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Deuterium enrichment of retinol in humans determined by gas chromatography electron capture negative chemical ionization mass spectrometry

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A new application of electron capture negative chemical ionization mass spectrometry method has been developed for detecting vitamin A enrichment in human serum. Octadeuterated all-trans retinyl acetate 1.5 mg was fed to a volunteer and blood samples were collected over a period of 32 days. Serum samples were extracted and isolated by high performance liquid chromatography to collect the fraction containing retinol. The retinol fraction was derivatized to a trimethylsilyl ether, which was analyzed by gas chromatograph (GC)/mass spectrometry using a capillary column coated with DB-1 followed by methane electron capture negative chemical ionization/mass spectrometry (ECNCI-MS). Although ECNCI-MS of derivatized retinol produced no molecular ion, it produced a single negatively charged fragment ion at m/z 268 for natural retinol (m/z 276 for octadeuterated retinol) due to loss of the silyl group. The serum enrichment of labeled retinol was detectable at 7 hours, reached a maximum of 2.6% at 24 hours, and declined thereafter but was still at 0.07% at 32 days. In 200 μL of serum, the minimum detectable enrichment of retinol-d_8 was 0.01%. The GC/ECNCI-MS method for detecting retinol in serum is at least 10 times more sensitive than any previously published mass spectrometry method. (J. Nutr. Biochem. 9:408–414, 1998) © *Elsevier Science Inc. 1998*

Keywords: octadeuterated retinyl acetate; octadeuterated retinol; human serum; electron capture negative ion chemical ionization mass spectrometry; gas chromatograph/mass spectrometry

Introduction

Vitamin A nutrition is essential for vision, growth, cellular differentiation and proliferation, reproduction, and the integrity of the immune system.[1] Vitamin A inadequacy is a health problem in many developing counties.[2] Blood retinol concentrations are homeostatically controlled in a well nourished population, unless vitamin A liver stores, where 90% of vitamin A in the body is stored, are depleted. Due to vitamin A storage in the liver (50–200 mg for a normal person), a daily dose of vitamin A (≤1.5 mg) will not significantly change the body pool of vitamin A. It has been a challenge to determine accurately the vitamin A status in humans over a large range of liver stores. The use of stable isotope labeled vitamin A overcomes the complications because it is able to trace newly administered vitamin A and to determine the dilution of labeled vitamin A by an existing body pool of vitamin A. The stable isotope method poses no risks to the volunteers and is minimally invasive compared with liver biopsy, which is the only direct method for measuring body vitamin A stores.

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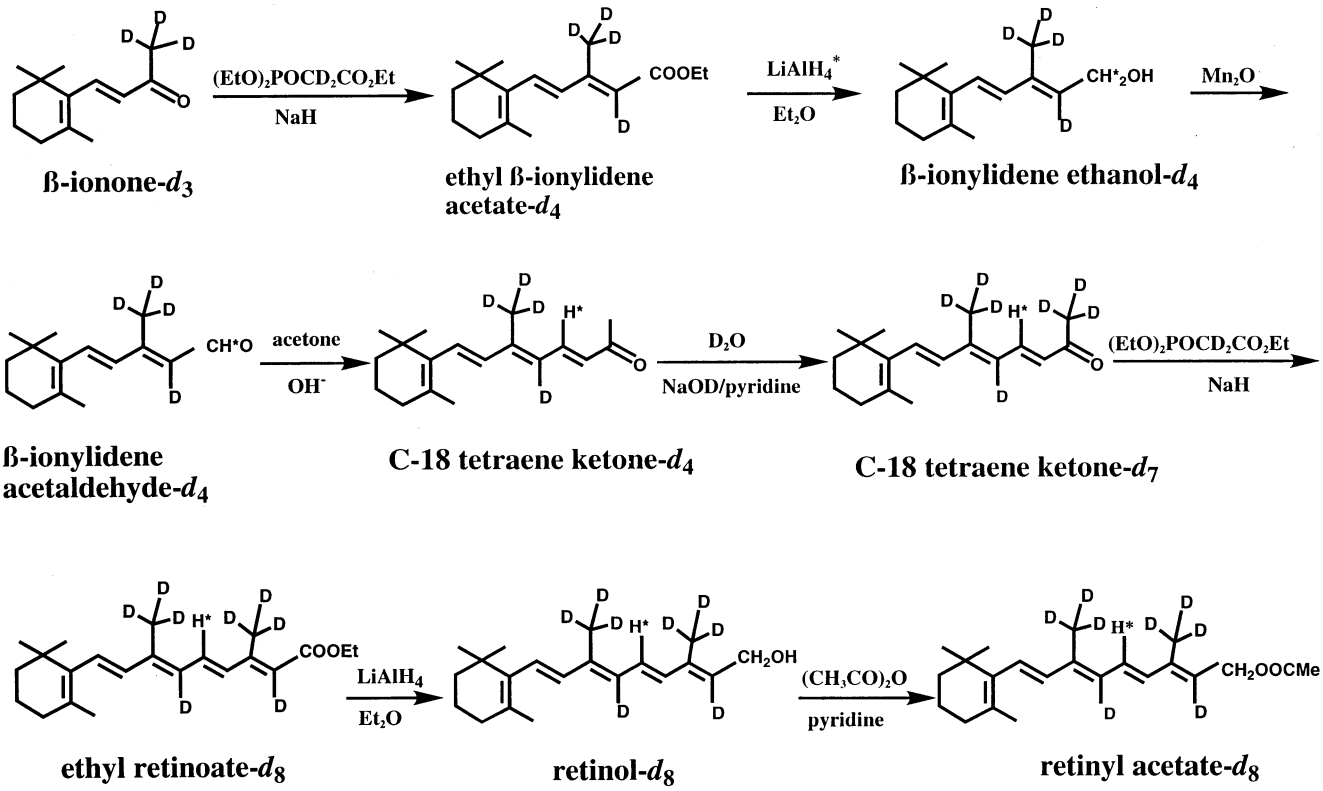

