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Determination of retinoids by reversed-phase capillary liquid chromatography with amperometric electrochemical detection

Jeffrey J. Hagen, Keith A. Washco[1], Curtis A. Monnig*

Department of Chemistry, University of California, Riverside, CA 92521-0403, USA

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Abstract

A method for separating and detecting retinoids by reversed-phase capillary liquid chromatography with amperometric electrochemical detection is described. Packed columns with an inner diameter of 180 μm were employed for the separation using a C_{18} stationary phase and a mobile phase containing acetonitrile–water–methanol (65:32.5:2.5, v/v/v) with 1% tetrabutylammonium perchlorate and 0.174 *M* acetate buffered at pH 5. The detection cell consisted of a carbon fiber barrel electrode held at 0.9 V versus an Ag/AgCl reference. Injection volumes of 2 μl produced detection limits of 2.73, 0.472, 0.428, and 0.267 fmol (or 410, 64.1, 60.9, and 38.2 pg ml^{-1}) for 13-*cis*-retinoic acid, all-*trans*-retinoic acid, retinaldehyde, and retinol, respectively. This represents an improvement in detection limits of at least three orders of magnitude for similar analyses using liquid chromatography and UV absorbance detection. The detector signal was linear over two orders of magnitude of analyte concentration. Retinoid concentrations in bovine serum were determined and found to be in good agreement with previously reported values.

Keywords: Retinoids; Retinoic acid; Retinaldehyde; Retinol

1. Introduction

The retinoids are a group of lipophilic polyenes, some members of which possess very strong biological activity. Retinoids play vital roles in signal transduction in the eye [1,2], maintenance of epithelial tissue [3], and regulation of the immune system response [4]. Similarly, retinoids are also being explored as therapeutic agents for diseases that involve pathogenic cell development [5]. One particularly important function of retinoids is their suspected role as regulators of cell differentiation in early embryonic development. The retinoids are known to have teratogenic activity [6], and retinoic acid in particular has been shown to cause developmental anomalies in prenatal systems [7]. Exposure of the anterior portion of a developing chick wing bud to retinoic acid, 3,4-didehydroretinoic acid, or 9-*cis*-retinoic acid results in mirror image digit formation [8–10], suggesting that these molecules are an important means of coordinating cellular development in this structure. Retinoids have also been linked to the establishment of the development axis for the central nervous system [10–12].

Although much indirect evidence suggests the

*Corresponding author.

[1]Present address: Matrix Pharmaceutical, Inc., 1430 O'Brien Drive, Menlo Park, CA 94025, USA.

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importance of retinoids in developmental processes, very few studies have directly measured and correlated retinoid concentrations with biological events. One difficulty with making these measurements is that the retinoids can assume many different forms that are readily inter-converted in vivo [13]. Further, the spatial regions over which the chemical gradients are believed to form are very small, often occupying sub-microliter volumes. In these regions, cellular development can be influenced by retinoic acid concentrations as low as 10 n*M* [14,15]. Under such conditions, the total quantity of analyte available for detection can be very small, often femtomoles or less. Further complicating these analyses, the samples may contain many components that can interfere with the measurement. For an analysis method to address these challenges, it must possess the selectivity to separate the major forms of retinoids and any potential interferences, while demonstrating the sensitivity necessary to monitor the retinoids at biologically relevant concentrations.

Current methods to determine retinoids usually employ normal- or reversed-phase liquid chromatography with UV–Vis absorbance detection [16–18]. Liquid chromatography can provide the selectivity required to isolate most forms of the retinoids, but UV absorbance detection provides only low picomole detection limits. When these instruments are used to monitor retinoids in embryonic tissue, heroic sample collection efforts are often required to obtain sufficient sample for analysis [19]. An attempt to overcome the problems of UV detection sensitivity by substituting electrochemical detection in place of spectroscopic detection produced some improvement in detection limits (82 pg, 269 fmol) [20], but not enough to overcome the sensitivity problems described earlier [21].

Fluorescence detection has also been investigated as a means of enhancing detection sensitivity for the retinoids. Laser-induced fluorescence detection of retinol in human serum provided excellent detection limits (10 fg, 32 amol) when the analyte was complexed with retinol-binding protein [22]. Unfortunately, the fluorescence signal was very dependent on pH, and other retinoids did not demonstrate the same level of fluorescence. As a result, detection sensitivity for these other species suffered. An attempt to enhance fluorescence detection by labeling retinoids with fluorescent molecules prior to their separation produced no tangible enhancement of the signal [23].

