methods

A sensitive and specific method for measurement of multiple retinoids in human serum with UHPLC-MS/MS

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Abstract Retinol (vitamin A) circulates at 1–4 μM concentration and is easily measured in serum. However, retinol is biologically inactive. Its metabolite, retinoic acid (RA), is believed to be responsible for biological effects of vitamin A, and hence the measurement of retinol concentrations is of limited value. A UHPLC-MS/MS method using isotope-labeled internal standards was developed and validated for quantitative analysis of endogenous RA isomers and metabolites. The method was used to measure retinoids in serum samples from 20 healthy men. In the fed state, the measured concentrations were 3.1 ± 0.2 nM for *at*RA, 0.1 ± 0.02 nM for 9-*cis*RA, 5.3 ± 1.3 nM for 13-*cis*RA, 0.4 ± 0.4 nM for 9,13-*dicis*RA, and 17.2 ± 6.8 nM for 4oxo-13-*cis*RA. The concentrations of the retinoids were not significantly different when measured after an overnight fast (3.0 ± 0.1 nM for *at*RA, 0.09 ± 0.01nM for 9-*cis*RA, 3.9 ± 0.2 nM for 13-*cis*RA, 0.3 ± 0.1 nM for 9,13-*dicis*RA, and 11.9 ± 1.6 nM for 4oxo-13-*cis*RA). 11-*cis*RA and 4OH-RA were not detected in human serum. The high sensitivity of the MS/MS method combined with the UHPLC separation power allowed detection of endogenous 9-*cis*RA and 4oxo-*at*RA for the first time in human serum.—Arnold, S. L. M., J. K. Amory, T. J. Walsh, and N. Isoherranen. **A sensitive and specific method for measurement of multiple retinoids in human serum with UHPLC-MS/MS. *J. Lipid Res.* 2012.** 53: **587–598.**

Supplementary key words retinoic acid • isomers • 4oxo-retinoic acid • MS/MS • metabolites

Retinoids are acquired from the diet as a proretinoid carotenoid or as retinol and retinyl esters (1). Retinol (vitamin A) is the main circulating retinoid, but it is biologically inactive and is metabolized in the body to active retinoic acid (RA). RA has been reported to exist as at least five isomers, including all-*trans* retinoic acid (*at*RA), 9-*cis*RA, 13-*cis*RA, 9,13-*dicis*RA, and 11-*cis*RA (2). These RA isomers are involved in numerous processes associated with vision, healthy skin, glucose regulation (3), embryonic development (4), stem cell differentiation (5), and spermatogenesis (6), although the exact role of each isomer is not fully understood. *at*RA has been shown to induce remission in patients with acute promyelocytic leukemia, and 13-*cis*RA (isotretinoin) is used for the treatment of acne and in children with high-risk neuroblastoma (7). 9-*cis*RA (alitretinoin) has been used to treat severe chronic hand eczema (8) and has been shown to have a role in regulating insulin-stimulated glucose secretion (3, 6, 9). In addition to the RA isomers, the oxidized metabolites of RA, including 4-hydroxy-RA (4OH-RA), 4oxo-RA, and RA-5,6-epoxide, possess biological activity in in vitro models (10). 4oxo-RA has also been shown to be teratogenic in animal models and to inhibit cell proliferation in a number of cell lines (11–13).

The biological activity of retinoids is mediated predominantly by their association with nuclear receptors, mainly the retinoic acid receptors (RARα, RARβ, and RARγ). However, binding of retinoids to retinoid X receptors (RXRα, RXRβ, and RXRγ) and peroxisome proliferator-activated receptors (PPARβ and PPARδ) has also been shown (14). Oxidative metabolism of the RA isomers can result in deactivation or activation of the parent retinoid, and isomerization of RA can change the specificity and activity of RA toward nuclear receptors. For example, 9-*cis*RA binds to RXR with a significantly higher affinity than *at*RA, and 4oxo-*at*RA and 4OH-*at*RA have been shown to bind to RAR with similar affinity as *at*RA (10, 12, 15).

This work was supported in part by an National Institutes of Health grants R01 GM081569 and GM081569-S1 and by the Eunice Kennedy Shriver National Institute of Child Health and Human Development, a division of the National Institute of Health through cooperative agreement #U01 HD060408. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Institutes of Health or other granting agencies.

Manuscript received 10 November 2011 and in revised form 9 December 2011.

Published, JLR Papers in Press, December 21, 2011
DOI 10.1194/jlr.D019745

Abbreviations: *at*RA, *all-trans*-retinoic acid; 9-*cis*RA, 9-*cis*-retinoic acid; 13-*cis*RA, 13-*cis*-retinoic acid; 4oxo-13-*cis*RA, 4oxo-13-*cis*-retinoic acid; 4oxo-*at*RA, 4oxo-*all-trans*-retinoic acid; 4OH-9-*cis*RA, 4-hydroxy-9-*cis*-retinoic acid; 4OH-*at*RA, 4-hydroxy-*all-trans*-retinoic acid; can, acetonitrile; APCI, atmospheric pressure corona ionization; CE, collision energy; CV, coefficient of variation; CXP, collision cell exit potential; DP, declustering potential; EP, entrance potential; UHPLC, Ultra-high-performance liquid chromatography; LXR, liver X receptor; LLOQ, lower limit of quantification; LLOD, lower limit of detection; RAR, retinoic acid receptor; RXR, retinoid X receptor; SRM, selected reaction monitoring; PPAR, peroxisome proliferator-activated receptor; QC, quality control.

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The major limitation in understanding the roles and importance of 4OH-RA, 4oxo-RA, and specific RA isomers in vivo has been the lack of knowledge on the concentrations of the specific isomers and metabolites in human tissues.

Analysis of RA isomers and their metabolites has been difficult due to their similar structures and low concentrations in human tissues. A lack of analytical separation power may have confounded individual isomer detection, whereas a lack of good MS/MS sensitivity has prevented selective quantification of individual retinoids. Previous methods have been developed using LC-MS/MS (9, 16–21), GC/MS (22), and LC/diode array detector-atmospheric pressure chemical ionization/MS/MS (23). However, none of these methods has incorporated measurement of hydroxylated RA metabolites at endogenous levels in serum. Endogenous *at*RA (4.6–5.8 nM), 13-*cis*RA (4.7–6 nM), and 4oxo-13-*cis*RA (8.1–9.8 nM) were measured in human serum using HPLC with UV detection (24, 25). However, 9,13-*dicis*RA and 9-*cis*RA were not measured, nor was separation of the main four isomers of RA demonstrated. Separation and quantification of *at*RA, 9-*cis*RA, 9,13-*dicis*RA, and 13-*cis*RA in mouse tissues was shown using LC-MS/MS analysis (9), but no measurement of the metabolites of RA was conducted.

The goal of this study was to develop a method for the simultaneous measurement of biologically active endogenous retinoids in human serum. Due to the challenges in separating the isomers of RA as well as the metabolites, chromatographic separation was optimized for UHPLC. The method was validated with spiked charcoal-treated blank human serum and used to determine retinoid concentrations in serum from healthy men. The developed assay is useful for characterization of retinoid disposition and endogenous retinoid homeostasis and for following therapeutic interventions with retinoids. The method can also be used in preclinical studies of compounds that target changing retinoid concentrations.

MATERIALS AND METHODS

Chemicals and reagents

*at*RA, 9-*cis*RA, 13-*cis*RA, and 9-*cis*-retinal were purchased from Sigma (St. Louis, MO). 4oxo-*at*RA, 4OH-*atRA*, and 9,13-*dicis*RA were synthesized as described below. 4oxo-13-*cis*RA, 4OH-9-*cis*RA, 4oxo-13-*cis*RA-d_3, 13-*cis*RA-d_5, and 11-*cis*RA were purchased from Toronto Research Chemicals (North York, Ontario). All compounds were stored in amber vials in ethanol as 1 mM stocks at −80°C. Acetonitrile, methanol, water, and formic acid used in the UHPLC-MS/MS method were from Fischer Scientific (Pittsburg, PA), and all were Optima LC/MS grade. Blank human serum (DC Mass Spect Gold MSG 4000) was purchased from Golden West Biologics (Temecula, CA). This serum has a normal range of triglycerides (30–200 mg/dl) and cholesterol (> 20 mg/dl) to mimic the extraction environment of clinical samples. This is important due to the matrix effects associated with lipids during extraction and analyte ionization.

