methods

Retinoid quantification by HPLC/MS^n

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Abstract **Retinoic acid (RA) mediates most of the biological effects of vitamin A that are essential for vertebrate survival. It acts through binding to receptors that belong to the nuclear receptor transcription factor superfamily (Mangelsdorf et al. 1994). It is also a highly potent vertebrate teratogen. To determine the function and effects of endogenous and exogenous RA, it is important to have a highly specific, sensitive, accurate, and precise analytical procedure. Current analyses of RA and other retinoids are labor intensive, of poor sensitivity, have limited specificity, or require compatibility with RA reporter cell lines (Chen et al. 1995. *Biochem. Pharmacol.* 50: 1257–1264; Creech Kraft et al. 1994. *Biochem. J.* 301: 111–119; Lanvers et al. 1996. *J. Chromatogr. B Biomed. Appl.* 685: 233–240; Maden et al. 1998. *Development.* 125: 4133–4144; Wagner et al. 1992. *Development.* 116: 55–66). This paper describes an HPLC/mass spectrometry/mass spectrometry product ion scan (HPLC/MS^n) procedure for the analysis of retinoids that employs atmospheric pressure chemical ionization MS. The retinoids are separated by normal-phase column chromatography with a linear hexane-isopropanol-dioxane gradient. Each retinoid is detected by a unique series of MS^n functions set at optimal collision-induced dissociation energy (30% to 32%) for all MS^n steps. The scan events are divided into three segments, based on HPLC elution order, to maximize the mass spectrometer duty cycle. The all-*trans*, 9-*cis*, and 13-*cis* RA isomers are separated, if desired, by an isocratic hexane-dioxane-isopropanol mobile phase. This paper describes an HPLC/MS^n procedure possessing high sensitivity and specificity for retinoids.**—McCaffery, P., J. Evans, O. Koul, A. Volpert, K. Reid, and M. D. Ullman. **Retinoid quantification by HPLC/MS^n. *J. Lipid Res.* 2002.** 43: **1143–1149.**

Supplementary key words retinol • retina • retinoic acid • didehydroretinoic acid • 4-oxo-retinoic acid • mass spectrometry • atmospheric pressure chemical ionization • high-performance liquid chromatography/mass spectrometry/mass spectrometry product ion scan

Vitamin A is a broad term encompassing all compounds that possess the biological properties of the parent compound, retinol (**Fig. 1**). The biological properties of vitamin A include the generation of visual pigment from the retinal product of retinol (7), while vitamin A also includes the retro-retinoid group of retinol derivatives that promote the proliferation of lymphocytes and fibroblasts or trigger cell death (8). The most extensive biological influences of vitamin A, however, occur via the oxidation of retinol to retinoic acid (RA), the transcriptionally active retinol metabolite. RA mediates most of the biological effects of vitamin A necessary for vertebrate development and survival (9). RA acts by binding to specific nuclear receptors of the steroid/thyroid hormone superfamily of transcriptional activators (1, 10). It can isomerize at several of its double bonds, and this isomerization is of biological relevance because the various isomers activate different receptors. The retinoic acid receptors (RARs) (alpha, beta, or gamma) are receptors activated by all-*trans* RA or 9-*cis* RA and the RXRs (alpha, beta, or gamma) are only activated by 9-*cis* RA. These two receptor classes bind to the DNA response element as homodimers, as RAR/RXR heterodimers, or as heterodimers with other nuclear receptors, such as thyroid hormone receptors, vitamin D receptors, or peroxisome proliferator-activated receptors (11). RA, hence, can modulate the action of a wide range of hormones and other nuclear receptor ligands to regulate the expression of an unusually large number of genes (12, 13).