One means of retaining the selectivity of liquid chromatography while enhancing detection sensitivity is to analyze the samples by capillary HPLC with a micro-scale electrochemical detector. The excellent performance of these instruments is the result of the enhanced separation efficiency provided by the capillary column format, and the selectivity and efficient detection provided by the electrochemical detector. Under optimized conditions, electrochemical detection provides attomole detection limits for catecholamine analysis [25]. This report describes the equipment and techniques required to utilize similar instrumentation to separate retinoids, and to detect femtomole and sub-femtomole quantities of these molecules.

2. Experimental

2.1. Chemicals

Ascorbic acid, tetrabutylammonium perchlorate, retinol, all-*trans*-retinoic acid, 13-*cis*-retinoic acid, retinaldehyde, and bovine serum were purchased from Sigma (St. Louis, MO, USA) and used without further purification. HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). All other chemicals were purchased from Fisher Scientific at the highest purity available.

All retinoid solutions were stored at 0°C in darkened vessels and handled in reduced light conditions to minimize photo- and heat-induced alterations of the analyte. To prevent oxidation, ascorbic acid (0.02 *M*) was added to all solutions. Just prior to use, samples were prepared from stock solutions containing between 0.3 and 0.6 m*M* of each retinoid in a solvent of acetonitrile and water (9:1, v/v). These stock solutions were diluted so that the samples contained retinoids at the desired concentration in a solvent consisting of acetonitrile–water (4:6, v/v).

2.2. Apparatus

The instrument used to analyze the retinoid solutions is depicted in Fig. 1. Capillary HPLC columns

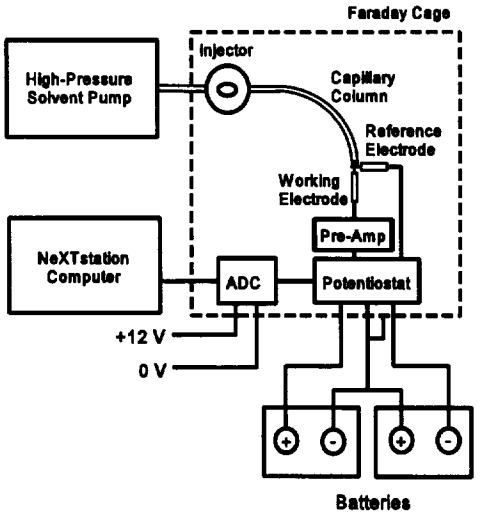

Fig. 1. Block diagram of the capillary HPLC instrument and electrochemical detector.

were manufactured from fused-silica capillaries obtained from Polymicro Technologies (Phoenix, AZ, USA). The separation capillary was formed by first inserting a small capillary (50 μm I.D., 150 μm O.D.) approximately 1.5 cm into a larger capillary (180 μm I.D., 360 μm O.D.) and the relative position of these two capillaries fixed by applying epoxy (No. 353ND, Epoxy Technology, Billerica, MA, USA) to the joint. A frit formed from glass filter paper (GF/A, Whatman International, Maidstone, England) was inserted into the larger capillary and forced against the smaller capillary with a stream of isopropanol. The stationary phase was packed against this frit by first suspending 0.1 g of the C_{18} support (3 μm particle diameter, ODS-AQ, YMC, Wilmington, NC, USA) in 3 ml of isopropyl alcohol, and then pumping this suspension into the larger capillary until a bed approximately 5 cm in length was formed. The final step in the preparation of the HPLC column was to trim the capillaries so that the larger and smaller diameter capillaries extended no more than 10 and 1.6 cm from the frit, respectively.

The mobile phase is supplied to the separation capillary at a flow of 4 μl min^{-1} (pressure of approximately 85 kg cm^{-2}) with a commercial HPLC pump (Model L-6200A, Hitachi Instruments, San Jose, CA, USA). Samples were introduced into the flow stream with a loop injector (Model 7125, Rheodyne, Cotati, CA, USA) equipped with a 2-μl loop. This injector was placed inside the Faraday cage to minimize the distance between the sample loop and the head of the separation capillary. All separations were performed under isocratic conditions with a mobile phase composed of acetonitrile–water–methanol (65:32.5:2.5, v/v/v) which contained 1% tetrabutylammonium perchlorate. The solution was buffered at pH 5 with acetic acid and sodium acetate (0.174 *M*).