Figure 1 Synthesis scheme of octadeuterated retinyl acetate. The asterisk (*) represents the deuteration at C11 position.

In the past, Furr et al.[3] reported the use of a gas chromatography/mass spectrometry (GC/MS) method to measure the enrichment of un-derivatized retinol in human blood samples. The results from the isotope dilution process were used to calculate the individual liver stores by using the Bausch and Rietz equation.[3,4] It required 2 mL of plasma to perform the analysis on human plasma collected after consumption of 45 mg of all-trans retinyl acetate-d_4. An improved method was developed recently by Handelman et al.[5,6] using 0.753 μmol retinyl acetate-d_4/kg body weight (12–25 mg per volunteer), in which retinol was derivatized to its tert-butyldimethylsilyl ether. It needed 1 mL of human plasma collected after a dose of all-trans retinyl acetate-d_4. However, the method of Handelman et al.[5] cannot detect enrichments of retinol-d_4 lower than 0.2% and amounts less than 1.2 ng. Because the detection limit was not low enough to determine low level of enrichments, all these experiments were performed after a pharmacologic dose of vitamin A (>5 times the daily intake). However, a pharmacologic dose of vitamin A will contribute substantially to the liver stores of vitamin A in humans. Thus, it may change the vitamin A status of the subject. To evaluate nutritional status of vitamin A under steady-state conditions, a more sensitive method is needed to analyze blood response to a physiologic dose of labeled vitamin A (≤1.5 mg/day).

In an effort to study human nutrition of vitamin A under physiologic conditions, we developed an on-column injection gas chromatograph/electron capture negative chemical ionization-mass spectrometry (GC/ECNCI-MS) combined with a gradient high performance liquid chromatography (HPLC) procedure to collect the retinol fraction and to analyze the enrichment of derivatized retinol in human serum samples.

Materials and methods

Standards

Retinyl acetate-d_8 (10, 14, 19, 19, 19, 20, 20, 20-$^2H_8$-retinyl acetate) was synthesized in this laboratory (*Figure 1*) by combining the synthetic procedures of 10,19,19,19-$^2H_4$-retinyl acetate and 14,20,20,20-$^2H_4$ published by Bergen et al.[7] The overall yield of raw retinyl acetate-d_8 was 28.6%. However, the production of purified retinyl acetate-d_8 was approximately 2% of raw retinyl acetate-d_8. The chemical purity of deuterated retinyl acetate is over 99%. The purity of retinyl acetate-d_8 was confirmed using HPLC and GC/MS (electron impact MS; *Figure 2*). The deuterated retinyl acetate showed the same retention time and absorption spectra as standard retinyl acetate (Sigma Chemical Co., St Louis, MO USA). The GC/MS (electron impact) analysis of retinyl acetate-d_8 showed that the deuterated fraction is greater than 99.9% (<0.01% is unlabeled retinyl acetate, retinyl acetate-d_0) with 72.8% in m/z 336 (octadeuterated retinyl acetate), 22.5% in m/z 335 (heptadeuterated retinyl acetate), and 4.7% in m/z 334 (hexadeuterated retinyl acetate).