Although RA is the major transcriptionally active retinoid in the embryonic mouse, in other species, additional active retinoids have been identified. Didehydroretinoic acid is the predominant active retinoid in the chick, where it is present in the embryonic limb bud (14), early trunk, developing spinal cord, and somites (5). This retinoid has been detected in both Xenopus and zebrafish embryos (15, 16), but has not been found in the mouse (17). In the mouse, 4-oxo-RA is considered one of the metabolites in the pathway of RA catabolism. Interestingly, in other species, this retinoid is known to regulate transcription. In the embryonic Xenopus (18), all-*trans* 4-oxo-RA regulates the development of the anteroposterior body

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Abbreviations: APCI, atmospheric pressure chemical ionization; CAD, collisional activation decomposition; RA, retinoic acid.

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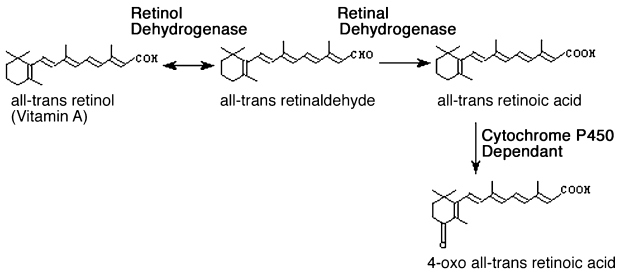

Fig. 1. Retinoic acid (RA) metabolism. The two-step synthesis of RA from retinol entails oxidation first to retinal catalyzed by a retinol dehydrogenase. Retinal is irreversibly oxidized to RA by a retinal dehydrogenase. Catabolism of RA likely involves oxidation on the 4-carbon to 4-oxo RA catalyzed by a cytochrome P450 enzyme such as CYP26.

axis and 9-*cis*-4-oxo-RA activates a unique RA receptor heterodimer combination (19).

The examination of retinoid biology in the embryo and adult has focused on the expression of the receptors (1, 20) and, more recently, on retinoid metabolic enzymes (21–24). Tissue retinoid levels have not had the same attention, in part because of the relative technical difficulty of quantifying these lipids at the nanomolar concentrations present in tissues (25). Further, retinoids are photosensitive, making them difficult compounds to isolate from tissue sources. A number of techniques have been developed in attempts to find reliable methods for their quantification.

Reversed-phase chromatography with gradient HPLC combined with spectrophotometric or photodiode-array detection has become the favored method of retinoid assay. Although isocratic conditions can resolve RA (26) and 4-oxo-RA (27, 28), gradient elution provides resolution of both polar and nonpolar retinoids (15, 17, 29–32). Electrochemical detection has also recently come into use, both for HPLC (33, 34) and capillary liquid chromatography (35). Specificity, however, is a critical issue with all of these techniques.

Normal-phase chromatography on silica columns (4, 32, 36–38) provides advantages over reversed-phase systems, such as the ability to include high concentrations of the antioxidant butylated hydroxytoluene. HPLC with online mass spectrometry product ion scan mass spectrometry (MS^n) detection of retinoids provides high specificity and sensitivity.

Combined HPLC/MS^n techniques are among the most sensitive and specific available for the analysis of minor components from complex biological extracts (39). Sensitivity and specificity are useful for the analysis of retinoids in extracts from tissues in which sample quantities are limited and their chemical and photochemical lability complicate their further isolation from crude extracts.

Atmospheric pressure chemical ionization (APCI) is a very sensitive technique for the ionization of lipids, including retinoids, which can provide efficient ion production for MS detection, and has been used for the analysis of RA in embryonic tissues (34) and the prostate gland (40). It still lacks, however, the specificity needed for the analysis of tissue extracts. The use of multiple steps of high efficiency (MS^n) is possible with quadrupole ion trap MS systems, which can provide the necessary high sensitivity and specificity for these analyses. Presented herein is a normal-phase HPLC/APCI/MS^n method for the analysis of retinol and its metabolic products, retinal, RA, didehydroretinoic acid, and 4-oxo-RA from crude tissue extracts using the external standards method for quantification.

MATERIALS AND METHODS

Materials

Solvents for extraction and chromatography were of HPLC grade (Fisher Scientific, Atlanta, GA).