2.3. Amperometric detection

Microelectrodes were manufactured by securing a 33-μm diameter carbon fiber (No. 800036-001, Textron Specialty Materials, Lowell, MA, USA) in a 0.68 mm I.D., 1.2 mm O.D. glass capillary (No. 6020, A-M Systems, Everett, WA, USA) with epoxy (No. 353ND, Epoxy Technology). The glass capillary is filled with mercury, then a 3-cm piece of nichrome wire is inserted into the open end of each tube so it is in electrical contact with the mercury (and carbon fiber). This nichrome wire was secured in position and the capillary sealed with a single drop of Duco cement (Devcon Corp, Wood Dale, IL, USA). Prior to using the electrode, a razor blade was used to trim the carbon fiber so that it extended no more than 5 mm from the glass capillary. The carbon fiber was then rinsed with toluene, suspended in the mobile phase and subjected to a 50-Hz waveform (−0.2 to 2.0 V, peak-to-peak) for 10 s to provide an electrochemically active electrode surface.

Reference electrodes were manufactured by placing a silver wire (Cat. No. 7830, A-M Systems) in 10% hydrochloric acid and applying a 10-V d.c. potential for 10 min. Prior to use, the silver wire was trimmed so that Ag/AgCl coating was no more than 1.5 cm in length. Under typical analysis conditions, the reference and working electrodes provided stable signals for extended periods, often as long as several weeks.

The electrochemical detection cell was formed by inserting the carbon-fiber working electrode (33 μm O.D.) approximately 3 mm into the outlet of the capillary HPLC column (50 μm I.D.). This configuration minimized the distance necessary for analyte to diffuse to the electrode surface, increasing the electrochemical current. The Ag/AgCl reference

electrode was positioned directly under the column outlet so that the column effluent would flow directly over its surface. A 4 mm wide, 3 cm long strip of filter paper (Cat. No. 1442 110, Whatman International) was saturated with mobile phase and inserted between the electrodes to maintain electrical contact and provide a constant flow of solvent over the two electrodes. For all of the analyses, the working electrode was held at 0.90 V relative to the Ag/AgCl reference electrode.

The two-electrode potentiostat used in these experiments was constructed in-house using a three-stage amplifier design. The combined gain of these amplifiers was $1.03 \cdot 10^9$ VA^{-1}, and the circuit had an effective time constant of 1.0 s. To minimize 60 and 120 Hz noise in the signal, the potentiostat was powered by two 12-V lead-acid batteries and shielded in a Faraday cage referenced to the battery common.

The signal from the potentiostat was digitized with a 16-bit analog-to-digital converter (XL-ADC2, Elexor Associates, Morris Plains, NJ, USA) at 500 Hz, and the average of 128 readings recorded as a single point in the chromatogram. Thus, the effective data acquisition rate was 3.45 Hz. This data was transferred to a NeXTstation workstation (NeXT, Redwood City, CA, USA) where an Objective C program displayed and stored the data. To further enhance the signal-to-noise characteristics of the data, the chromatograms were subjected to a 0.333-Hz low-pass digital filter. Quantitative estimates of the analyte are based on peak-height measurements, and separation efficiency was determined by measuring the statistical moments of the peaks using previously published procedures [24].

2.4. Sample preparation

The bovine serum samples were prepared by mixing 200 μl of acetonitrile and 10 μl of 0.02 *M* ascorbic acid with 100 μl of serum, and the resulting solution was centrifuged at 16 000 *g* for 5 min to sediment protein aggregates. The supernatant was mixed with 200 μl of deionized water for a final acetonitrile–water concentration of 4:6 (v/v). A 2-μl aliquot of this solution is introduced into the chromatography column.

3. Results and discussion

Separation and detection of retinoids can exploit many advantages provided by capillary HPLC with electrochemical detection. Retinoids are readily oxidized with carbon electrodes at potentials greater than 0.8 V versus Ag/AgCl. Although each retinoid has slight differences in their oxidative formal potential, all species provide significant Faradaic current at potentials greater than 0.85 V. The primary electrode reaction is believed to be the oxidation of the conjugated double bonds common to all retinoids [20].

Initial attempts to separate and detect retinoids by capillary HPLC provided neither the chromatographic resolution nor the detection limits required to monitor these molecules in embryonic tissues. To minimize shifts in the signal baseline and enhance separation resolution, isocratic separation conditions were employed. Separation under the initial set of isocratic conditions (acetonitrile–water (65:35, v/v) with 1% tetrabutyl ammonium perchlorate and 0.1% acetic acid) resulted in an overlap of the all-*trans*-retinoic acid and retinol peaks. Increasing the pH of the mobile phase to 5.0 decreased the retention of retinoic acid and eliminated the peak overlap. Substitution of methanol for some acetonitrile in the mobile phase improved the separation of the retinoic acid isomers, similar to the improvements previously reported for retinol isomers [20]. Complete replacement of acetonitrile with methanol rapidly decreased the observed electrochemical signal in addition to causing a slight increase in background current, so the minimum amount of methanol necessary to provide the desired resolution was used for these analyses.