4OH-RA and 4oxo-RA were synthesized according to previously published methods (26, 27). In brief, the methyl ester of *at*RA was generated using trimethylsilyldiazomethane. Methyl 4oxo-retinoate was prepared using activated MnO_2 in anhydrous dichloromethane. The 4oxo-RA-methyl ester was hydrolyzed using 2 M KOH in methanol, and 4oxo-*at*RA was extracted using ethyl acetate and crystallized. 4OH-*at*RA was synthesized from 4oxo-RA using two equivalents of $NaBH_4$ in methanol. The product was extracted with 1:1 mixture of ethyl acetate and ether, evaporated to dryness, and crystallized.

9,13-*dicis*RA was synthesized as previously published (28). In brief, 100 mg of 9-*cis*-retinal was dissolved in 20 ml of methanol. Eight milliliters of Tollens Reagent (equal volumes of 10% $AgNO_3$ and 10% NaOH mixed and titrated with ammonium hydroxide until precipitate dissolved) was added to the solution, and the mixture was stirred at 37°C for 6 h. The reaction was quenched with 4 N HCl on ice and filtered, and the products were extracted with hexane and evaporated to dryness. The mixture of 9-*cis*RA and 9,13-*dicis*RA was purified using silica gel chromatography and ethyl acetate:hexane mobile phase. The fraction containing 9,13-*dicis*RA was quantified by NMR by calculating the ratio between the area of the 9-*cis*RA doublet at 6.09 ppm (H in C10) to the corresponding doublet from 9,13-*dicis*RA at 6.2 ppm. The identity of the 9,13-*dicis*RA was also confirmed by the ratio of the 9,13-*dicis*RA doublet at 7.7 ppm (H at C12) to the corresponding doublet in 9-*cis*RA at 6.3 ppm. Based on NMR quantification, the reaction yielded 20% 9,13-*dicis*RA and 80% 9-*cis*RA.

Extraction of retinoic acid isomers and 4oxo-retinoic acid

All sample processing, preparation, and extraction was conducted on ice under red light. 9-*cis*RA, 13-*cis*RA, *at*RA, 4oxo-13-*cis*RA, 4oxo-*at*RA, 4OH-*at*RA, and 4OH-9-*cis*RA were spiked into blank serum. Into 500 μl of serum, 10 μl of a 60:40 ACN:MeOH mixture with 1 μM 13-*cis*RA-d_5 and 2 μM 4oxo-13-*cis*RA-d_3 was added as internal standards. All compounds were extracted according to previously described method for extraction of RA isomers (29). In brief, 1 ml ACN and 60 μl 4 N HCl were added to each sample, the samples were vortexed, and retinoids were extracted twice with 5 ml of Hexanes. The organic phase was separated by centrifugation at 1000 rpm for 3 min and evaporated to dryness under nitrogen stream at 32°C. The dry residue was reconstituted in 50 μl of 60:40 ACN:H_2O and transferred to an amber autosampler vial.

Serum retinoids could also be measured with no extraction using a simple protein precipitation with ACN. In this method, 50 μl of ACN is added to 50 μl of serum followed by centrifugation at 2000 rpm for 10 min at 4°C to pellet serum proteins. The supernatant is transferred to a 96-well plate, and 20 μl is injected into the UHPLC/MS/MS for analysis.

UHPLC MS/MS analysis

The retinoids were separated using an Agilent 1290 UHPLC (Santa Clara, CA) equipped with a Sigma (St. Louis, MO) Ascentis Express RP Amide column (2.7 μm; 150 mm × 2.1 mm). Gradient elution with a flow rate of 0.5 ml/min using water (A) and acetonitrile (B) with 40% methanol and 0.1% formic acid in A and B was used. The gradient was from initial 60% (A) for 2 min to 45% (A) over 8 min and then to 10% (A) over 7 min. The column was then washed with 95% (B) for 3 min and returned to initial conditions. The column heater was set to 40°C. Samples were kept in the autosampler at 4 °C with the light turned off. Ten microliters of sample was injected for analysis. Analytes were detected using an AB Sciex 5500 qTrap Q-LIT mass spectrometer (Foster City, CA) operated in positive ion APCI mode. The compound independent MS parameters were curtain gas: 20; collision gas: low; nebulizer current: 5; temperature: 350 °C; ion source gas 1:80. The final compound dependent parameters used for analysis are summarized in **Table 1**. The specific MS/MS transitions for each analyte were optimized using the Analyst software (Applied Biosystems, Foster City, CA) using direct infusion with

TABLE 1. Compound dependent mass spectrometer parameters used for detection of the retinoids

Analyte	Q_1 *m/z*	Q_3 *m/z*	msec	DP	EP	CE	CXP
RA (all isomers)	301	205	100	80	10	17	10
4OH-RA	299	157	100	59	10	16	15
4oxo-RA	315	159	100	66	10	23	16
4oxo-RA-d_3	300	226	100	71	10	35	2
13-*cis*RA-d_5	306	116	100	97	10	97	6

CE, collision energy; CXP, collision cell exit potential; DP, declustering potential; EP, entrance potential.

and without the addition of the LC solvents. Initially, MS/MS transitions were identified for each analyte using the automatic optimization features of the Analyst software. These transitions were compared with those from a manual optimization, and the five most abundant fragments for each analyte found in both the automatic and manual process were optimized independently for their declustering potential (DP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP). All five identified potential MS/MS transitions for each analyte were included in a method that was used to analyze blank, spiked, and normal human serum to determine interference, matrix effects, and signal-to-noise ratios of each MS/MS transition. The MS/MS transitions that showed the least amount of matrix interference in the blank serum and the highest signal-to-noise ratio in spiked samples were chosen for the final analysis.

Assay validation

The assay was validated according to the published guidelines for bioanalytical method validation (30, 31). The on-column lower limit of detection (LLOD) determined as signal-to-noise ratio >3 and the on-column lower limit of quantification (LLOQ) determined as signal-to-noise ratio >9 were measured for each compound as standard solution. The linearity of response was determined using standard curves generated over four orders of magnitude (0.0001–1.0 μM). For quantification, blank serum was spiked at six concentrations between 0.1 nM and 20 nM for RA isomers and between 0.5 nM and 20 nM for the metabolites to construct standard curves for each analyte. The peak area ratios of analyte/internal standard were plotted as a function of concentration. 13-*cis*RA-d_5 was used as the internal standard for quantification of *at*RA, 9-*cis*RA, 13-*cis*RA, and 9,13-*dicis*RA, and 4oxo-13-*cis*RA-d_3 was used for metabolite quantification. Each retinoid, with the exception of 9,13-*dicis*RA, was quantified using standard curves of the same compound. 9,13-*dicis*RA concentrations were measured using the standard curve for 9-*cis*RA after confirming that the MS/MS responses were identical for the two compounds using the NMR quantified standard. Quality control (QC) samples were extracted along with each standard curve and run at the beginning, middle, and end of each run. All QC samples had accuracy and precision within published guidelines for bioanalytical method validation (30, 31).

To determine the intraday and interday coefficients of variation (CVs), the samples were extracted on three separate days. The CV was measured at the LLOQ for serum of each compound and at 7.5 nM of RA isomers and 10 nM of 4oxo-RA metabolites spiked into blank serum. The LLOQ in serum was determined by spiking 20 ml of blank serum with RA isomers and RA metabolites at concentrations of 0.05 nM (RA isomers) or 6 nM (metabolites). Aliquots of the QC samples were frozen and analyzed on three separate days. Due to their poor extraction recovery, the LLOQ (5 nM spiked in blank serum) of 4OH-*at*RA and 4OH-9-*cis*RA was determined after precipitation of serum proteins with acetonitrile, centrifugation of precipitated proteins, and direct analysis of the supernatant as described above for retinoid extraction.