Extraction of retinoids from rat liver

To minimize photoisomerization of retinoids, acquisition of tissue specimens and all subsequent processing steps were performed in a room with only a low-intensity yellow light source. Rat liver was obtained from 8-week-old male Sprague-Dawley rats on a diet of Prolab RMH 3000 (22% protein and 5% fat) from PMI Nutrition Inc. (Bretwood, MO). Liver tissue was minced and separated into three weighed portions. Each liver sample was homogenized in a glass homogenizer fitted with a glass pestle in ethanol-isopropanol 2:1 (v/v) (1 ml/100 mg wet weight of tissue). The extraction solvent contained butylated hydroxytoluene (1 mg/ml) as an antioxidant. The homogenates were centrifuged in the cold and the supernatants were stored at −20°C.

Extraction of retinoids from retina supernatant

Retinae were dissected from 12 embryonic day-13 and 12 embryonic day-15 mouse eyes. These were combined, incubated for 12 h in 200 μl tissue culture medium (CO_2 modified L15 with 1% FBS) and then centrifuged to provide a supernatant fraction. The isolated supernatant was extracted in 200 μl of hexane-dioxane-isopropanol 25:5:0.5 (v/v/v), dried under nitrogen, and reconstituted in 50 μl of the injection solvent and analyzed by HPLC/MS. Pellet protein content was determined by colorimetric analysis using the Pierce BCA kit.

HPLC/MS^n of retinoids

At the time of analysis, standard mixtures or isolated retinoid fractions were warmed to 0°C and 3 μl of sample were drawn into an aluminum foil-sleeved syringe and injected into the silica column. HPLC separation of retinoids utilized normal-phase chromatography performed on a normal-phase (Inertsil, Keystone Inc., Bellefonte, PA) column (150 × 2 mm; 5 μm particle size). Retinoids were eluted (flow rate 0.2 ml/min) with an 18.9 min linear gradient from 90% solvent A (hexane)-10% solvent B (hexane-dioxane-isopropanol, 40:8:2, v/v/v) to 58% solvent A-42% solvent B. At the end of the gradient run, the mobile-phase composition was returned to initial conditions over 1 min. The flow rate was then increased to 1 ml/min over 4 min, and initial conditions were held for an additional 6 min at this flow rate. At the end of that time, the flow rate returned to initial flow rate conditions.

If desired, all-*trans*, 9-*cis*, and 13-*cis* RA isomers were separated on the same column utilizing a 14 min isocratic elution (flow rate: 0.2 ml/min) with hexane-dioxane-isopropanol, 70.00:25.71:4.29 (v/v/v) as the mobile phase.

Retinoid standards in the column effluent were monitored in series first by UV detection at 354 nm using an in-line Spectra-

physics UV 2000 and then by MS. For tissue analysis, standard and liver retinoids in the column effluent were detected by various APCI/MS^n scan events using a quadrupole ion trap mass spectrometer (Finnigan LCQ, San Jose, CA). APCI was performed with the HPLC eluate flowing into the ion source at 200 μl/min, with the vaporizer at 375°C, the nitrogen sheath gas flow set at 35%, the source current at 5 μA, and the heated capillary at 150°C. The mass spectrometer was auto-tuned on the MH^+ ion for RA (m/z 301) during a constant infusion of a 1.0 μg/ml solution in hexane at 200 μl/min. Each analyte was detected by a unique series of MS^n functions, each at optimal collision-induced dissociation energy (30–32%) for all MS^n steps. The scan events for different retinoids were divided into three time segments based on HPLC elution order to maximize the mass spectrometer duty cycle. The MS^n scan functions, including precursor ions and product ions used for quantification, were unique for each analyte. Only retinal was detected in the first time segment (0–6 min), utilizing an MS^3 scan function with m/z 285 (MH^+) selected as the precursor ion in the first step and its m/z 193 product ion as the precursor in the final (MS^3) step. The final efficiency for this was 12%. Peak areas from ion plots of the resulting m/z 175 MS^3 product ion were used for its quantification. The second time segment (5–12 min) contained the scan events for two analytes. The first of these, didehydroretinoic acid, was detected by an MS^3 scan event selecting m/z 299 (MH^+) as the MS^2 precursor ion, m/z 243 as the MS^3 precursor ion, and using m/z 225 product ion plot peak areas for quantification. The final efficiency for this was 35%. The second set of analytes included in the segment were RA isomers that were detected by an MS^3 scan event utilizing m/z 301 (MH^+) as the MS^2 precursor ion, m/z 205 as the MS^3 precursor ion, and m/z 159 product ion plots for quantification. The final efficiency for this was 1.3%. Time segment three (12–20 min) also contained two scan events. The first was for 4-oxo-RA, which was detected by an MS^4 scan event utilizing m/z 315 (MH^+) as the MS^2 precursor ion, m/z 297 as the MS^3 precursor ion, m/z 279 as the MS^4 precursor ion, and m/z 209 product ion plot peak areas for quantification. The final efficiency for this was 0.9%. The second analyte included in the third segment was retinol, which was detected by an MS^3 scan event utilizing m/z 269 (MH^+-H_2O) as the MS^2 precursor ion, m/z 213 as the MS^3 precursor ion, and m/z 157 product ion plot peak areas for quantification. The final efficiency for this was 3.8%.

