Chapter 8

HPLC / MSN Analysis of Retinoids

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Abstract

This protocol describes a highly sensitive and selective method to quantify retinoids using normal-phase HPLC with online APCI MSN. The retinoids are key regulators of gene expression, retinol being oxidized via a retinaldehyde intermediate to retinoic acid (RA) which activates specific nuclear receptors, the signalling of which is turned off by oxidative inactivation of the ligand to 4-oxo-RA and other metabolites. Many of these retinoids are present only transiently at low concentrations in tissues and during analysis are labile to heat, light, and oxygen. HPLC with online APCI MSN provides a rapid technique to quantify these retinoids simultaneously. Techniques to extract the retinoids and prevent their degradation are described, with an emphasis on transcriptionally active RA. RA controls patterning of gene expression in the embryo, organizing embryonic morphology in the central nervous system. Similarly, a patterned distribution of RA controls function of the adult CNS, a tissue particularly difficult to analyse for RA because of its high lipid content. To understand how these patterns are organized in the brain and change over time, it is essential to determine the concentration of RA in small areas of tissues, and techniques of exquisite sensitivity are indispensable.

Key words: Retinoic acid, retinol, retinaldehyde, 4-oxo retinoic acid, high-performance liquid chromatography, mass spectrometry, brain.

1. Introduction

1.1. Why Assay for Retinoic Acid?

Vitamin A is defined as the group of compounds derived from C20-beta-ionone that demonstrate the biological activity of all-trans retinol (1). It is a member of the ‘retinoids’ family, themselves defined as a group of compounds comprising of four isoprenoid units joined head to tail (2). The major active members of this group are retinol itself and its oxidative metabolites retinaldehyde and retinoic acid (RA) (Fig. 8.1).
The biological activity of vitamin A includes support of visual transduction via the requirement of 11-cis retinaldehyde as the light-responsive component of rhodopsin. Retinol itself can function as a cofactor for a number of kinases and derivatives such as retroretinol and anhydroretinol may act on similar intracellular signalling pathways (3). The best understood retinoid signalling pathway required for a great number of physiological events is RA’s regulation of transcription via its binding to specific nuclear receptors. This is required for events such as organ development in the embryo and, in the adult, control of epithelial cell differentiation and proliferation, lung, bone, skin, and brain function (4). As a consequence of these functions the storage and/or transport forms of the retinoids, such as retinol and retinyl esters, are naturally present at high concentration (μM). Their more polar metabolic products, which are produced locally to function as transient signalling molecules, as well as their inactive metabolites, are inevitably present at much lower concentrations (nM) and are thus more difficult to detect.

The brain is a region that has been a topic of recent interest with regard to RA function (5–7) and, for instance, brain-related defects are among the first symptoms of vitamin A deficiency in chick, specifically ataxia, a loss of balance and coordination with unsteady gait, which can be reversed (although not always completely), with RA treatment (8). The brain, however, is a prime example of one of the great problems in retinoid research; it is very difficult to quantify and localize the transcriptionally active metabolite of the retinoids, RA. Unlike proteins, which can be pinpointed using antibodies, or mRNA, localized with antisense, no such probes exist for RA. Particularly in the brain, where RA acts within very discrete regions in a concentration-dependent manner, knowledge of RA’s distribution within the many sub-compartments of the brain is essential to understand its function. At present, the most effective way to determine RA distribution is
through the localization of the protein required for RA synthesis, specifically the enzyme retinaldehyde dehydrogenase (RALDH) which catalyses the last step of RA synthesis and whose pattern of expression corresponds very well with the distribution of RA (9–11) (Fig. 8.1). However, to quantitatively determine how retinoids, or RA specifically, change over time, condition, or vary between tissue or species, their direct measurement is necessary.

1.2. Methods of Retinoid Detection

1.2.1. Problems with the Fragility of RA

The five or more double bonds present in retinoids make them easily oxidized in air, isomerized by light, and altered by heat; this is particularly the case when concentrations are low. Thus, it is essential that these conditions are controlled through use of antioxidants such as butylated hydroxytoluene in solvents and maintaining samples under an inert atmosphere using argon or nitrogen gas, avoiding high temperatures, and preparing samples under long-wavelength gold or yellow light and/or low-light conditions. The retinoids are also labile to strong acids particularly in anhydrous conditions, while during preparation there may be adsorption of retinoids to glass or plastic containers or steel components of the HPLC setup (4, 12).