To determine whether matrix effects altered the ionization and detection of retinoids in serum, four serum samples were extracted and reconstituted in duplicate with 50 μl of 60:40 ACN/H_2O mixture containing 5 nM and 10 nM 13-*cis*RA-d_5. In parallel, the same ACN/H_2O mixture with 5 nM and 10 nM 13-*cis*RA-d_5 was added into glass tubes with no extracted samples. After a quick vortex, all samples were transferred into amber vials and analyzed by LC-MS/MS. The response was quantified and compared between the matrix containing samples and the clean standards.

Subjects

Serum samples were collected from 20 healthy men between the ages 18 and 65 who were enrolled in a study examining the relationship between retinoids and spermatogenesis. The study was approved by the Institutional Review Board at the University of Washington, and all subjects gave written informed consent before any study procedures. All subjects exhibited normal blood chemistry, liver function, hematology, and hormones. Subjects were excluded from the study if they used anabolic steroids or illicit drugs, ingested more than four alcoholic drinks a day, or were being treated with ketoconazole, finasteride, dutasteride, methadone, or lithium. The first set of blood was collected between 8 AM and 12 PM during a fed state within 2–6 h of breakfast. The second blood draw occurred at least 7 days after the first one, and blood was collected between 8 AM and 12 PM after an overnight fast of at least 8 h. Blood samples were collected from the subject's antecubital vein, and blood tubes were immediately wrapped in aluminum foil to minimize light exposure (light-protected samples). The blood was allowed to clot at 4 °C and then spun for 20 min at 3,000 *g*. Serum was aliquoted into amber sample tubes and stored at –80 ° C until analysis. Samples were also collected without the aluminum foil wrapping and light-protected vials. Five of these samples were extracted and analyzed together with the light-protected samples.

Results are expressed as mean ± SD. All statistical analysis was done using GraphPad Prism (La Jolla, CA). Due to nonnormality, comparisons of retinoids between the fasting and fed states were performed using a Wilcoxon signed-rank test. Correlation between data sets was tested with linear regression. For all comparisons, a *P* value of 0.05 was considered significant.

Analysis of the human serum samples and confirmation of analyte identity

All the serum samples were analyzed using the described method. The identities of the quantified retinoids were confirmed by collecting MS/MS spectra of each analyte. For this analysis, four serum samples were extracted with hexanes as described above, and the hexane phases were combined. After drying under nitrogen flow, the sample was reconstituted in 50 μl of 40:60 H_2O:ACN, and 20 μl was injected into the UHPLC-MS/MS. The UHPLC conditions were identical to those described for quantitative analysis, and the same MS/MS parent-fragment pairs used for quantification were recorded to detect the analytes and trigger MS/MS spectrum acquisition. Once the signal for the MS/MS transition exceeded a threshold, a fragment ion scan for the same parent ion was triggered using positive ion APCI and with collision energy spread of 15 from a set value of 35. A dynamic fill time, which allows for the maximum amount of ions to be collected in the linear ion trap for best sensitivity, was used to collect the fragment ion spectra.

To confirm that each quantified peak for the detected RA isomers in serum represented only a single compound, two

independent MS/MS transitions from the extracted serum samples were monitored, and the response ratio across the peak was recorded. If a peak consists of two compounds, the ratio between the two transitions usually changes across the peak. Both transitions used parent ion *m/z* 301, and the fragment ions monitored were *m/z* 205 and *m/z* 123. These fragment ions were chosen based on their signal-to-noise ratios from spiked serum and retinoic acid standards. The MS parameters for the *m/z* 301 > 123 transition were DP:62, CE:23, EP:10, CXP:14. The *m/z* 301 > 123 MS/MS fragmentation of RA is most likely a result of a cleavage of the bond between carbons C6 and C7, resulting in the β-ionone-ring fragment with an *m/z* 123 as shown previously (32). The structure of the *m/z* 301 > 205 fragment could not be assigned due to a likely rearrangement of the retinoid structure during mass spectrometry. However, a corresponding fragment at *m/z* 306 > 210 was detected from RA-d_5, showing that this fragment also retained the β-ionone-ring (data not shown). The corresponding fragments (*m/z* 301 > 205 and *m/z* 305 > 209) were detected previously from *at*RA and $^{13}C_4$-*at*RA, respectively, providing additional confidence for the reproducibility of this fragmentation (32).

Despite extensive efforts, 11-*cis*RA could not be separated chromatographically from the 9,13-*dicis*RA. To determine whether the retinoid detected in human serum was 9,13-*dicis*RA or 11-*cis*RA, palladium(II)nitrate was used to convert 11-*cis*RA to *at*RA according to a published method (33). Four light-protected serum samples were pooled to obtain a total volume of 2.5 ml of serum. 13-*cis*RA-d_5 was spiked into the sample to a final concentration of 20 nM as an internal standard. A 1.1 ml aliquot was removed from the pooled sample, and 5 nM 11-*cis*RA was spiked into the sample. Two 500 μl aliquots were collected from the 11-*cis*RA spiked serum and from the remaining pooled control serum. The aliquots were extracted as described for serum samples. All four of the samples were reconstituted in 500 μl ACN. A solution containing a 1:4.8 ratio of palladium(II)nitrate to Triethylamine in ACN was added to one sample with 11-*cis*RA spiked in and to one sample without added 11-*cis*RA. The final Palladium(II)nitrate to retinoic acid molar ratio was approximately 50,000:1. The reaction was allowed to proceed at 50°C for 5 h in the dark, after which the ACN was removed under a stream of dry nitrogen. The samples were reconstituted in ethyl acetate and washed twice with water to remove the Palladium reagent. Samples were then dried again under nitrogen and reconstituted in 50 μl of a 60:40 ACN/H_2O solvent.

RESULTS

Optimization of chromatographic separation and MS/MS detection of retinoids

The ionization and fragmentation of the retinoids were evaluated using electrospray and APCI in both the positive and negative ion modes. The negative ion mode provided approximately 10-fold lower sesitivity than the positive ion mode (data not shown); hence, the positive ion mode was chosen for further evaluation. The fragmentation and specific MS/MS ion transitions (SRM transition) for each RA isomer, for the isotope-labeled internal standards, and for the metabolites were optimized for maximum sensitivity. The MS/MS fragmentation patterns of the protonated molecules ($[M+H]^+$s) for representative RA, 4oxo-RA, and 4OH-RA isoform are shown in **Fig. 1**. The protonated molecules ($[M+H]^+$s) of 4OH-*at*RA and 4OH-9-*cis*RA were not stable enough in the positive ion mode to isolate for MS/MS analysis. This was due to the apparent loss of water (−18) from the 4OH-RA $[M+H]^+$ protonated molecule (*m/z* 317) resulting in a base peak at *m/z* 299. In negative ion mode, an $[M-H]^-$ ion could be detected for the 4OH-RA compounds, confirming that they were stable during chromatography and unstable in the mass spectrometer (data not shown).

To achieve separation of the five RA isomers (*at*RA, 9-*cis*RA, 9,13-*dicis*RA, 11-*cis*RA, and 13-*cis*RA), 4OH-RA isomers, and 4oxo-RA isomers, numerous C18 and C8 columns with UHPLC capability were evaluated as well as several chiral columns with mobile phases, including methanol, water, acetonitrile, acetic acid, and formic acid. Significant differences in the separation capacity between the different columns was observed, and in most columns the 13-*cis*RA and 9,13-*dicis*RA were not separated, whereas separation between 13-*cis*RA and *at*RA was obtained in virtually all columns tested. Baseline separation of *at*RA, 9-*cis*RA, 9,13-*dicis*RA, and 13-*cis*RA was achieved only by using the amide column described, and separation of the oxidized RA metabolite isomers was satisfactory (**Fig. 2**). The 11-*cis*RA isomer could not be separated from the 9,13-*dicis*RA regardless of the analytical conditions (vide infra). Optimal separation of the oxidized metabolite isomers was achieved using a C18 column, but the separation achieved using the amide column was sufficient for quantification.