RESULTS

Chromatography of retinoids

A standard mixture containing retinal, 13-*cis*-RA, didehydroretinoic acid, all-*trans*-RA, 9-*cis*-RA, retinol, and all-*trans*-4-oxo-RA was chromatographed under the gradient conditions described in Materials and Methods (**Fig. 2**). Baseline resolution was obtained for retinol, retinal, and all-*trans*-4-oxo-RA. Near-baseline resolution was obtained with 13-*cis*-RA, didehydroretinoic acid, all-*trans*-RA, and 9-*cis*-RA.

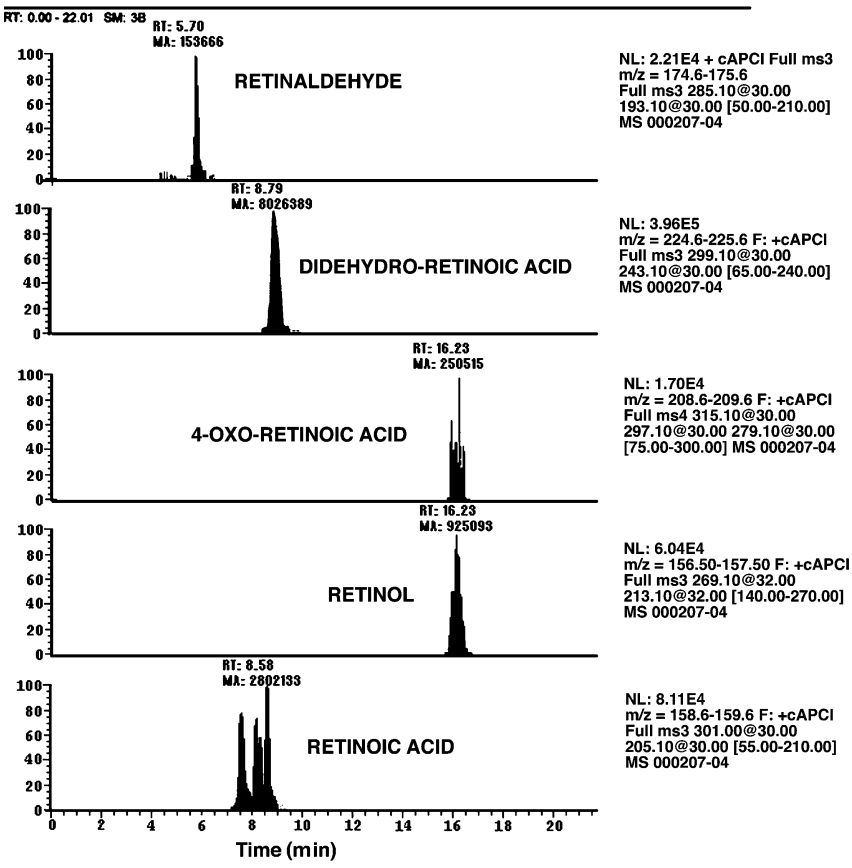

Fig. 2. HPLC/mass spectrometry/mass spectrometry product ion scan (HPLC/MSn) resolution of retinoid standards. Resolution of 1,000 fmol of a standard mixture of retinoids, as indicated, was performed on a normal-phase (Inertsil, Keystone Inc., Bellefonte, PA) column (150 × 2 mm; 5 μ particle size). They were eluted (flow rate 0.2 ml/min) with an 18.9 min linear gradient from 90% solvent A (hexane)-10% solvent B (hexane-dioxane-isopropanol, 40:8:2) to 58% solvent A-42% solvent B.

Selection of product ions

MS^3 product ion scans were used to gain high specificity for the detection of low levels of the retinoids in these extracts. The MS^2 collisional activation decomposition (CAD, He collision gas) of the MH^+ ions (MH^+-H_2O for retinol) resulted from APCI of the analyses in the HPLC eluate. CAD was optimized to produce an intense product ion, which was then isolated in the ion trap and subjected to an additional round of CAD to produce a product ion that was used for detection and quantification of the analytes. For retinal, the *m/z* 285 MH^+ ion was decomposed (through a presumed rearrangement and neutral loss of toluene) to produce an *m/z* 193 product ion as the most intense ion in its MS^2 spectrum. This ion was