1.2.2. Solvent Extraction of RA from Tissue

The retinoids in general are insoluble in water and soluble in organic solvents. Certain retinoids have a solvent preference; for instance relatively polar solvents like methanol and ethanol are excellent for retinol and RA but very poor for retinyl esters, which prefer non-polar solvents like hexane while solvents such as diethyl ester, chloroform, dichloromethane, dimethyl sulfoxide, and ethyl acetate are excellent for most retinoids (13). Therefore the choice of solvents is dependent on the retinoid to be extracted. A further useful property of the solvent is its relative volatility if the sample is to be concentrated for later analysis. Obviously, when extracting any retinoid from tissues, a significant amount of water will also be released and solvents that are water-immiscible will not be efficient in extraction. One key step of extraction is the release of RA from the binding proteins, cellular retinoic acid-binding proteins I and II (CRABPI and II), which carry it in the cell; this can be brought about by precipitation in ethanol or methanol.

1.2.3. Chromatography

The earliest methods to detect RA included paper and thin layer (14) chromatography. Lipids extracted with diethyl ether would be separated on paper strips and zones of lipid migration cut out under ultraviolet (UV) light and determined for colour reaction with antimony chloride (8). Alumina and silicic acid were among the first type of material to be used for column chromatography (15, 16) as well as ion exchange chromatography (17) but such
methods would not provide complete resolution of retinoids (18, 19) and were also prone to generation of artefacts (20, 21). The use of Sephadex™ gel filtration chromatography improved resolution and also gave good recovery of retinoids (22). The next step in development was high-performance liquid chromatography (HPLC), initially using normal phase with silica columns (23), but this had some difficulties because of the presence of water from the sample, and reverse-phase chromatography with, for instance, Spherisorb ODS provided good resolution and could be completed over a relatively short period of time (24). Use of gradient elution with reverse-phase HPLC could separate the full spectrum of retinoid isomers (25), important, for instance, because the differing isomers of RA have very different affinities for the RA receptors. Modern high-purity silica-based chromatographic materials are low in metal contaminants (binding sites for retinoids at low levels and promoting their isomerization and oxidation) and are also more robust; thus effective normal-phase chromatography is now achievable (26). Overall, reverse-phase HPLC is often preferred for biological samples because separations are generally less sensitive to slight changes in mobile-phase composition. They also equilibrate more rapidly for gradient separation and thus can be useful for simultaneous runs of different retinoid classes. However, normal-phase (adsorption) chromatography is often better at separating closely related compounds such as cis/trans retinoid isomers.

Techniques to obtain the required specificity for low-level retinoid assay through cleanup before analysis by HPLC with UV or fluorescence detection were essential. This was particularly the case for tissues with a high lipid content such as liver, kidney, and brain and the difficulty in separating these lipids from retinoids with similar chromatographic properties. However, the problem with extensive preparation before analysis is the greater possibility for losing sensitive, low-level retinoids during the process. Use of column-switching (27) and online solid-phase extraction (28) has enabled efficient enrichment of samples. Solid-phase extraction based on aminopropyl columns has been found to be helpful with analysis of both polar and apolar retinoids from samples with a high lipid content (29). The importance of such cleanup techniques though has been reduced with the development of detection methods of ever-increasing specificity.

Common HPLC detection methods have included fluorescence detection (for retinol and its esters) and UV absorption. The characteristic UV absorption spectra of the different retinoids have led to the use of diode-array detection allowing identification of the resolved retinoids. Mass spectrometry (MS), however, has led to a large improvement in the capacity to specifically detect and identify low levels of retinoids. One of the first uses of MS was
in conjunction with gas chromatography; however, this was not ideal because of the necessity for derivatization to form volatile, thermally stable derivatives and the sensitivity of retinoids to disruption by the high temperatures necessary for their GC elution (30). These factors lead to extensive cis/trans isomerization of retinoids and losses due to oxidation. Use of negative ion electrospray ionization (ESI) mass spectrometry (31) allows direct analysis of carboxyl bearing retinoids (RA) with little sample handling but does not allow sensitive detection of neutral retinoids. Atmospheric pressure chemical ionization (APCI)-mass spectrometry provides efficient ionization of both acidic and nonionic retinoids such as retinol and retinyl palmitate directly from a reverse-phase column, avoiding the problem associated with gas chromatography (32). Recent studies have relied upon positive ion APCI MS to quantify simultaneously retinol and RA with better sensitivity than ESI, as well as negative ion APCI, and also to provide simultaneous determination of retinoids (33). These findings were confirmed by Kane et al. (34) who also demonstrated the increased detection specificity that can be obtained with crude extracts by the use of tandem MS (MS/MS).