Selectivity of the method for endogenous retinoids

Initially, the five MS/MS parent-fragment pairs with the greatest signal magnitude were chosen for the assay, and each of these SRM transitions (MS/MS parent-fragment pairs monitored) was tested in blank human serum for the existence of interference from the matrix. For several of the mass transitions, endogenous interference was detected, including *m/z* 301 > 161, *m/z* 301 > 159, *m/z* 301 > 91, and *m/z* 301 > 105 for RA isomers; *m/z* 299 > 91, *m/z* 299 > 128, and *m/z* 299 > 115 for 4OH-RA; and *m/z* 315 > 120 for 4oxo-RA. The *m/z* 315 > 297 MS/MS transition was not considered for quantification of 4oxo-RA due to the lack of specificity of a loss of water fragment. An alternative fragment that reduced interference from the matrix was chosen for quantitative analysis for each analyte as summarized in Table 1. Due to matrix interference, fragments with the highest abundance were generally not the MS/MS transitions used for final analysis. Close to the retention time of *at*RA-d_5, a significant interfering peak was detected (Fig. 2E) at the relevant SRM channels of *m/z* 306 > 131, *m/z* 306 > 116, *m/z* 306 > 210, *m/z* 306 > 96, and *m/z* 306 > 154 at 13.9 min. Although the interference somewhat separated from *at*RA-d_5 (Fig. 2C and E), its abundance interfered with the quantification of *at*RA-d_5. Therefore, 13-*cis*RA-d_5 was chosen as the internal standard for RA isomers (Fig. 2E). No interference was detected at the retention time of 13-*cis*RA-d_5.

Validation of the method for serum samples

The on-column LLOD and LLOQ for clean standards for each analyte are shown in **Fig. 3**. Standard curves of *at*RA, 9-*cis*RA, 13-*cis*RA, 4oxo-*at*RA, and 4oxo-13-*cis*RA were

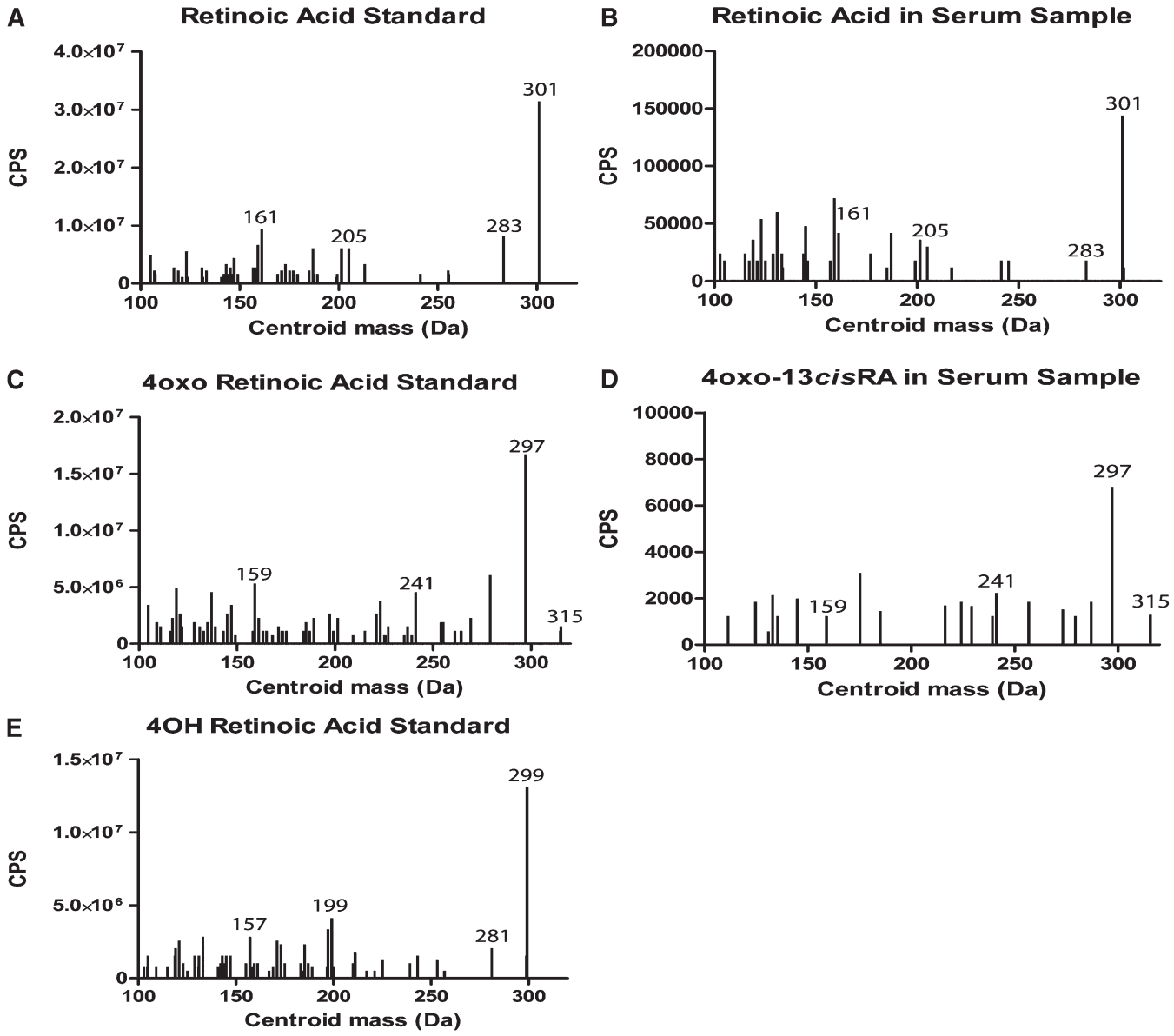


Fig. 1. Representative MS/MS spectra of RA, 4oxo-RA, and 4OH-RA standards and of RA and 4oxo-RA detected in serum samples. The MS/MS spectra of RA isomers and metabolites as clean standards and from serum samples were collected as described in Materials and Methods. The MS/MS spectra representative of all detected isomers are shown for *at*RA (A), 4oxo-13-*cis*RA (C), and 4OH-*at*RA (E) standards. Similar MS/MS spectra were generated for each quantified retinoid in a sample of pooled human serum, and representative MS/MS spectra for *at*RA (B) and for 4oxo-13-*cis*RA (D) are shown.

generated in blank human serum and extracted along with the study samples. The generated standard curves were linear, and the equations with r^2 values are shown in **Table 2**. The validation data at the LLOQ for serum (0.05 nM for RA isomers and 6 nM for 4oxo-13-*cis*RA) and at the mid to high concentration (7.5 nM for RA isomers and 10 nM for 4oxo-13-*cis*RA) are shown in Table 2. All of the measured concentrations were within 15% of the true value (accuracy) for all analytes at the mid-high QC and within 20% of the true value at LLOQ. The LLOD in serum for 4OH-RA isomers was 2 nM when the samples were analyzed after protein precipitation with acetonitrile. The retention times of the analytes were reproducible between days and within days with variability in the retention times, being <0.5% for RA isomers and <0.8% for the metabolites.

A lack of significant ion suppression or other matrix effects on retinoid detection was determined by comparing the peak areas for 13-*cis*RA-d_5 standard without matrix and 13-*cis*RA-d_5 in the sample spiked after extraction (100% recovery) at three different concentrations. The matrix-containing samples were not different in terms of peak area or retention time from the samples without matrix.

