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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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A B S T R A C T

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 (α RP) 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 pseudo-molecular 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.

Keywords:

α -Retinyl palmitate
 α -Retinol
Retinyl esters
Provitamin A carotenoids
Synthesis
HPLC–MS/MS

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: α RP, 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 (α RP, 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 α RP standard

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

2.3. HPLC-MS/MS method for separation of α RP and RP

Separation of α RP and RP standards was achieved using a C30 column (4.6 mm \times 250 mm, 3 μ m 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/H₂O with 0.1% formic acid (v/v), and solvent B: 78:20:2 MTBE/MeOH/H₂O 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 μ L 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 α RP 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 α RP 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 α RP, 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.

esters were quantitated using RP. The fatty acid moiety of each ester is lost in-source providing the same parent ion (and thus same response) for α -retinol and retinol, respectively (as previously confirmed with the authentic retinyl esters used for identification).

3. Results and discussion

3.1. HPLC-MS/MS method development

Identification and quantitation of α -retinyl ester and retinyl ester pairs presents an analytical challenge due to their structural similarity and isobaric nature. By coupling HPLC and MS/MS, we were able to chromatograph and use unique fragmentation patterns to differentiate the α -retinyl products from their retinyl counterparts.

Chromatographic separation of α RP and RP was achieved by modifying the gradient and increasing the polarity of the non-eluting solvent of the method outlined by Kopec et al. [18]. As a result, α RP eluted immediately before RP, while still maintaining peak resolution. This elution pattern was observed for all esters of α -retinol and retinol. C30 columns, widely used for carotenoid and retinoid separation, were tested at varying lengths (150 mm, 250 mm) and particle sizes (3 μ m, 5 μ m) to improve resolution. A column length of 250 mm with 3 μ m particle size gave the best separation.

UV-vis spectral characteristics were also helpful in distinguishing α -retinyl esters from retinyl esters. The movement of a double bond out of conjugation in the ϵ -ring of α RP causes a hypsochromic shift, resulting in a λ_{\max} of 311 nm (as compared a λ_{\max} of 325 nm for RP) (Fig. S2) [19]. α RP retains the 3-pronged fine-structure characteristic of carotenoids, with shoulders clearly visible at 298 and 325 nm, while RP has no fine structure (as commonly observed for short β -ring apo-carotenoids and other vitamin A derivatives) [19]. Furthermore, fatty acid acylation has no impact on the λ_{\max} of

α -retinol and retinol [20]. α -Retinol and retinol eluted at 3.62 and 3.76 min respectively.

By extending the gradient, we observed and separated myristate, linoleate, oleate, and stearate esters of both α -retinol and retinol in addition to palmitate in the TRL extracts (Fig. 1). Identities were confirmed in ESI positive mode (Fig. S3), where each of the esters formed a radical cation which fragmented to yield a dehydrated retinol fragment at m/z 269.2. The same pseudomolecular ion has been described previously for retinyl esters [4]. Transitions monitored are listed in Table 1 and a mass chromatogram is shown in the supplementary material.

An APCI source operated in positive ion mode proved to be more sensitive for the detection of α RP and other α -retinyl esters as compared to an ESI source, confirming previous reports for retinoids [5,18,21]. Further signal enhancement was achieved by replacing the mobile phase additive ammonium acetate with formic acid. α RP and retinyl ester standard solutions confirmed APCI in-source fragmentation to produce a parent ion at 269.2 m/z (representing the neutral loss of the fatty acid moiety and water, $[M+H-fatty\ acid-H_2O]^+$). This observation is consistent with other reports of retinyl palmitate analysis by MS [5,18,21,22]. Notably, the dehydrated α -retinol species (containing an ϵ -ring) and retinol species (containing a β -ring) have different fragmentation patterns, which simplified the identification of the α -analogues in the TRL extracts. In addition, these fragmentation patterns mirror the fragmentation of the parent provitamin A carotenoids α -carotene (containing an ϵ -ring and a β -ring) and β -carotene (containing two β -rings) [19,23]. The parent-daughter pair m/z 269.2 > 123.1, characteristic of ϵ -ring carotenoids, was the strongest transition observed for α -retinol species and is thought to correspond to the ϵ -ring moiety itself [19,23]. This daughter was also observed for retinol, but at lower levels, and thus was selected for quantification of α RP. In contrast, m/z 269.2 > 239.1 showed exceptional selectivity for the retinyl esters and was not produced from fragmentation of α -retinol. This fragment was summed with m/z 145.1 (also