To shield the detection system from environmental noise sources, the detection cell and electronics were positioned inside a Faraday cage, and this cage referenced to the common of the batteries used to power the potentiostat. The analog-to-digital converter was also positioned inside the Faraday cage and powered with an external d.c. power supply. Although the analog filters in the potentiostat significantly enhanced the quality of the analytical signal, digital filtering further improved the signal. A low-pass Fourier transform digital filter with a time constant of 3 s was applied to all of the data to

reduce the contribution of high-frequency noise. Under these conditions, the electrochemical cell generated noise with a standard deviation of 0.39 pA.

Despite extensive efforts to minimize fluctuations in the signal baseline, significant deviations were still observed. These fluctuations were the result of the buildup and loss of mobile phase at the column outlet. The surface area of the electrode in contact with the mobile phase changes as these droplets grow and fall, which produces a fluctuating current in the electrode. Fortunately, the buildup of solvent on the electrode was easily prevented by inserting a small strip of filter paper between the working electrode and the reference electrode so that excess solvent was drawn away by capillary action. Addition of the filter paper reduced the standard deviation of the background signal from 4.17 to 1.34 pA.

The chromatogram for the separation of 13-*cis*-retinoic acid (49.6 fmol), all-*trans*-retinoic acid (87.6 fmol), retinol (70.0 fmol), and retinaldehyde (137 fmol) is shown in Fig. 2a. It is important to note the excellent signal-to-noise characteristics of this chromatogram. Although the quantity of each component in the sample is below the detection limit for previously reported methods for HPLC determination of retinoids, the signal is much greater than the detection limit for this instrument. In Fig. 2b, the chromatogram for a solution containing the retinoids at a lower concentration is displayed. This sample contained 2.48 fmol of 13-*cis*-retinoic acid, 4.40 fmol of all-*trans*-retinoic acid, 3.51 fmol of retinol, and 6.84 fmol of retinaldehyde. For these measurements, separation efficiency was approximately 3500 theoretical plates with capacity factors between 8.90 (13-*cis*-retinoic acid) and 16.17 (retinaldehyde).

The detection limits (signal-to-noise ratio of 3) for the retinoids were determined to be 2.7, 0.47, 0.43, and 0.27 fmol (or 410, 64, 61, and 38 pg ml^{-1}) for 13-*cis*-retinoic acid, all-*trans*-retinoic acid, retinaldehyde, and retinol, respectively. These values correspond to improvements of approximately three orders of magnitude over previously published chromatographic determinations.

An examination of the peak height as the analyte concentration was varied demonstrated that the analytical signal increased linearly with the quantity of the analyte to levels approximately two orders of magnitude greater than the detection limit (i.e., 49.6 fmol of 13-*cis*-retinoic acid, 87.6 fmol of all-*trans*-retinoic acid, 70.0 fmol of retinol, and 137 fmol of retinaldehyde). There was no evidence of rollover in these calibration plots so the linear response of the detector could extend to even higher levels. However, the instrument response at these higher levels was not measured as these quantities can be monitored by previously described procedures.