The effect of light exposure during sample collection to retinoid measurements was tested by comparing measurements from light-protected samples with measurements in samples that were not collected in light-protected conditions. All of the samples showed significant decreases (≈70%) in the concentrations of RA isomers, demonstrating that protecting the samples from UV light already during sample collection is critical. No decrease in the concentrations of

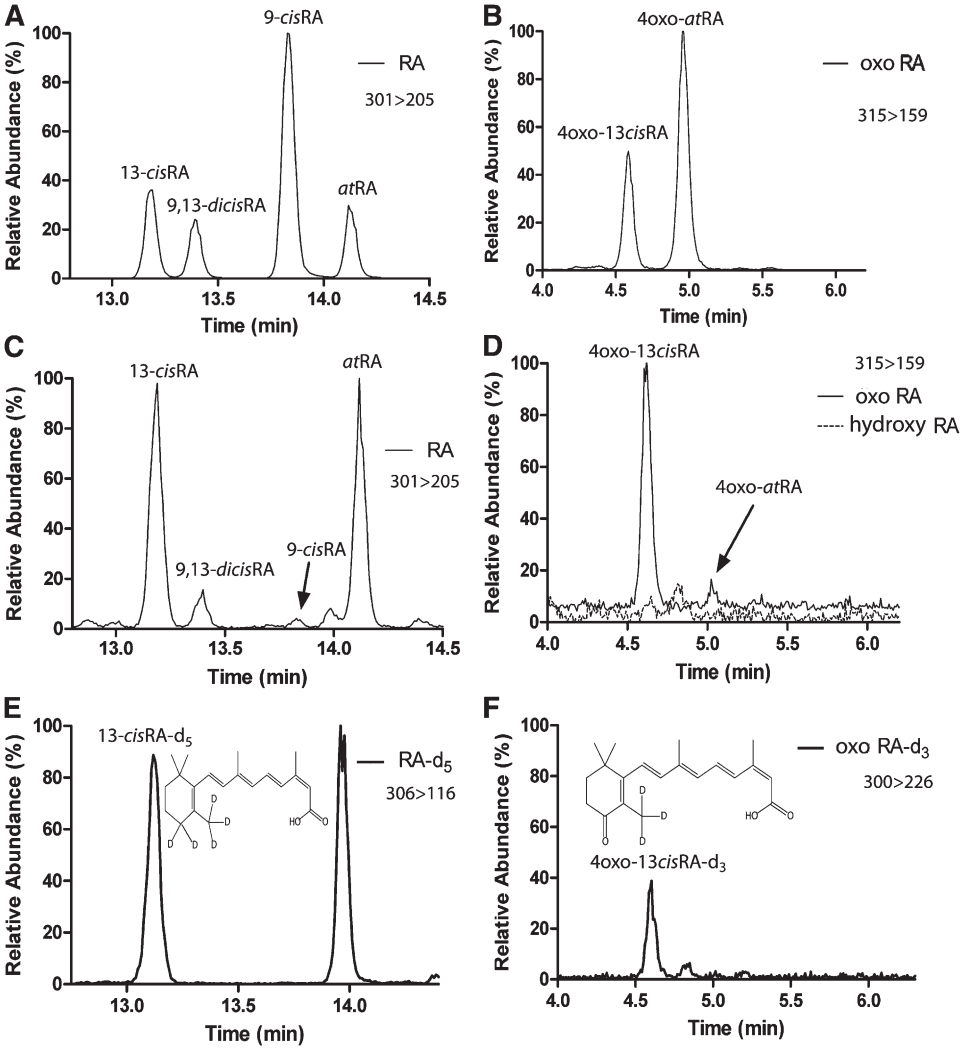


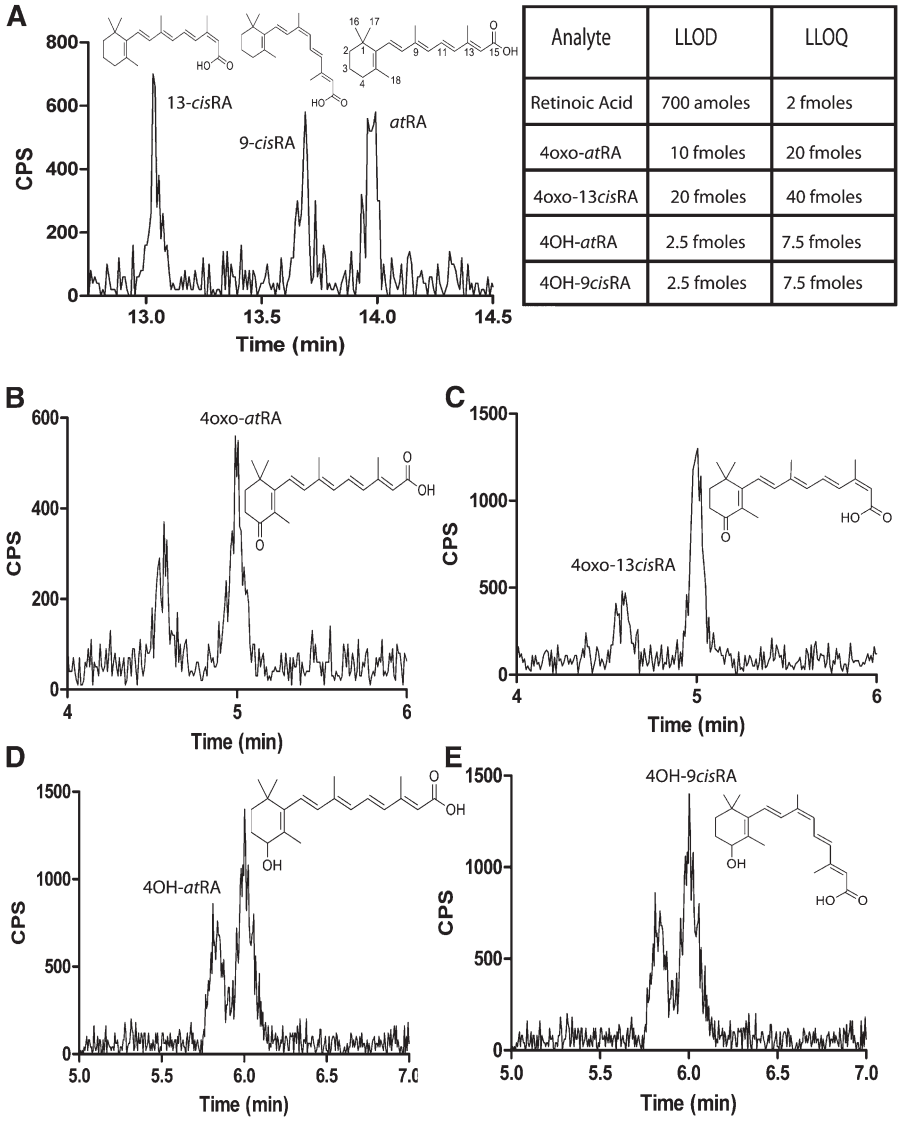
Fig. 2. Separation of retinoic acid and 4oxo-RA isomers and detection of the retinoids and labeled internal standards in serum. Separation of *at*RA, 9,13-*dicis*RA, 9-*cis*RA, and 13-*cis*RA standards (A) and detection of the same isomers in serum (C). (B) Separation of 4oxo-*at*RA and 4oxo-13-*cis*RA and (D) detection of these compounds in serum. The dotted line in panel (D) also shows the lack of detection of 4OH-RA in extracted serum. All serum samples were prepared by liquid-liquid extraction and analyzed as described in Materials and Methods. (E) Detection of 13-*cis*RA-d_5 internal standard (at 13.2 min) extracted from serum together with the structure of the labeled compound. The peak at 13.9 min is not a retinoid but a matrix peak. (F) Detection of 4oxo-13-*cis*RA-d_3 internal standard in serum together with the structure of the internal standard. Panels E and F also show the lack of isomerization of the internal standards in serum. The insets on each panel specify the MS/MS transition used for the specific analyte.

4oxo-13*cis*RA was detected in response to light exposure (data not shown).