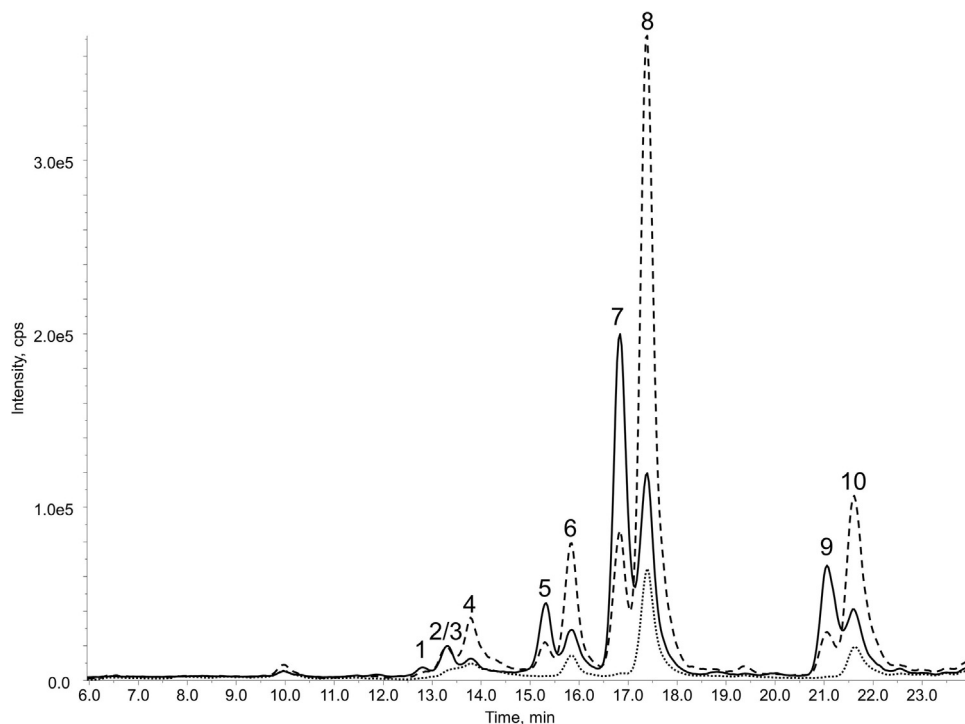


Fig. 1. HPLC-MS/MS chromatogram of a TRL extract from a representative subject 6 h post-prandial, utilizing the HPLC method separating all esters and the MS equipped with an APCI+ source. MRM transitions specific for α -retinol and α -retinyl esters (269 m/z > 123 m/z -) and retinol and retinyl esters RP (269 m/z > 145 m/z ----, 269 m/z > 239 m/z ...) are displayed. Peak numbers correspond to those detailed in Table 1.

representative of retinol) for quantitation. Fig. 1 demonstrates a mass chromatogram of a TRL extract with these characteristic transitions selected. Using these aforementioned transitions, this method was determined to have a limit of quantitation (LOQ, defined as signal to noise = 10) of 4.8 and 29.5 nM for α RP and RP, respectively, and a limit of detection (LOD, defined as signal to noise = 3) of 1.4 and 8.9 nM for α RP and RP, respectively. In total, the 3 MS/MS transitions chosen were continuously monitored to provide more confidence in the identities of α -retinyl and retinyl esters by comparing ratio intensities.

4. Conclusions

Without proper tools, the contribution of α -retinyl esters to the newly circulating retinyl ester pool can be easily overlooked. Herein, we have provided a descriptive method of α -retinol and α RP synthesis, and detailed HPLC-MS/MS methods that allow for the direct characterization and quantification of α -retinyl esters (both intact and fragmented) in biological samples. We anticipate that these methods will aid in the accurate quantification of vitamin A derived from α -carotene sources, to better assess its vitamin A potential.

Author disclosures

The authors have no conflicts of interest to disclose.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jchromb.2016.06.043>.

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