1.2.5. Example of Detection of Retinoids in Tissue, the Brain

Detection of retinoids in the brain has proven particularly difficult; the myelin that surrounds all nerves in the CNS results in a large amount of lipid in brain extracts, from which the retinoids need to be distinguished. To provide a useful measurement of RA in the brain, the sensitivity has to be sufficient to determine concentration within brain subregions. Determination of RA levels in whole rat brain obtained values of 6.3 pmol/g tissue, the lowest value in all regions tested compared to, for example, testes, liver, fat, and pancreas, the latter particularly high at 29.3 pmol/g tissue (35). It has been proposed that most of the RA in the brain (as well as the liver) was simply derived from the low concentrations (1.8 pmol/g) present in the circulation, in experiments infusing radiolabelled RA at steady state (35). However, quite different results for RA levels in the CNS were found in a study on retinoids in the vitamin A-deficient rat treated with physiological levels of radiolabelled retinol. Of all organs examined the region of the cortex and hippocampus had the highest ratio of RA to retinol, and with regard to total RA only liver and kidney contained higher amounts of RA per gram tissue than brain. Despite the earlier finding that the majority of RA in the brain derived from plasma it was found that this transport was no greater in the brain than any other tissue. One potential explanation for the exceptionally high levels of RA found in the brain in this study is the use of vitamin A-deficient animal models which may result in a compensatory increase in the enzymes that generate RA. If the brain has a particularly high requirement for RA this may result in a greater elevation of these synthetic enzymes in the brain and,
when radiolabelled retinol is added, this results in greatly elevated levels of RA.

1.2.6. Current Method

This chapter describes our use of normal-phase silica gel-based HPLC with online APCI MSN for the analysis of crude tissue and media extracts allowing the sensitive analysis of a broad range of biologically important retinoids in a single assay (26). The use of a low binding site high-purity silica gel (low trace metal) based HPLC column packing allows trace levels of sensitive retinoids to be assayed without significant losses. Positive ion APCI provides for efficient ionization of all the retinoids as opposed to ESI that only provides sensitive ionization for retinoids that contain free carboxyl groups. Quadrupole ion trap mass spectrometry provides unique capabilities for multistage tandem MS (MSN) which allows a number of sequential stages of precursor–product ion MS/MS to be performed. This is key to obtaining high specificity while maintaining high sensitivity. The high specificity of MSN (MS3 and MS4) used here allows the analysis of low-level retinoids in crude lipid extracts without interference from other sample components. This ability to directly analyse crude extracts without multiple steps of prefractionation prevents sample handling losses that would otherwise be unavoidable. Methods described below are designed to provide high recovery of native retinoids with sensitive, specific, and accurate quantification.

2. Materials

All solvents used were HPLC grade or higher.

2.1. Extraction of Retinoids from Tissues

1. Extraction solvent: ethanol–isopropanol (2:1, v/v) containing 1 mg/ml butylated hydroxytoluene antioxidant (>99%, Sigma Chemical Company).

2. Dounce glass homogenizer (7 ml, Wheaton, Millville, NJ).

2.2. Extraction of Retinoids from Media and Biofluids

Hexane–dioxane–isopropanol (50:5:1, v/v/v) containing 1 mg/ml butylated hydroxytoluene antioxidant (>99%, Sigma Chemical Company).

2.3. HPLC Materials

1. Inertsil silica normal-phase HPLC column (150 × 2 mm, 5 μm particle, 5 μm particle; Keystone Scientific, Inc., Bellefonte, PA). This column was chosen for its high-purity silica gel packing and 2 mm ID that has an optimum flow rate of ~200 μl/min for high APCI sensitivity on the Thermo LCQ mass spectrometry system used. The HPLC
pumping system used was a Leap Technologies Rheos Flux 2000 ternary gradient system with a Rheodyne 7125 injector equipped with a 10 μl PEEK sample loop. All connection tubing was 0.005 in. ID PEEK.