Detection and identification of endogenous retinoids in human serum

The detection and separation of the endogenous RA isomers in serum is shown in Fig. 2C and 2D, with the identified retinoids indicated. As shown in **Fig. 4C** with 11-*cis*RA spiked serum, 11-*cis*RA could not be separated from 9,13–*dicis*RA. However, treatment of serum with palladium(ii) nitrate converted the spiked 11-*cis*RA quantitatively to *at*RA (Fig. 4D). In the nonspiked sample (Fig 4A, B), no decrease in the 9,13-*dicis*RA peak area or increase in *at*RA peak area was detected, confirming that 11-*cis*RA is not present in human serum at quantifiable concentrations.

The identitiy of each detected retinoid was confirmed by obtaining an MS/MS spectrum of the compound in serum (Fig. 1). There were no detectable differences between the MS/MS spectra of 13-*cis*RA, 9,13-*dicis*RA, *at*RA, and 9-*cis*RA or between the 4oxo-13-*cis*RA and 4oxo-*at*RA. Analysis of the human serum samples allowed collection of an MS/MS spectrum for each RA isomer, confirming the identity of the detected analytes. A representative MS/MS spectrum from human serum is shown in Fig. 1B. Additionally, the fragmentation for the endogenous compound 4oxo-13-*cis*RA (Fig. 1D) was similar to the standard. Peak purity was confirmed by monitoring two separate SRM transitions for the RA isomers. The difference in the ratio between the two transitions (m/z 301 > 205 and m/z 301 > 123) remained constant across each peak in the standard and analyte in serum.



Analyte	LLOD	LLOQ
Retinoic Acid	700 amoles	2 fmoles
4oxo-*at*RA	10 fmoles	20 fmoles
4oxo-13*cis*RA	20 fmoles	40 fmoles
4OH-*at*RA	2.5 fmoles	7.5 fmoles
4OH-9*cis*RA	2.5 fmoles	7.5 fmoles

Fig. 3. Chromatograms of the detected retinoids as standards at determined LLOD. The on-column LLOD and LLOQ values for each compound are shown in the inset table. All retinoids were measured using the MS/MS transitions summarized in Table 1, and the corresponding SRM channels (*m/z* 301 > 205 for RA isomers, *m/z* 315 > 159 for 4oxo-RA isomers, and *m/z* 299 > 157 for 4OH-RA isomers) are shown.

The hydroxylated metabolites 4OH-9-*cis*RA and 4OH–*at*RA were not detected in the samples extracted and analyzed using the method optimized for RA isomers (Fig. 2). To determine whether this was due to poor extraction recovery of 4OH-RA isomers, serum was analyzed after precipitation of proteins with ACN followed by centrifugation. The 10 serum samples analyzed had no quantifiable levels of 4OH-9-*cis*RA and 4OH-*at*RA (**Fig. 5**), showing that the concentrations of these metabolites, if they are present in human serum, are <2 nM. It is possible that the two peaks detected in the serum (Fig. 5C) are 4OH-RA isomers, but this could not be confirmed because experiments to obtain MS/MS spectra on these peaks were unsuccessful.

Quantification of retinoic acid isomers and metabolites in human serum

Serum samples collected after an overnight fast and 2–6 h after normal breakfast from 20 healthy men were analyzed to determine the normal concentrations of endogenous RA isomers and metabolites in human serum. All four RA isomers and 4oxo-13-*cis*RA were detected in all samples. 4oxo-*at*RA could also be detected in 18 of the 40 samples, but the concentrations were below LLOQ. The concentration of 4oxo-13-*cis*RA could not be quantified in one subject at the fed state and in another subject in the fasted state, 9-*cis*RA could not be quantified in one sample from fed state, and 9,13-*dicis*RA could not be quantified in samples from two subjects in the fasted state because concentrations were below the LLOQ. The mean concentrations of the four RA isomers and 4oxo-13-*cis*RA are shown in **Table 3**, and a box and whiskers plot representing the measured concentrations for each retinoid in the 20 volunteers is shown in **Fig. 6**. The most abundant RA isomer in human serum was 13-*cis*RA, although *at*RA concentrations were only about 30–40% lower. The concentrations of *at*RA and 13-*cis*RA were about 10-fold higher than

TABLE 2. Validation data for the analyzed retinoids

Analytes	Equation for Calibration Curve	r^2	LLOQ (intraday, interday CV%)[a]	Middle LOQ (intraday, interday CV%)[b]
*at*RA	Y = 1.67x − 0.12	0.995	5.4, 15.0	3.6, 6.9
9-*cis*RA	Y = 1.43x − 0.18	0.996	14.3, 19.6	2.1, 4.9
13-*cis*RA	Y = 1.96x + 0.18	0.994	7.3, 13.7	3.2, 3.3
4oxo-*at*RA	Y = 2.41x + 0.55	0.982	15.8, 17.9	4.0, 13.8
4oxo-13*cis*RA	Y = 7.64x − 0.42	0.997	10.8, 18.8	6.5, 6.7

[a] The LLOQs were determined at 0.05 nM RA isomers and 6 nM 4oxo-RA isomers.
[b] The LOQs were determined at 7.5 nM RA isomers and 10 nM 4oxo-RA isomers.

9,13-*dicis*RA concentrations and 30- to 50-fold higher than 9-*cis*RA concentrations. Two of the RA metabolites (4oxo-13-*cis*RA and 4oxo-*at*RA) were also detected in human serum, and the concentrations of 4oxo-13-*cis*RA exceeded those of 13-*cis*RA by 3-fold, making this compound the most prevalent retinoid measured. No statistically significant differences ($P > 0.05$) were found between the fed and fasted states for any of the detected analytes (Table 3).

Linear regression analysis was used to determine whether correlations exist between the serum concentrations of the detected analytes. There was a significant correlation between the fed and fasted concentrations of 13-*cis*RA ($P < 0.001$), 9,13-*dicis*RA ($P < 0.001$), and 4oxo-13-*cis*RA ($P < 0.001$), whereas the concentrations of *at*RA and 9-*cis*RA did not correlate between the fed and fasted states ($P > 0.05$). In the fed state, there were significant correlations between 9-*cis*RA and 13-*cis*RA ($P < 0.001$), between 9-*cis*RA and 9,13-*dicis*RA ($P < 0.001$), between 13-*cis*RA and 9,13–*dicis*RA ($P < 0.05$), and between 13-*cis*RA and 4oxo-13-*cis*RA ($P < 0.05$). No correlation ($P > 0.05$) between *at*RA concentrations and any of the other retinoids was found in the fed state and in the fasted state *at*RA concentrations correlated only with 9-*cis*RA ($P < 0.01$). In addition, in the fasted state, similar to the fed state, there were significant correlations between 9-*cis*RA and 13-*cis*RA ($P < 0.001$), between 9-*cis*RA and 9,13-*dicis*RA ($P < 0.05$), and between 13-*cis*RA and 9,13-*dicis*RA ($P < 0.05$) concentrations.

DISCUSSION

The described UHPLC MS/MS method is to date the most sensitive method to selectively monitor bioactive retinoids. The selectivity for RA isomers and metabolies is achieved using a combination of UHPLC, specfic solvents, and an amide column that provides the specific separation capacity needed for each isomer. Surprisingly, positive ion APCI ionization produced the best signal-to-noise ratio for the RA isomers despite the readily ionizable carboxylic acid group in the molecule. However, the RA metabolites exhibit better sensitivity using electrospray ionization and negative ion detection. For the quantification of the retinoids in serum, positive ion APCI was chosen because matrix interference was observed using electrospray