subsequently isolated and decomposed through CAD to produce an *m/z* 175 ion (presumed dehydration) as the base ion in the MS^3 product ion spectrum that was used for retinal detection and quantification. Similarly, the *m/z* 301 MH^+ ion from RA was subjected to CAD to produce an *m/z* 205 in high yield (presumed rearrangement and loss of phenol), which was subsequently isolated and decomposed to produce an *m/z* 159 ion (loss of C_2H_6-O) that was used for quantification. For 4-oxo-RA, a very high efficiency loss of water occurs from CAD of the *m/z* 315 MH^+ ion to produce a *m/z* 297 product that subsequently efficiently produces a *m/z* 279 MS^3 product ion through an additional loss of water. Because two successive dehydrations do not provide high specificity for detection, the high efficiency of these dehydrations allows an additional MS^4 step to be performed using the *m/z* 279 MS^3 product ion to produce a *m/z* 209 product in good yield.

Linearity

Standard mixtures of retinol, retinal, all-*trans* RA, didehydroretinoic acid, and 4-oxo-RA were injected into the HPLC column, and the column effluents were monitored by MS as described in Materials and Methods. Duplicate injections of standard mixtures ranging in concentration from 63 to 1,995 fmol on-column were used to determine mass spectrometer response linearity (**Fig. 3**). All stan-

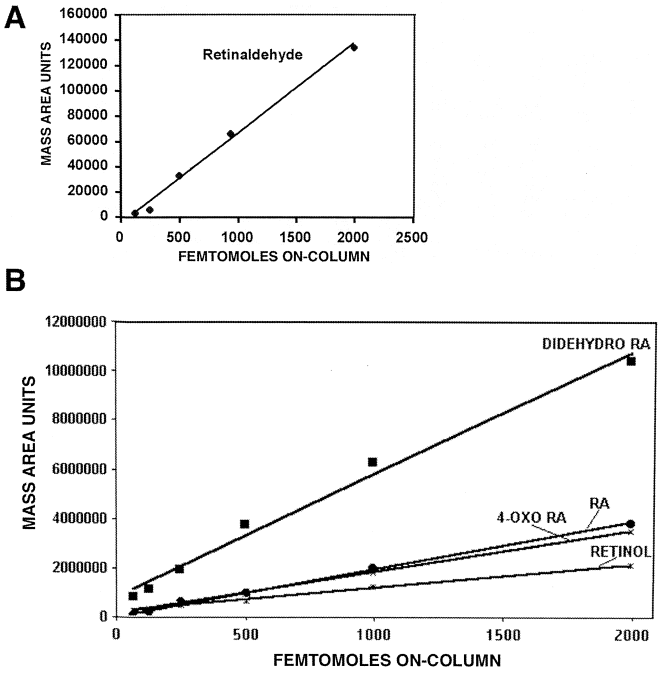

Fig. 3. Linearity of analysis of retinaldehyde (A) and other retinoids (B). Three microliters of a mixture of retinoid standards was injected into a normal-phase column and separated, detected, and ion-intensity determined by $HPLC/MS^n$ (Materials and Methods). Concentrations for the standards ranged from 67.5 fmol to 1,995 fmol on-column.

