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Short communication

An HPLC–MS/MS method for the separation of α-retinyl esters from retinyl esters

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ABSTRACT

Enzymatic cleavage of the nonsymmetric provitamin A carotenoid α-carotene results in one molecule of retinal (vitamin A), and one molecule of α-retinal, a biologically inactive analog of true vitamin A. Due to structural similarities, α-retinyl esters and vitamin A esters typically coelute, resulting in the overestimation of vitamin A originating from α-carotene. Herein, we present a set of tools to identify and separate α-retinol products from vitamin A. α-Retinyl palmitate (αRP) standard was synthesized from α-ionone following a Wittig-Horner approach. A high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) method employing a C30 column was then developed to separate the species. Authentic standards of retinyl esters and the synthesized α-RP confirmed respective identities, while other α-retinyl esters (i.e. myristate, linoleate, oleate, and stearate) were evidenced by their pseudomolecular ions observed in electrospray ionization (ESI) mode, fragmentation, and elution order. For quantitation, an atmospheric pressure chemical ionization (APCI) source operated in positive ion mode was used, and retinol, the predominant in-source parent ion was selected and fragmented. The application of this method to a chylomicron-rich fraction of human plasma is demonstrated. This method can be used to better determine the quantity of vitamin A derived from foods containing α-carotene.

1. Introduction

Vitamin A deficiency remains a significant problem worldwide, with deficient regions obtaining a majority of their vitamin A needs from provitamin A carotenoids found in fruits and vegetables [1,2]. The vitamin A capacity of carotenoids is dictated by the presence of an unsubstituted β-ionone ring in conjugation with a polyene chain [3]. While numerous analytical methods have been developed for the determination of vitamin A delivery from a provitamin A rich meal [4–6],most studies focus on the provitamin A carotenoid β-carotene. The symmetric structure of β-carotene yields two molecules of retinal after central cleavage making it the most potent provitamin A precursor. However in nature, β-carotene is often found concurrent with nonsymmetric provitamin A carotenoids like α-carotene. Analogous to β-carotene, α-carotene is enzymatically cleaved at the central double bond, yet only produces one molecule of retinal. The remaining product, α-retinal, contains an ε-ring, and possesses only 2% of the bioactivity of vitamin A in animal models [7–10]. Both α-retinal and retinal are reduced to α-retinol and retinol, respectively, then esterified to fatty acids, before being packaged and released in blood chylomicrons. The structural similarity between the isobars α-retinol and retinol, with only a difference in the placement of a single double bond, causes difficulty resolving α-retinol/α-retinyl esters from analogous retinol/retinyl esters, respectively, using traditional C18 reversed-phase chromatography. Due to this coelution, it is likely that reported levels of newly formed vitamin A after the consumption of an α-carotene-rich meal have been overestimated.

A few articles have reported tentative identification of α-retinyl esters in animal tissues and blood after the feeding of α-carotene or α-retinol [11–15]. Collectively, these studies identified α-retinyl esters based upon anticipated retention time, expected UV–vis spectra, and disappearance after sample saponification [11–15]. In

Abbreviations: αRP, α-retinyl palmitate; APCI, atmospheric pressure chemical ionization; ESI, electrospray ionization; HPLC, high performance liquid chromatography; MeOH, methanol; MTBE, methyl *tert*-butyl ether; NMR, nuclear magnetic resonance; PDA, photodiode array; RP, retinyl palmitate; MS/MS, tandem mass spectrometry.

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one study, the presumed resulting α-retinol was isolated from tissues and then used to quantify α-retinol from the same tissues [11]. Another study required double-analysis of both a saponified and unsaponified sample, in addition to a calculation, to estimate α-retinyl ester contribution [12]. Saponification of retinol species prior to analysis requires more preparatory time, increases potential for degradation of these sensitive compounds, and does not allow for the differentiation of the circulating esters [12,14]. Additionally, without authentic standards, reports of intact α-retinyl esters in animal tissues are tentative [15]. Indeed, the lack of authentic standards has prevented unequivocal identification of α-retinyl esters for the past 20 years.

The objective of this work was to provide a tool to better measure the vitamin A potential of α-carotene-containing foods. α-Retinol was synthesized and esterified to palmitic acid (αRP, the presumed predominate acylated form of α-retinol [16]). Together with retinyl palmitate (RP), and other authentic retinyl ester standards, a high-performance liquid chromatography-photodiode array-tandem mass spectrometry (HPLC-PDA-MS/MS) method was developed. The method was then applied to differentiate α-retinyl esters from retinyl esters found in the chylomicron-containing fraction of human blood plasma.

2. Materials and methods

2.1. Reagents

Ammonium acetate was purchased from J.T. Baker (Phillipsburg, NJ, USA). HPLC grade methyl *tert*-butyl ether (MTBE), Optima grade water, methanol (MeOH) and formic acid were purchased from Fisher Scientific (Pittsburgh, PA, USA).

2.2. Synthesis and purification of all-trans αRP standard

A detailed description of αRP synthesis and purification is provided in the supplementary material and is summarized in Fig. S1.

