetyl retinyl amine and retinal acetylhydrazzone sheds some light on their possible mechanism of action. Both of these retinoids have been shown to have vitamin A-like activity in reversing the keratinization of the vitamin A-deficient hamster tracheal epithelium grown in organ culture (23). The relative activity of these 2 retinoids in tracheal organ culture is of the same order of magnitude as their relative liver storage in the form of retinyl esters following p.o. dosing. It must therefore be considered that these 2 synthetic retinoids might be converted to a physiological metabolite by cleavage of the end group derivative prior to exerting their biochemical action. The amount of liver storage resulting from dosing with these 2 synthetic retinoids relative to that from animals dosed with retinyl acetate was about 3 times greater in another experiment in which animals were given a daily dose of 1 mg retinoid for each of 5 days. This may indicate an inductive effect of the enzymes involved in the cleavage of these 2 derivatives.

In summary, it is imperative that synthetic retinoids being developed for long-term clinical application in the prevention of epithelial cancers (24) be screened for their ability to promote liver storage of retinyl esters, as excessive liver stores are a potential contributor to toxic effects. The sensitive assay described here would be particularly useful for this and for other general pharmacological studies, since it requires only a small amount of retinoid for testing and provides information on all species of retinoids present in the tissues.

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