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Original Article

# A simple and robust quantitative analysis of retinol and retinyl palmitate using a liquid chromatographic isocratic method

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ABSTRACT

Vitamin A is a vital nutritional substances that regulates biological activities including development, but is also associated with disease onset. The extent of vitamin A intake influences the retinoid content in the liver, the most important organ for the storage of vitamin A. Measurement of endogenous retinoid in biological samples is important to understand retinoid homeostasis. Here we present a reliable, highly sensitive, and robust method for the quantification of retinol and retinyl palmitate using a reverse-phase HPLC/UV isocratic method. We determined the impact of chronic dietary vitamin A on retinoid levels in livers of mice fed an AIN-93G semi-purified diet (4 IU/g) compared with an excess vitamin A diet (1000 IU/g) over a period from birth to 10 weeks of age. Coefficients of variation for intra-assays for both retinoids were less than 5%, suggesting a higher reproducibility than any other HPLC/UV gradient method. Limits of detection and quantification for retinol were 0.08 pmol, and 0.27 pmol, respectively, which are remarkably higher than previous results. Supplementation with higher doses of vitamin A over the study period significantly increased liver retinol and retinyl palmitate concentrations in adult mice. The assays described here provide a sensitive and rigorous quantification of endogenous retinol and retinyl palmitate, which can be used to help determine retinoid homeostasis in disease states, such as toxic hepatitis and liver cancer.



## 1. Introduction

Vitamin A is essential for maintaining normal growth, development, vision, immunity, and neurogenesis [1]. Both retinoid excess and deficiency can have adverse effects, such as abnormal fetal development and disruption to energy balance regulation [2,3].

Some studies have examined the effects of excess vitamin A intake on serum and tissue retinoid levels in rodents, which were often fed standard laboratory rodent chow or were treated by injection [4–8]. However, vitamin A

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injection paradigms independent of ingestion are difficult for study compared with ingesting pellets containing high concentrations of vitamin A because vitamin A is easily absorbed during or after a meal. Standard laboratory rodent chow comprises natural ingredients, which vary in lot-to-lot reproducibility, and also contains copious amounts of nutrients, including vitamins. In contrast, a semi-purified diet provides a consistent nutrient composition in amounts that meet National Research Council recommendations for rodent nutrient intake, avoiding the delivery of excessive vitamins that can confound studies of nutrient functions. The American Institute of Nutrition (AIN) has determined that the minimum amount of vitamin A necessary for rodents is 2.9 IU/g diet, and recommends an intake of 4 IU/g diet to provide a margin of safety, such as the AIN-93G diet [9,10]. The upper limit of vitamin A consumption in humans is suggested as being no more than three times the recommended dietary allowance [11]. The amount of vitamin A in standard rodent chow diet is approximately five times (20 IU/g diet) that of the semi-purified AIN-93G diet recommended for rodents.

Quantifying circulating retinoid concentrations reflects whole-body vitamin A status, and can provide insights into retinoid homeostasis and metabolism. Being able to measure endogenous retinoid levels in serum and tissues is pivotal to elucidate the regulatory mechanisms that maintain retinoid homeostasis and, eventually, to overcome many pathological conditions and diseases associated with alterations in retinoid metabolism [12–15].

HPLC is one of the best analytical techniques available to assess and characterize retinoid concentrations in biological samples. However, the different chemical properties of retinoid metabolites do not allow for accurate quantification of each retinoid in a single chromatographic run [16]. In addition, levels of retinoic acid in biological samples are extremely low, thus requiring sophisticated methods for their accurate quantification [17,18].

Many assays have been developed to analyze retinol and retinyl ester levels in biological samples. Some previously described HPLC gradient methods focus on separation of the different molecular species [7,16,17,19,20].

In this study, we describe a reverse-phase HPLC isocratic method that allows for the simple separation and quantitation of retinol and retinyl palmitate and has high reproducibility and sensitivity. As an example of the application of the method, we analyzed the effects of chronic intake of excess dietary vitamin A on retinoid accumulation in the mouse liver, the most important organ for the storage of vitamin A.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Chemicals used, their purity grade, and the source of purchase were: methanol-HPLC grade (Cat. No. 21929-23), ethanol-HPLC grade (Cat. No. 14741-41), hexane-HPLC grade (Cat. No. 17929-53) from Nacalai Tesque (Kyoto, Japan); retinol (Cat. No. R7632-100MG), retinyl acetate (Cat. No. 46958) from Sigma Aldrich (St. Louis, MO, USA); and distilled water-HPLC grade (Cat. No. 042-16973) and retinyl palmitate (Cat. No. 188-01331) from Wako Chemicals (Osaka, Japan).

### 2.2. Animal and vitamin A supplementation experiments

Five pregnant ICR mice were obtained from CLEA (CLEA Japan, Inc., Tokyo, Japan). On the day of birth (day 0), the litter size was standardized to eight pups to avoid fluctuations in the pups' dietary intake. From gestation until weaning, all dams and their pups were fed AIN93G semi-purified diet (Oriental Yeast Co., Ltd., Osaka, Japan) containing sufficient vitamin A (4 IU/g diet) in the form of retinyl palmitate. After weaning on postnatal day 21, male mice were randomly assigned to AIN-93G diet (control) or modified excess vitamin A diet groups. The control group (two litters per dam) was maintained on the AIN93G semi-purified diet. The excess vitamin A group (two litters per dam) was changed to the AIN93G semi-purified diet containing modified excess vitamin A (1000 IU/g diet). The doses used were part of a study aimed to observe the effects of high vitamin A intake on leptin expression in mice adipose tissue samples [4] or on fatty acid composition of phospholipids in mice [21], as previously reported. Both groups were fed each diet for a period from 3 to 10 weeks of age in home cages at 23 ± 2 °C on a 12-h light/dark cycle (lights on from 8:00 to 20:00). Food and water were provided *ad libitum* during the experiment.

All experiments were performed in accordance with National Institutes of Health (USA) guidelines for animal experiments and were approved by the Animal Care Committee of Ohu University (approval No. 2015-64).

At postnatal days 70 and 72, tissue samples were obtained under sodium pentobarbital euthanasia. All efforts were made to minimize suffering. At the time of euthanasia, each mouse was weighed, blood was taken from the inferior vena cava, and the remnant liver was removed under a dim yellow safety light to prevent photoisomerization and photodegradation of retinoid [16,22–24]. Dissected livers were rapidly frozen in liquid nitrogen and stored at −80 °C. Tissues were stored continuously without thawing at −80 °C under nitrogen gas until analysis.

### 2.3. Preparation of standard stock solutions

All laboratory manipulations involving retinoids were performed in dark rooms under dim yellow light. Stock solutions of retinoids were prepared by dissolving 1 mg (for retinol and retinyl acetate) and 400 μg (for retinyl palmitate) in 1 mL ethanol. After degassing using nitrogen gas, solutions were kept in brown sample vials at −30 °C until use.

### 2.4. Analytical performance for calibration and system validation

Samples were directly injected into a HPLC/UV apparatus (PU-2080 plus chromatography pump, UV-2075 plus ultraviolet detector, and 807-IT integrator; Jasco, Tokyo, Japan) equipped with a Mightysil RP-18 GP column (Cat. No. 25416-96, 150 × 4.6 mm, 5-μm particle size; Kanto Chemical Co., Inc., Tokyo, Japan). The column was protected by a guard column

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