2.3. HPLC–MS/MS method for separation of αRP and RP

Separation of αRP and RP standards was achieved using a C30 column (4.6 mm × 250 mm, 3 μm particle size, YMC, Allentown, PA)). A 1200 SL series HPLC system with a 60 mm path length 1260 PDA (Agilent Technologies, Santa Clara, CA) was employed. A gradient of solvent A: 90:10 MeOH/H_2O with 0.1% formic acid (v/v), and solvent B: 78:20:2 MTBE/MeOH/H_2O with 0.1% formic acid (v/v) was as follows: 30% B, followed by a linear gradient to 50% B over 18 min, holding at 100% B for 2 min, and re-equilibrating at 30% B for 3.5 min. The column was held at 40 °C, with a flow rate of 1.3 mL/min, and 40 μL injection volumes. The HPLC-PDA was interfaced with a QTRAP 5500 mass spectrometer (AB Sciex, Foster City, CA) using an APCI source in positive ion mode for αRP and RP quantitation. The source parameters were as follows: curtain gas: 30 psi, heated nebulizer temperature: 450 °C, nebulizer gas: 45 psi, declustering potential: 100 V, entrance potential: 10 V, and collision cell exit potential: 11 V. Both esters afforded strong in-source retinol fragments that were utilized for MS/MS detection. The parent-daughter transitions which both (1) displayed distinctly different MRM ratios for α-retinyl and retinyl esters and (2) provided optimal intensities for quantitation were chosen (Table 1). Peak areas were integrated with Analyst 1.5.1 (AB Sciex).

2.4. Biological sample preparation

Triglyceride-rich lipoprotein (TRL) fractions of human plasma containing newly formed chylomicrons were isolated [17] and extracted as published previously [18].

2.5. HPLC-PDA-MS/MS method to separate total α-retinyl esters and retinyl esters in biological samples

To distinguish the non-palmitate esters of α-retinol and retinol found in biological samples (i.e. myristate, linoleate, oleate, and stearate), the HPLC gradient described above was extended as follows: beginning at 30% B, followed by a linear increase to 55.6% B over 23 min, holding at 100% B for 2 min, and re-equilibrating at 30% B for 3.5 min. To detect the intact parent α-retinyl esters and retinyl esters, the HPLC was interfaced with the mass spectrometer via an ESI probe operated in positive ion mode. Source parameters, as described in Section 2.3, were applied, with the exception of the source temperature which was increased to 525 °C and collision energies of 17.5 and 40 V for the α- and retinyl esters, respectively. Identification of αRP and retinyl esters in the TRL sample was based upon parent ion masses, UV–vis spectra, and retention time coincident with authentic standards (Table 1). The non-palmitate α-retinyl esters were tentatively identified by expected retention order relative to the corresponding retinyl ester, UV–vis spectra, parent ion mass, and expected daughter ratios of the in-source α-retinol parent. An APCI source (with parameters provided in Section 2.3) was used for analyte quantitation. For all MS/MS experiments, MRM mode was used with maximized 70 ms dwell times per transition. All α-retinyl esters were quantitated using αRP, and all retinyl

Table 1
MS/MS parameters used for the identification and quantitation of α-retinyl esters and retinyl esters.

No.	Retention time (min)	Compound identity	HPLC-PDA spectrum (nm)	HPLC-APCI(+)-MS/MS *m/z* parent ion > *m/z* daughter ion	APCI collision energies (volts)[e]	HPLC-ESI(+)-MS/MS *m/z* parent ion > *m/z* daughter ion	ESI collision energies (volts)[e]
1	12.8	α-Retinyl myristate	298,311[a],325	269.2 > 123.1[b]	27.5	496.6 > 145.0	17.5
2	13.2	Retinyl myristate	325[a]	269.2 > 239.1[c,d], 145.1[d]	35.0, 25.0	496.6 > 197.0	40
3	13.2	α-Retinyl linoleate	298,311[a],325	269.2 > 123.1[b]	27.5	548.6 > 145.0	17.5
4	13.9	Retinyl linoleate	325[a]	269.2 > 239.1[c,d], 145.1[d]	35.0, 25.0	548.6 > 197.0	40
5	15.4	α-Retinyl oleate	298,311[a],325	269.2 > 123.1[b]	27.5	550.6 > 145.0	17.5
6	15.9	Retinyl oleate	325[a]	269.2 > 239.1[c,d], 145.1[d]	35.0, 25.0	550.6 > 197.0	40
7	16.9	α-Retinyl palmitate	298,311[a],325	269.2 > 123.1[b]	27.5	524.6 > 145.0	17.5
8	17.3	Retinyl palmitate	325[a]	269.2 > 239.1[c,d], 145.1[d]	35.0, 25.0	524.6 > 197.0	40
9	21.0	α-Retinyl stearate	298,311[a],325	269.2 > 123.1[b]	27.5	552.6 > 145.0	17.5
10	21.6	Retinyl stearate	325[a]	269.2 > 239.1[c,d], 145.1[d]	35.0, 25.0	552.6 > 197.0	40

[a] Denotes λ_{max}.
[b] Daughter with the strongest transition for α-retinol derivatives, chosen for quantitation.
[c] Daughters unique to retinol derivatives only.
[d] Daughters chosen for quantitation of retinol derivatives.
[e] Collision energy used for each respective daughter follows the order in which they are listed in the previous column.