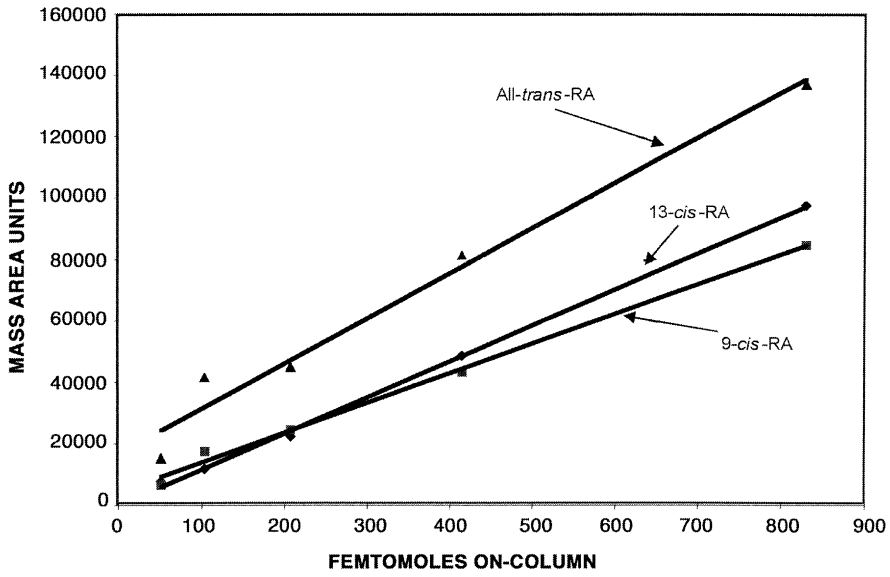

Fig. 4. Linearity of RA isomer analysis. Three microliters of a mixture of RA standards was injected into a normal-phase column and separated, detected, and ion-intensity determined by $HPLC/MS^n$ (Materials and Methods). Concentrations for the standards ranged from 50 fmol to 840 fmol on-column.

dards provided a linear response over the concentration range. Regression analysis gave the following values for R^2: retinal, 0.989; all-*trans* RA, 0.998; didehydroretinoic acid, 0.989; retinol, 0.998; 4-oxo-RA, 0.999.

Several isomers of RA exist that are of biological significance. In addition to all-*trans* RA, which activates the RAR class of receptors, 9-*cis* RA is considered to be a physiological activator of the RXR class (1) and 13-*cis* RA is a physiological compound of indeterminate function (41). Both 9-*cis* RA and 13-*cis* RA can be identified in tissues (40). All-*trans*, 9-*cis*, and 13-*cis* RA can be separated on the same column rapidly with an isocratic method. Duplicate injections of standard mixtures ranging in concentration from 50 to 840 fmol on-column were used to determine mass spectrometer response linearity (**Fig. 4**). Regression analysis gave the following values for R^2: all-*trans* RA, 0.9780; 9-*cis* RA, 0.9948; 13-*cis* RA, 0.9988. The intensity of the *m/z* 301 product ion differed between the isomers with the highest response from the *trans* isomer.

Reproducibility and sensitivity

Repetitive injections (N = 4) of the 66.5 nmolar standard provided coefficients of variation of: retinol, 5%; retinal, 13%; all-*trans* RA, 12%; didehydroretinoic acid, 9%; 4-oxo-RA, 9%. The lower limit of detection, defined as a signal-to-noise ratio of 2:1, was approximately 50 fmoles on-column for each standard, with the exception of retinaldehyde, which was approximately 100 fmoles on-column.