2. Gradient elution mobile phases for separation of a broad spectrum of retinoids. Solvent A: n-hexane. Solvent B: n-hexane–dioxane–isopropanol (40:8:2, v/v/v). HPLC mobile phases were continuously sparged with helium (99.9%) to remove oxygen that could oxidize retinoids before and during analyses.


4. Retinaldehyde, all-trans, 9-cis, and 13-cis RA and retinol (Sigma Chemical Company) and didehydro-RA and 4-oxo-RA (gift from Dr. J Grippo, Hoffmann-La Roche, Inc., Nutley, NJ, USA) stock standards dissolved in DMSO at 0.1 M (see Note 1).

3. Methods

3.1. Tissue Extraction

All extraction procedures were performed under low-intensity yellow light.

1. All tissues were collected as fresh as possible and stored at −80°C until extraction (see Notes 2 and 3).

2. The fresh or freshly thawed tissue was deposited into the cold homogenizer containing 1 ml of extraction solvent per 100 mg wet weight tissue and the tissue homogenized.

3. The homogenate was centrifuged at 3000 × g for 10 min at 3°C and the supernatants collected and stored in the dark at −80°C until analysis.

3.2. Media and Biofluid Extraction

The cold fluid (0°C, 200 μl) was vortexed with 200 μl of cold extraction solvent in a Teflon™ sealed tube under nitrogen for 1 min and centrifuged at 3000 × g for 10 min at 3°C. The supernatants were collected, briefly stored in the dark at −20°C, and analysed as soon as possible (see Note 4).

3.3. HPLC/MSN Analysis of Retinoids

The methods presented here utilized a Thermo LCQ 3D ion trap mass spectrometer with the ability to perform MS3 or 4 experiments. These MSN capabilities are used to provide high-specificity analyses while maintaining the necessary sensitivity for low-level retinoid quantification. While many of the ion trap instruments that are currently available have these capabilities,
current-generation linear ion traps can be expected to provide higher sensitivities (compared to 3D ion traps) for MSN analyses due to their better ion trapping efficiencies, higher ion capacities, and more sensitive detection. Two HPLC/MSN methods are presented here. One utilizes gradient solvent elution to provide analysis of the full spectrum of retinoids in less than 20 min. The other is an isocratic method that provides improved resolution of all-trans, 9-cis, and 13-cis RA isomers for their individual quantification.

3.3.1. Gradient Elution
Normal-Phase HPLC

Under low-level yellow light, extracts were warmed to 0°C immediately before analysis and 3 μl of the extract (see Note 5) was drawn into a cold 10 μl gas-tight Hamilton glass syringe and rapidly loaded and injected onto the column at initial solvent conditions (90% solvent A–10% solvent B flowing at 200 μl/min). Immediately after injection a 18.9 min linear gradient to 58% solvent A–42% solvent B was initiated, also at 200 μl/min.

3.3.2. APCI MSN
Analysis of Major Retinoids

The column was directly connected to the APCI source of the mass spectrometer and the source operated with the vaporizer at 375°C, the nitrogen sheath gas flow at 35% (relative), the source current at 5 μA, and the heated capillary at 150°C. A constant infusion of 1.0 μg/ml RA in n-hexane at 200 μl/min was used to optimize the APCI source and auto-tune the mass spectrometer using the m/z 301 ion (MH+) (see Note 6). Each analyte separated by HPLC was detected by a unique series of MSN scan functions that were optimized by trial of multiple precursor/product ion combinations and collision energies to provide maximum selectivity and sensitivity as listed in Table 8.1 and illustrated in Fig. 8.2 (see Note 7). The isolation width used was adjusted to provide the precursor ion free of any adjacent ions while maintaining maximum ion current. Examples of the spectra for predominant retinoids, retinaldehyde, RA, and retinol, are shown in Fig. 8.3.

3.3.3. Isocratic HPLC
APCI MSN Analysis of Retinoic Acid Isomers

This mode of operation is preferred when RA isomers are the only analytes of interest. All operating conditions were the same as described above except for the mobile phase and the scan functions that were used. The isocratic mobile phase results in elution of all three isomers in less than 14 min. The scan function used was the one listed in Table 8.1 for RA isomers (m/z 301, 205, and 159). Improved chromatographic resolution resulted from the isocratic separation and the dedicated scan mode used under these conditions lead to improved quantification for the RA isomers and provided shorter overall analysis times because of the faster elution of RA’s and lack of a requirement for column re-equilibration following each injection.
### Table 8.1
Scan functions and approximate HPLC retention times for analytes

