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Quantitation of retinaldehyde in small biological samples using ultrahigh-performance liquid chromatography tandem mass spectrometry

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ABSTRACT

We report an ultrahigh-performance liquid chromatography tandem mass spectrometry (UHPLC–MS/MS) method to quantify all-*trans*-retinal in biological samples of limited size (15–35 mg), which is especially advantageous for use with adipose. To facilitate recovery, retinal and the internal standard 3,4-didehydroretinal were derivatized in situ into their *O*-ethyloximes. UHPLC resolution combined with high sensitivity and specificity of MS/MS allowed quantification of retinal-*O*-ethyloximes with a 5-fmol lower limit of detection and a linear range from 5 fmol to 1 pmol. This assay revealed that extraocular concentrations of retinal range from approximately 2 to 40 pmol/g in multiple tissues—the same range as all-*trans*-retinoic acid. All-*trans*-retinoic acid has high affinity ($k_d \leqslant 0.4$ nM) for its nuclear receptors (RARα, -β, and -γ), whereas retinal has low (if any) affinity for these receptors, making it unlikely that these retinal concentrations would activate RAR. We also show that the copious amount of vitamin A used in chow diets increases retinal in adipose depots 2- to 5-fold relative to levels in adipose of mice fed a vitamin A-sufficient diet, as recommended for laboratory rodents. This assay also is proficient for quantifying conversion of retinol into retinal in vitro and, therefore, provides an efficient method to study metabolism of retinol in vivo and in vitro.

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The dioxygenase BCO1 catalyzes central cleavage of β-carotene and approximately 60 other carotenoids to generate the vitamin A (all-*trans*-retinol) precursor all-*trans*-retinaldehyde (retinal) [1,2]. Cellular retinol binding protein type 2 (Crbp2) sequesters retinal generated in the intestine and directs it to reduction into retinol, which is esterified and transported for storage, mostly in liver [3]. Generating the visual pigment chromophore 11-*cis*-retinal accounts for the quantitatively major use of vitamin A. The isomerohydrolase Rpe65 produces 11-*cis*-retinal from all-*trans*-retinyl esters in a concerted reaction [4]. 11-*cis*-Retinal forms a Schiff's base with rhodopsin to generate the protein opsin. Photoactivation of opsin isomerizes 11-*cis*-retinal into all-*trans*-retinal, which prompts a neuronal signal and release of all-*trans*-retinal. The released all-*trans*-retinal undergoes reduction into all-*trans*-retinol, which undergoes reesterification for reuse in the visual cycle. Thus, the eye consumes most of an organism's vitamin A through production of retinal isomers to support the visual cycle.

The aldehyde functional group of retinal isomers reacts readily in biological matrices to form Schiff's bases, which degrades extraction efficiency. To circumvent this impediment, retinal isomers have been routinely converted into oxime derivatives before extraction from tissues [5–7]. This generates *syn* and *anti* forms, not always in a consistent ratio. The *anti*-all-*trans*-retinal oxime coelutes with retinol and/or as a broad asymmetric peak. This issue has been resolved by converting retinal isomers in situ into their *O*-ethyloxime derivatives [8,9]. Resolution of retinal isomers has been achieved with both normal- and reverse-phase high-performance liquid chromatography systems, which have been applied mostly to evaluating retinal isomers in the visual cycle [10–14].

Retinal also serves as the intermediate for biosynthesis of the autocoid all-*trans*-retinoic acid (atRA)[2] from retinol [15,16].

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[2] *Abbreviations used:* atRA, all-*trans*-retinoic acid; UHPLC, ultrahigh-performance liquid chromatography; MS/MS, tandem mass spectrometry; LC, liquid chromatography; MS, mass spectrometry; IS, internal standard; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; APCI, atmospheric pressure chemical ionization; MRM, multiple reaction monitoring; HPLC, high-performance liquid chromatography; UV, ultraviolet; RA, retinoic acid; LOD, limit of detection; LOQ, limit of quantification; RAR, retinoic acid receptor.

Although this pathway seems quantitatively minor relative to vitamin A use in the visual cycle, all vertebrates require atRA for reproduction and embryonic development, growth, and throughout postnatal life to maintain neurological function, energy balance, and immune system function [17,18]. Although atRA has been established as the activated retinol metabolite that regulates the systemic functions of vitamin A [19], several other metabolites have been proposed to contribute to the systemic physiological actions of vitamin A. Relatively recently, a hypothesis has been proposed that all-*trans*-retinal has signaling actions distinct from atRA that attenuate weight gain in females through effects in adipose and liver [20]. All-*trans*-retinal concentrations in extraocular tissues occur in nanomolar (nM) levels [9]. Extraction and efficiency of oxime formation are relatively low in samples of adipose tissue large enough to quantify by liquid chromatography/ultraviolet. Therefore, we developed an assay based on ultrahigh-performance liquid chromatography tandem mass spectrometry (UHPLC/MS/MS) to quantify all-*trans*-retinal in small biological samples to facilitate study of its extraocular functions. This assay has the highest sensitivity reported to date for quantifying all-*trans*-retinal in multiple biologic media.