Reproducibility was estimated by analyzing retinoids from 8-week-old Sprague-Dawley male rat liver samples as described in Materials and Methods. Analyses were performed on 3 μl of the sample extract supernatant. Because of the highly polar nature of the isopropanol/ethanol extraction solvent, retinoid retention times were dependent on the volume of sample injected (**Fig. 5**). Slightly larger volumes could be injected, if necessary, providing the mass spectrometer segments were adjusted accordingly. Alternatively, extracts could be dried and reconstituted in a smaller volume. The rat livers were analyzed for both retinol and RA. The concentration of retinol was 13.32 (SD 0.06) nmol/g, and that of RA was 0.98 (SD 0.06) nmol/g in triplicate runs. Comparing the concentrations of retinoids in two other 8-week-old Sprague-Dawley male rats, we found, respectively, 22.39 and 16.62 nmol/g (wet weight) retinol and 2.18 and 1.89 nmol/g (wet weight) RA. The ion chromatograms for retinol and RA are shown in **Fig. 6**. In a second experiment to analyze embryonic tissue, embryonic retinae were prepared and analyzed. We have previously demonstrated that cultured embryonic retinae are able to release RA into the surrounding media, but this has not been quantified other than by a semiquantitative bioassay technique (42). Retinae from 24 embryonic day 13–15 mouse eyes were combined, incubated for 24 h in 200 μl tissue culture and then centrifuged to provide a supernatant fraction. The isolated supernatant was hexane-dioxane-isopropanol extracted and analyzed by HPLC/MS. Triplicate analysis of the supernatant showed the all-*trans*-RA concentration to be 20.75 (SD 2.04) pg/μg retina protein.

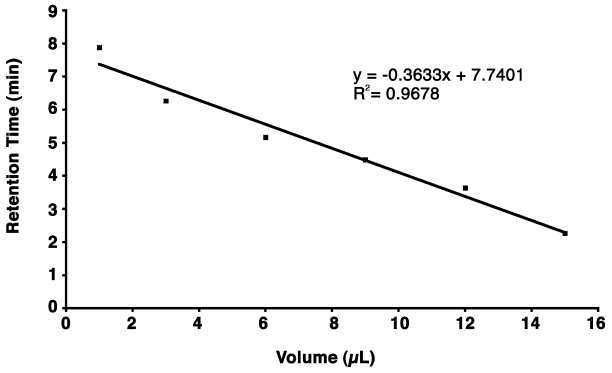

Fig. 5. Effect of injection volume on retinoid retention times. Solutions of all-*trans*-RA (0.64 pmol/μl) in 1, 3, 6, 12, and 15 μl of ethanol-isopropanol 2:1 (v/v) were made and then chromatographed using the gradient HPLC method. As the injection volume increased, retention time decreased because of the high solvent strength of the ethanol-isopropanol solvent, which hindered chromatofocusing of the analyte.

DISCUSSION

This normal-phase HPLC/MS^n technique is a departure from spectrophotometric procedures used for retinoid analysis in which detection limits are approximately 0.7 pmol (43) and in which confirmation of compound identity requires derivitization of the eluted material (2–6). Reporter cell assays have limits of detection in the picomolar concentration range for all-*trans* RA. Sensitivity is severely decreased for other isomers, however, and reagents that are toxic to the cells cannot be used (6). Other HPLC/MS procedures (44–46) do not provide the specificity of MS^n techniques. This HPLC/MS^n procedure provides both quantitative measurement and a high degree of specificity for multiple retinoids. The method affords ex-