<table>
<thead>
<tr>
<th>Time segment (min)</th>
<th>Retinoid</th>
<th>Retention time (min)</th>
<th>MS2 Precursor m/z (MH⁺)</th>
<th>Collision energy</th>
<th>MS3 Precursor m/z</th>
<th>Collision energy</th>
<th>MS4 Precursor m/z</th>
<th>Collision energy</th>
<th>Quantification ion (m/z)</th>
<th>Overall efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–6</td>
<td>Retinaldehyde</td>
<td>5.7</td>
<td>285.1</td>
<td>30</td>
<td>193.1</td>
<td>30</td>
<td>175.1</td>
<td>12.0</td>
<td>175.1</td>
<td>12.0</td>
</tr>
<tr>
<td>5–12</td>
<td>RA isomers</td>
<td>7.5–8.6</td>
<td>301.1</td>
<td>30</td>
<td>205.1</td>
<td>30</td>
<td>159.1</td>
<td>1.3</td>
<td>159.1</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>Didehydro-RA</td>
<td>8.8</td>
<td>299.1</td>
<td>30</td>
<td>243.1</td>
<td>30</td>
<td>225.1</td>
<td>35.0</td>
<td>225.1</td>
<td>35.0</td>
</tr>
<tr>
<td>12–20</td>
<td>Retinol</td>
<td>16.2</td>
<td>269.1</td>
<td>32</td>
<td>213.1</td>
<td>32</td>
<td>156.0</td>
<td>3.80</td>
<td>156.0</td>
<td>3.80</td>
</tr>
<tr>
<td></td>
<td>4-oxo-RA</td>
<td>16.2</td>
<td>315.1</td>
<td>30</td>
<td>297.1</td>
<td>30</td>
<td>279.1</td>
<td>30</td>
<td>209.1</td>
<td>0.90</td>
</tr>
</tbody>
</table>
3.3.4. Results

The mass spectrum obtained of RA, retinaldehyde, and retinol analysed at each stage of MS$^N$ analysis illustrates the selection of product ions available at each MS$^N$ stage for selection as precursors for subsequent utilization as shown in Fig. 8.3.
Fig. 8.3. Representative APCI MSN spectra of retinoids. The precursor and quantitation product ions chosen for analysis of retinaldehyde, retinoic acid, and retinol are shown.
4. Notes

1. RA standards are relatively stable at high concentrations (e.g. 0.1 M in DMSO). Low-concentration standards (μM and below) should be diluted immediately before use. DMSO is preferable to ethanol as a solvent as it is less volatile and thus less is evaporated when flushed with argon or nitrogen.

2. The high sensitivity of retinoids to oxidation, light, and heat is still the case even when present in tissue, and best results are always obtained when the time between removal of tissue and analysis is kept as short as possible, i.e. the same day. If this is not possible, the tissue should be snap frozen in liquid nitrogen and kept no longer than a week, preferably flushed with argon or nitrogen, and the container in which it is kept sealed.

3. A tissue with high retinoid content to function as a positive control is the eye, which is simple to dissect and retinoids are easy to extract with its lower lipid content compared to other CNS regions.

4. Retinoids have a high affinity for the silanol groups of glass and glassware is best considered disposable. Glassware can be silanized with a 5% (w/v) solution of dichlorodimethylsilane in toluene followed by washing with a 1:1 (v/v) mixture of methanol and acetone.

5. Injection volumes of the ethanol–isopropanol extracts must be less than 3 μl to avoid shortening of retention times and degradation of chromatographic resolution by the relatively polar solvent. Because extraction efficiency of RA is decreased when lower polarity solvents are used and that serious losses are encountered when concentration is attempted we have not been able to increase the tissue equivalent amount of extract injected.

6. It is important to tune the APCI ion source and ion optics using a solution of a standard analyte (RA) infused at chromatographic flow rate in a solvent similar to the chromatographic solvent to obtain the highest possible sensitivity. This will assure optimal ionization efficiency and ion transmission with minimal ion losses in the high-temperature and high-pressure regions of the mass spectrometer.

7. In most cases MS3 product ion scans were used to provide high-specificity detection of retinoids; however, for 4-oxo-RA the first two successive high-efficiency collisional
activation decomposition products were through loss of water, which do not impart high detection specificity. Hence an additional MS4 step from the m/z 279 MS3 product ion was used to give a good yield of a m/z 209 product.

References