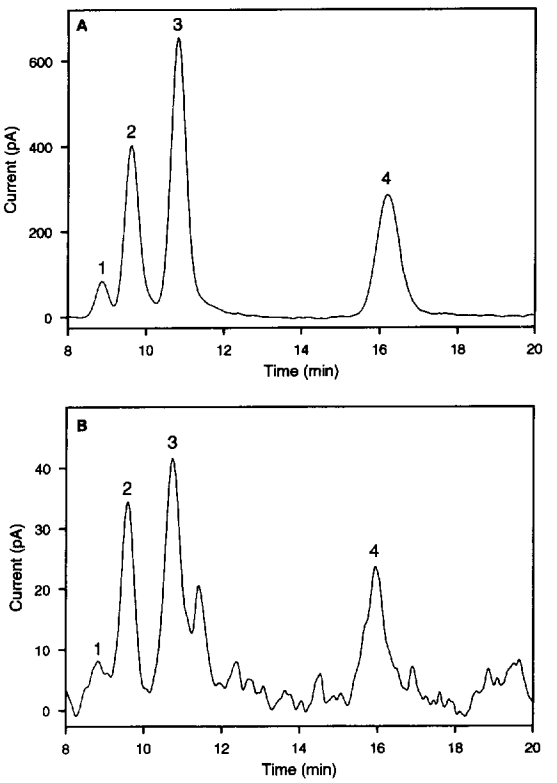

Fig. 2. Chromatograms of (1) 13-*cis*-retinoic acid, (2) all-*trans*-retinoic acid, (3) retinol, and (4) retinaldehyde. In (A) the quantity of analyte introduced into the separation capillary is 49.6, 87.6, 70.0, and 137 fmol, respectively. In (B) the quantity of analyte is 2.48, 4.40, 3.51 and 6.84 fmol, respectively. Analysis conditions: acetonitrile–water–methanol (65:32.5:2.5, v/v/v) as mobile phase which contains 1% tetrabutylammonium perchlorate and 0.174 *M* acetate buffer adjusted to pH 5. Detection was at 0.90 V versus the Ag/AgCl reference electrode.

The utility of this instrument for analysis of retinoids was confirmed by determining retinoid concentrations in bovine serum. A chromatogram for the retinoids in a bovine serum extract is shown in Fig. 3. Sample constituents with retention characteristics identical to those of retinol, 13-*cis*- and

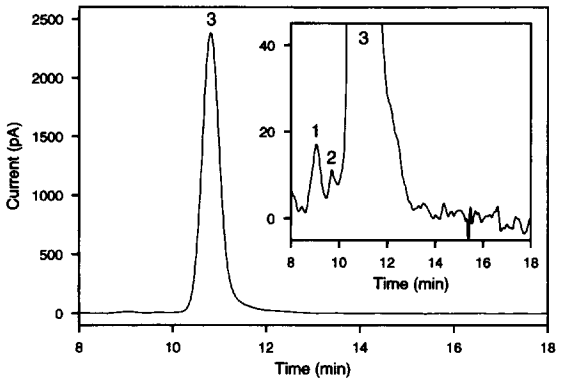

Fig. 3. Chromatogram of the extract from the bovine serum sample. The inset is an expanded view of this chromatogram. The peaks are identified as follows: (1) 13-*cis*-retinoic acid, (2) all-*trans*-retinoic acid, and (3) retinol. Analysis conditions are identical to those listed in Fig. 2.

all-*trans*-retinoic acid were identified and measured. There was no detectable signal which corresponded to retinaldehyde. The average retinoid concentrations determined for three serum samples were 4.7±1.12 μg l^{-1} for 13-*cis*-retinoic acid, 340±62 ng l^{-1} for all-*trans*-retinoic acid, and 180±10.3 μg l^{-1} for retinol. This concentration of retinol is in good agreement with the value 300 μg l^{-1} previously reported for bovine serum [26]. Retinol and retinoic acid isomer concentrations are also in agreement with the concentrations reported for these same species in human plasma [18]. The small differences in these numbers could result from natural heterogeneity of the animal population, incomplete extraction of the sample, and degradation of the analytes during prolonged storage of the bovine serum samples.

Day-to-day variations in the electrochemical response of the electrochemical cell can be a source of error in these measurements. Daily pretreatment of the carbon fiber electrode with a 50-Hz waveform with an amplitude of 2.2 V (peak-to-peak) and a d.c. offset of 1.1 V provides a clean electrochemical surface and minimized changes in electrochemical response. When the electrochemical response degrades during the analysis (e.g., by irreversible adsorption of sample constituents on the electrode surface), this treatment can be performed before each analysis to improve reproducibility. Use of internal standards can provide an additional means of minimizing the influence of variations in electrode response on the analytical measurement as well as compensate for analyte loss during sample preparation or unintentional changes in chromatographic conditions. Retinaldehyde was found to be an excellent internal standard because its chromatographic and electrochemical characteristics are similar to other retinoids, and because it is present in negligible concentrations in most tissue samples.

4. Conclusions

Capillary HPLC with amperometric detection is both a selective and sensitive means of monitoring retinoids. Isocratic chromatographic conditions allowed baseline resolution for a mixture of 13-*cis*-retinoic acid, all-*trans*-retinoic acid, retinol, and retinaldehyde. Amperometric detection provides subfemtomole detection limits in sample volumes as large as 2 μl. Further, this detection method should be amenable to other chromatographic conditions (e.g., normal-phase chromatography). Reducing the volume of the samples to values less than 2 μl could further improve separation efficiency and allow the monitoring of retinoids in single or small groups of cells [27,28]. We are presently exploring this possibility in embryonic tissue samples.

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