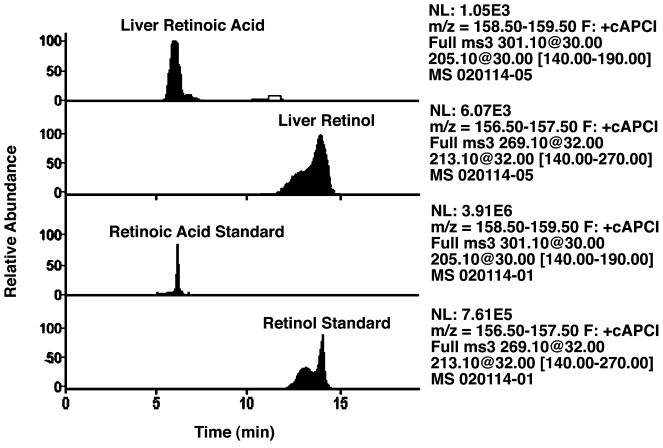

Fig. 6. HPLC/MS^n resolution of liver extract and RA and retinol standards. Resolution of liver RA and retinol, compared to a standard mixture of retinoids, as indicated, was performed on a normal-phase (Inertsil) column (150 × 2 mm; 5 μm particle size). They were eluted (flow rate 0.2 ml/min) with an 18.9 min linear gradient from 90% solvent A (hexane):10% solvent B (hexane-dioxane-isopropanol, 40:8:2) to 58% solvent A-42% solvent B.

cellent sensitivity and is linear over a broad and physiologically relevant concentration range.

Particular attention must be given to the sample volume injected when using the 30 min gradient system. When sample volume varied from 3 μl, retinoid retention times were altered. Apparently, the high solvent strength of the ethanol/isopropanol solvent hindered chromatofocusing of the retinoids at the head of the column. If larger sample equivalents are needed, the extraction may be performed with slightly less extraction volume of the ethanol/isopropanol solvent, larger sample volumes can be injected using different timing segments in the mass spectrometer, or the sample may be applied to the column by diluting the sample with a less-polar solvent (e.g., hexane or *iso*-octane) to chromatofocus the analytes at the head of the column prior to initiation of the gradient elution. Although the gradient elution provides near-baseline resolution of RA isomers, the mass spectrometric analysis of these isomers is performed using an isocratic gradient that provides improved resolution.

The external standards method was used for quantification. The extraction procedure used in this study (47) provides excellent recoveries so that internal standards, which are not readily commercially available, are not needed. Further, preliminary studies using D5-RA (a generous gift from Dr. J. Napoli University of California, Berkeley) as an internal standard gave more variable results than those obtained with the external standards procedure. Apparently, the time required for added D5-RA to equilibrate with endogenous protein-associated RA permitted degradation and/or isomerization of the retinoids.

During the course of this work, reversed-phase HPLC was also attempted. Although retinoid resolution could have been affected, results were not as reproducible as in the normal-phase procedure, perhaps because high (1 mg/ml) butylated hydroxy toluene could have been included in the extraction procedure for normal-phase HPLC, thus diminishing the potential for oxidative degradation of the retinoids.

The liver analysis provided retinol concentrations that were similar to those described for rats of the same sex, strain, and age by Wang et al. (48) (26.7 nmol/gm wet weight of liver for 8-week-old male Sprague-Dawley rats). Variations were evident among the livers of different rats, presumably because of variation such as the time of day or prior food consumption by the individual rat. The liver RA concentrations, however, were found to be up to 20 times higher than those published by Wang et al. This difference may be largely attributable to extraction conditions. Wang et al. used chloroform-methanol 2:1 (v/v) to extract retinoids, which is commonly used to extract highly lipophilic retinoids such as retinol and retinyl esters but, at neutral pH, is not ideal for anionic RA. We selected our ethanol/isopropanol technique to maximize the extraction of retinol as well as RA and its derivatives. Although this method broadly extracts the majority of lipids, which would be a problem with some detection techniques, use of HPLC/MS provides the specificity required to resolve RA in a complex mixture. It will be of interest to revisit the RA concentration in other organs using this extraction technique. This procedure will be applicable to a broad range of tissues. For example, we currently use it for the analysis of retinoids from postnatal day one rat cerebellum and cortex. The assay sensitivity and specificity will be useful in the determination of tissue retinoid responses to developmental and environmental cues. This will ultimately lead to a greater understanding of retinoid mechanisms of action and its possible involvement in disease.

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