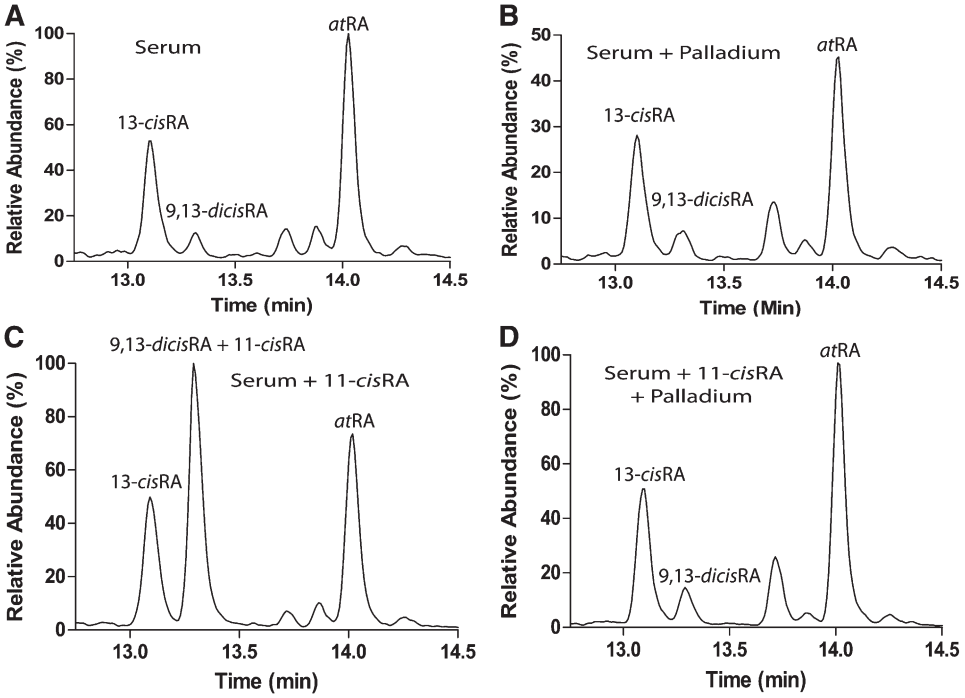


Fig. 4. Evaluation of the presence of 11-cisRA in serum. (A) Light-protected serum sample without the addition of 11-*cis*RA and palladium(II)nitrate extracted as described in Materials and Methods. (C) Chromatogram of an aliquot of the same extracted sample spiked with 11-cisRA. Fractions of both samples were treated with palladium(II)nitrate. (B and D) Chromatograms of the serum samples after the reaction. No decrease in 9,13-*dicis*RA peak between panel A and B is detected, and no increase in *at*RA peak suggesting that 11-*cis*RA was not present in serum. In the control reaction, the spiked 11-*cis*RA was quantitatively converted to *at*RA (panel C vs. D).

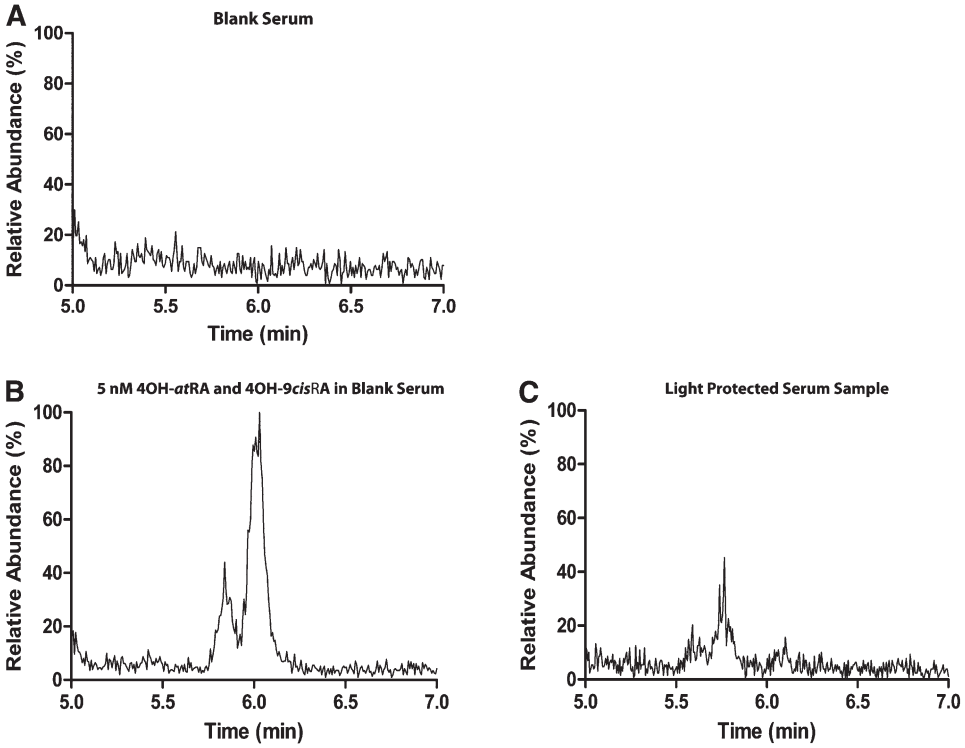


Fig. 5. Detection of 4OH-RA isomers in human serum using an ACN protein precipitation and centrifugation for sample preparation. Blank serum (A), 5 nM 4OH-*at*RA and 4OH-9-*cis*RA spiked into blank serum (B), and a representative human serum sample (C) are shown. The peak at 5.75 min in panel C is likely 4OH-*at*RA, but the peak area is below LLOQ for this compound.

ionization with negative ion, and limited amount of biological samples may require a single analysis for all relevant bioactive retinoids. The best sensitivity for retinoid detection was obtained using a liquid-liquid extraction with hexanes, and the method was validated for serum using this extraction method. However, the recovery of the hydroxylated metabolites is poor using the extraction method, and a direct protein precipitation with acetonitrile is recommended for analysis of hydroxylated metabolites of RA. A similar extraction method as shown here for serum samples has been previously used for detection of *at*RA, 13-*cis*RA, and 9,13-*dicis*RA in mouse tissues, and hence the sample preparation is expected to be applicable for tissue analysis (9). However, for any given tissue, especially the eye, a confirmatory analysis should be conducted to determine the potential presence of 11-*cis*RA and 9,13-*dicis*RA in the samples. In addition, special attention should be paid to the potential interfering compounds in the matrices analyzed. As demonstrated here, significant interference is present in many of the SRM transitions examined, and it is expected that abundance and identity of interfering matrix compounds varies between tissues. Because blank tissues are generally unavailable for retinoid analysis, simple transfer of a method validated for one tissue to another should be exercised with caution.

TABLE 3. Concentrations of the quantified retinoids in serum from 20 healthy men

Analyte	Mean ± SD (nM)	Minimum (nM)	Median (nM)	Maximum (nM)	Wilcoxon Signed-Rank TestFed vs. Fast *P* value
*at*RA					
Fed	3.1 ± 0.9	1.7	2.9	5.2	0.82
Fasted	3.0 ± 0.6	1.9	3.1	4.4	
13-*cis*RA					
Fed	5.3 ± 5.7	2.4	3.9	29.0	0.27
Fasted	3.9 ± 1.0	2.7	3.8	7.3	
9-*cis*RA					
Fed	0.1 ± 0.1	0.05	0.09	0.49	0.14
Fasted	0.08 ± 0.02	0.05	0.09	0.15	
9,13-*dicis*RA					
Fed	0.4 ± 0.4	0.1	0.3	1.8	0.09
Fasted	0.3 ± 0.1	0.1	0.2	0.7	
4oxo-13*cis*RA					
Fed	17.8 ± 31	7.1	10.2	146	0.59
Fasted	12.2 ± 7.2	6.8	10.7	40.4	

One advantage of the described method is the use of isotope-labeled internal standards for quantification of endogenous RA and its metabolites. The use of deuterium-labeled internal standards is important because they are likely to mimic the extraction efficiency of RA isomers and metabolites. The results of this study show that the extraction characteristics of retinoids, even within a closely chemically related group (e.g., RA, 4oxo-RA, and 4OH-RA), can be very different, and hence using a chemical analog as an internal standard may confound quantification results. In addition, binding of the analytes to cellular retinoic acid-binding proteins and fatty acid-binding proteins may affect recovery and may be corrected by isotope labeled internal standards. Finally, a significant advantage for the use of deuterated retinoids as internal standards is that it allows monitoring of isomerization during sample