Materials and methods

Materials

Liquid chromatography/mass spectrometry (LC/MS)-grade acetonitrile, formic acid ($\geqslant$95%), all-*trans*-retinal ($\geqslant$98%), and O-ethylhydroxylamine ($\geqslant$97%) were purchased from Sigma–Aldrich. 3,4-Didehydro retinal was purchased from Toronto Research Chemicals. LC/MS-grade water and other solvents were purchased from Fisher Scientific. Retinoid standards were prepared on the day of use. Concentrations were determined spectrophotometrically using published ε values [21,22].

Animals and tissues

One group of male C57BL/6 mice (originally purchased from Jackson Laboratory) was fed an AIN93G diet with 4 IU/g vitamin A (retinyl palmitate) over 10 generations. Another group of male C57BL/6 mice (Jackson Laboratory) was fed a stock diet (Teklad Global 18% protein rodent diet, 15 IU vitamin A acetate/g). Tissues were dissected under yellow light and frozen in liquid nitrogen immediately after harvest. Samples were kept frozen at −80 °C until assay.

Retinal derivatization and extraction

Tissue samples were homogenized on ice using a Duall size 21 glass homogenizer (Kontes), either manually or with a Heidolph motorized homogenizer (280 rpm), in cold 0.9% saline to generate 10% homogenates. Serum was recovered by centrifuging clotted blood for 10 min at 10,000g (4 °C). After the addition of the internal standard (IS: 10 µl of a 50-nM solution of all-*trans*- 3,4-didehydroretinal), 1 ml of methanol and 2 ml of 0.1 M O-ethylhydroxylamine in 100 mM Hepes (pH 6.5) were added to the homogenates (typically 300 µl) or sera (100–200 µl) samples to convert retinals into their O-ethyl oxime derivatives. The resulting suspension was mixed thoroughly (vortex) and incubated for 20 min at room temperature. Retinal O-ethyloximes were extracted with 10 ml of hexane. After centrifuging, the hexane (upper) layer was transferred to a glass tube and evaporated under nitrogen with gentle heating at 25 to 30 °C in a water bath (model N-EVAP 112, Organomation Associates, Berlin, MA, USA). The residue was suspended in 40 µl of acetonitrile, and 5 µl was used for UHPLC–

MS/MS analysis. Typically, 15 to 35 mg of tissue or 100 to 200 µl of serum from adult mice generated reliable data.

Liver microsomes

Wild-type C57BL/6 mice fed a purified AIN93G diet ad libitum were sacrificed at 11 am. Liver was homogenized in a buffer of 10% sucrose, 10 mM Tris–HCl, 1 mM ethylenediaminetetraacetic acid (EDTA), and 1.5 mM dithiothreitol (DTT) at pH 7.4 using a Heidolph motorized homogenizer at 1240 rpm for 6 strokes, 8 to 10 s each, while on ice. The homogenate was centrifuged for 10 min at 2000g (4 °C). The resulting supernatant was centrifuged for 1 h at 100,000g (4 °C). The microsomal pellet was resuspended in homogenizing buffer and frozen.

Retinal biosynthesis assay

Assay buffer (20 mM Hepes, 150 mM KCl, and 2 mM EDTA, pH 7.4, with 2 mM freshly added DTT), protein, and retinol substrate (delivered at 1:1000 ratio in dimethyl sulfoxide) (total volume of 0.5 ml) were preincubated for 5 min at 37 °C in a water bath shaker at 65 rpm. NAD$^+$ and NADP$^+$ cofactors were added in assay buffer to a final concentration of 1 mM each. At the end of the assay, the IS was added and O-ethyloximes were formed by adding 1 ml of methanol and 1.5 ml of 0.1 M O-ethylhydroxylamine in 100 mM Hepes (pH 6.5).

External calibration

A stock standard solution was prepared by dissolving all-*trans*-retinal in absolute ethanol. Absorption was measured spectrophotometrically at 383 nm. Samples were diluted with ethanol to an absorbance value of approximately 0.5. Concentrations of stock solutions were calculated according to Bier's law using an ε value of 42,880 [21,22]. Retinal was converted into its O-ethyl oxime derivative by reaction with 0.1 M O-ethylhydroxylamine in 100 mM Hepes (pH 6.5) as described above. After hexane extraction, the organic phase was evaporated under nitrogen and the residue was dissolved in acetonitrile. The solution was diluted into a series of working solutions with acetonitrile and applied directly to UHPLC–MS/MS to generate a calibration curve.

Variation

To determine coefficients of variation, 1.2 g of liver was homogenized in 12 ml of saline. Then, 600 µl of 50 nM 3,4-didehydroretinal was added and the mixture was divided into 12 aliquots, 6 of which were assayed immediately. The other 6 aliquots were stored at −80 °C and then assayed individually over 6 consecutive days to determine interassay variation.

Recovery

Briefly, 10 µl of 500 nM 3,4-didehydroretinal was added to 1 ml of 10% liver homogenate or 1 ml of saline (6 repeats for each group). The samples were derivatized and analyzed as described above. Recovery was determined by comparing 3,3-didehydroretinal oxime measured in tissues with the amounts quantified in saline blanks.

Resolution

All-*trans*-retinal-O-ethyloxime or all-*trans*-3,4-didehydroretinal-O-ethyloxime was resolved via reverse-phase chromatography with an Agilent 1290 system (Santa Clara, CA, USA) equipped with