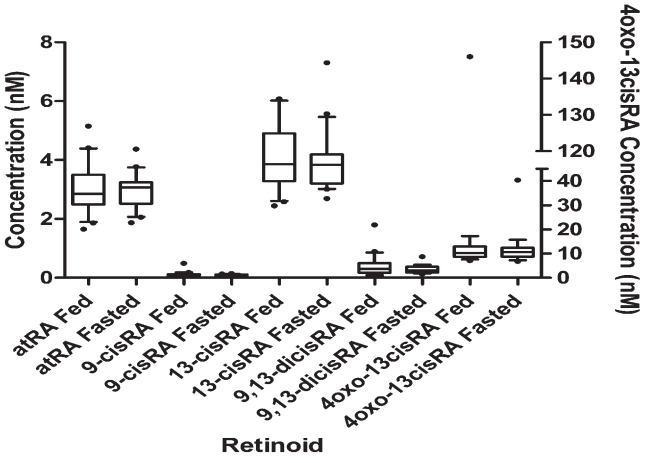


Fig. 6. Box and whiskers plots for endogenous retinoid concentrations in serum. The box represents the 25th and 75th percentiles of each group. The whiskers are determined based on 10th and 90th percentiles, and outliers are calculated as 1.5 times the mean of the sample set. All of the retinoid concentrations, with the exception of 4oxo-13-*cis*RA, are plotted against the left *y* axis. The 4oxo-13-*cis*RA concentrations are plotted against the right hand *y* axis.

preparation and analysis. When serum samples were exposed to light in this study, isomerization of the internal standard could be detected (data not shown). Deuterated standards can also be added into the samples during sample collection to control for stability during storage and freeze-thaw. Despite the fact that only one isomer internal standard was used in this study, one may choose to add individual isomer standards to samples to confirm the retention times of individual analytes.

The retinoid concentrations reported here are likely to represent average concentrations in healthy adult humans. Previous studies monitoring endogenous retinoid concentrations in the serum of men and women have not noted any differences between the two (24, 25). The observed concentrations of *at*RA are lower and concentrations of 4oxo-13-*cis*RA are higher than what has been previously reported. The endogenous concentrations measured in this study are approximately 40% lower for *at*RA and about 80% higher for 4oxo-13-*cis*RA than previously reported (24, 25). This may be due to improved assay selectivity in comparison to previous studies that used LC-UV, which may not have separated all RA and its metabolite isomers, and matrix interference. As shown in this study, a significant interference was detected eluting close to *at*RA, but it was mainly detected at the mass transitions relevant for *at*RA-d_5. However, this interference would likely confound quantification in LC-UV assays.

The fact that 9-*cis*RA can be detected in human serum is of interest because 9-*cis*RA activates RAR and RXR receptors and may have specific biological functions. In previous studies, endogenous 9-*cis*RA has not been detectable in human or mouse serum (8, 9, 29), and dosing with alitretinoin (9-*cis*RA) was required to detect 9-*cis*RA in human serum (8). However, 9-*cis*RA was quantifiable in all but one of the samples in this study, most likely due to the increased sensitivity of the UHPLC/MS/MS method. The identity of the detected 9-*cis*RA was confirmed by obtaining an MS/MS spectrum of the detected 9-*cis*RA. This detection of 9-*cis*RA is unlikely to be an artifact of the assay or a result of isomerization because no isomerization of the internal standards was detected and because the ratio between isomers did not change when samples were reanalyzed. In addition, 9-cisRA was detected in the samples after ACN precipitation as well as after extraction, providing additional support for 9-*cis*RA being present in human serum. The 9-*cis*RA has also been detected in mouse pancreas and has been shown to regulate glucose homeostasis (3).

The ratio of RA isomers determined in human serum was different than what has been previously reported in mouse serum (9). In mouse serum, *at*RA was the most abundant retinoid, with 9,13-*dicis*RA being present at similar concentrations. The concentrations of 13-*cis*RA were much lower than the two other detected retinoids. In contrast, in human serum 9,13-*dicis*RA was a minor circulating species, and 13-*cis*RA was the most abundant retinoid. This demonstrates a significant species difference between the isomers. It is unlikely that the observed difference is due to isomerization in the two studies because the extraction methods were similar and both studies used LC-MS/MS. In addition, analysis of the human serum samples using the ACN precipitation protocol decribed here resulted in an identical ratio of RA isomers as seen with the extraction method. The precipitation method eliminates the potential for isomerization, which may result from the extraction process and which requires multiple pieces of glassware and the addition of acid. It is likely that the species difference is due to differences in the age and/or diet of the mice versus humans. The biological importance of this interspecies variability is not known, and further studies in species- and age-dependent changes in retinoid profiles are warranted.

The results presented show no significant differences in retinoid serum levels after at least 8 h of fasting despite the fact that a previous study discovered decreasing concentrations of *at*RA and 13-*cis*RA during fasting (25). However, the previous study was conducted after a 5-day fast, suggesting that the length of the fasting period has an effect on the retinoid concentrations. One of the subjects in this study showed extremely high levels of 13-*cis*RA and 4oxo-13-*cis*RA compared with the mean. It was confirmed that this individual was not taking exogenous retinoids during the study. Although he was administered prostaglandin F2α eyedrops, it is unlikely that these eyedrops affected 13-*cis*RA concentration in serum. The possibility of 13-*cis*RA and 4oxo-13-*cis*RA contamination in the samples is low because his serum concentration was measured multiple times on different days. In studies involving isotretinoin treatment (80 mg m^{-2} bd), the average concentration of 4oxo-13-*cis*RA was calculated to be approximately 4.7 μM (7), which is over 100-fold higher than the concentrations observed in this study. In a previous study, the effect of diet on *at*RA levels in serum was tested, and *at*RA levels were shown to increase 100% after the consumption of carrot juice (34). However, no large

increases in 13-*cis*RA concentrations were reported. The lack of correlation between *at*RA concentrations and the concentrations of other RA isomers suggests that measurement of at least *at*RA and 13-*cis*RA separately is necessary to characterize an individual's RA status. Because the different isomers have different pharmacological activity, separation of *at*RA and 13-*cis*RA from the other isomers is important in biological assays.

The 4oxo-*at*RA was detectable in over half of the human serum samples, but the concentrations were too low to be quantified. In agreement with published results (24, 25), 4oxo-13-*cis*RA was the retinoid with highest concentration in human serum. 4oxo-*at*RA has properties that overlap with *at*RA, such as activation of RARβ, but it also shows weak activation of RXRα (35). In addition, treatment of MCF7 breast cancer cells with 4oxo-*at*RA results in inhibition of proliferation (12), demonstrating that 4oxo-RA isomers may contribute to the biological activity of RA. Due to the activity of the 4oxo-RA compounds, future studies, including accurate quantification in tissues, are needed to determine their overall biological importance.

The significantly higher concentrations of 4oxo-13-cis-RA in comparison to 4oxo-*at*RA are unexpected, based on the similar levels of *at*RA and 13-*cis*RA in serum. Pharmacokinetic studies to determine the clearance of *at*RA, 4oxo-*at*RA, 13-*cis*RA, and 4oxo-13-*cis*RA are needed to better understand the reason for the large difference in the ratios between the RA isomers and their corresponding 4oxo-metabolites. These studies would need to include monitoring glucuronidation of the retinoids in serum and urine and the determination of accurate clearance values. Although the 4OH-RA preferentially is glucuronidated at the hydroxy position, RA and 4oxo-RA are known to undergo glucuronidation at the carboxyl function (26).

In conclusion, a UHPLC-MS method of retinoid measurement in serum was developed and validated. The method enabled quantification of four major RA isomers in serum as well as quantification of 4oxo-13-*cis*RA. The use of isotope labeled internal standards and the careful evaluation of matrix interference provided increased confidence for the quantification of the important retinoids. The developed method can be used in future studies to correlate specific retinoid concentrations in human tissues to pharmacological effects and in evaluating the relationships between disease states and retinoid concentrations.

The authors thank Drs Hua-Fen Liu and Loren Olson from AB Sciex, Foster City, CA, for their help in the initial method development and Dr. Jakob A. Shimshoni for his contribution to initial method development and compound synthesis.

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