Retinyl acetate-d_4 (11,19,19,19-$^2H_4$-retinyl acetate) was synthesized by a modified procedure described above for the synthesis of retinyl acetate-d_8. The deuterium at the C11 position is introduced by a reduction using lithium aluminum deuteride ($LiAlD_4$). The retinol-d_4 was produced by the reduction of retinyl acetate-d_4 using lithium aluminum hydride ($LiAlH_4$) and was derivatized for MS analysis as a reference to unlabeled retinol (*Figure 3* and *Figure 4*).

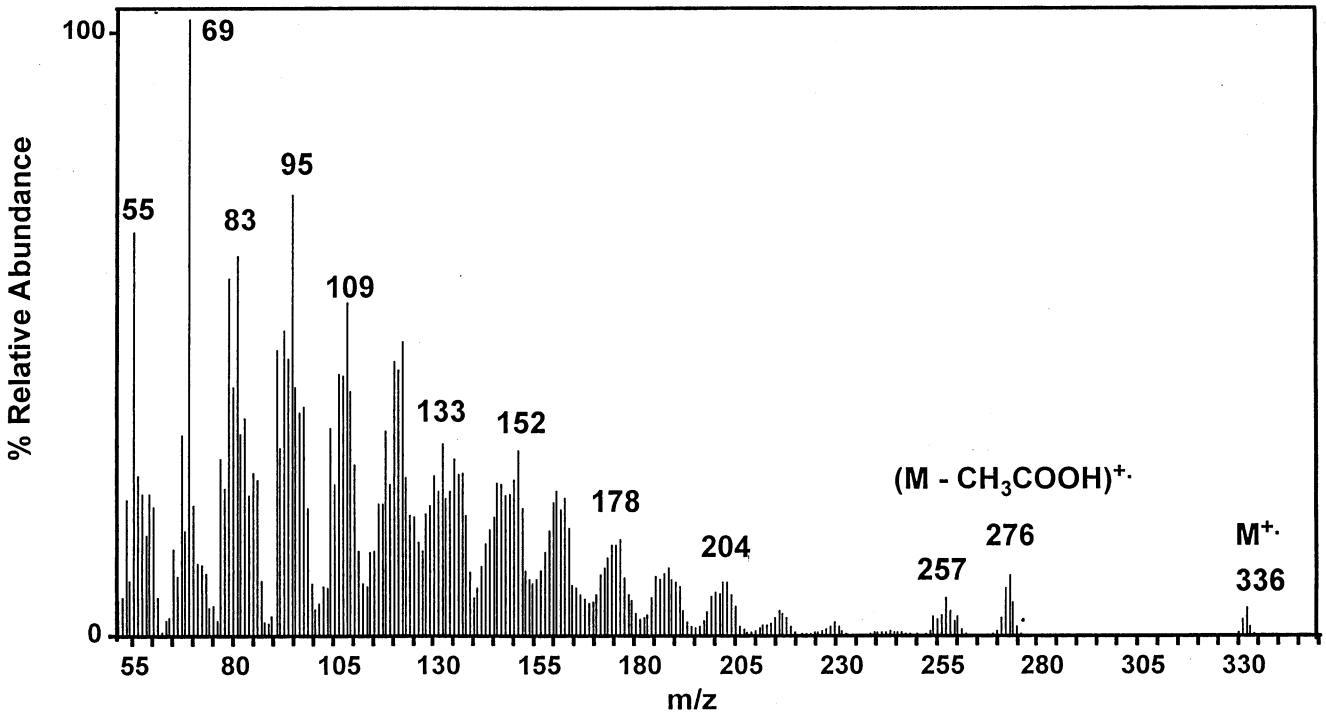

Figure 2 Mass spectrum of octadeuterated retinyl acetate.

Sample preparation

Blood sample collection. Blood sampling followed the regulations of the Human Investigation Committee at Tufts University. After an overnight fast, a healthy volunteer (male, 58 years-old, 95 kg) consumed a gelatin capsule containing 1.5 mg (1277 retinol equivalence) of retinyl acetate-d_8 in 88 mg corn oil with a 500 kcal meal of liquid diet (Promod, Ross Laboratories, Columbus, OH USA). Thereafter, the subject consumed regular meals. Whole blood (10 mL) was collected into a Vaccutainer (no additive) at 0, 1, 3, 5, 7, 9, 11, and 13 hours and fasting samples were collected at 1, 2, 3, 4, 5, 6, 12, 19, 26, and 32 days. Blood samples were kept at room temperature for 30 minutes and then centrifuged with Sure-sep II (Organon Teknika Corp., Durham, NC USA) at 4°C and 800 g for 15 minutes. Serum was aliquoted into 2 mL vials and stored at −70°C.

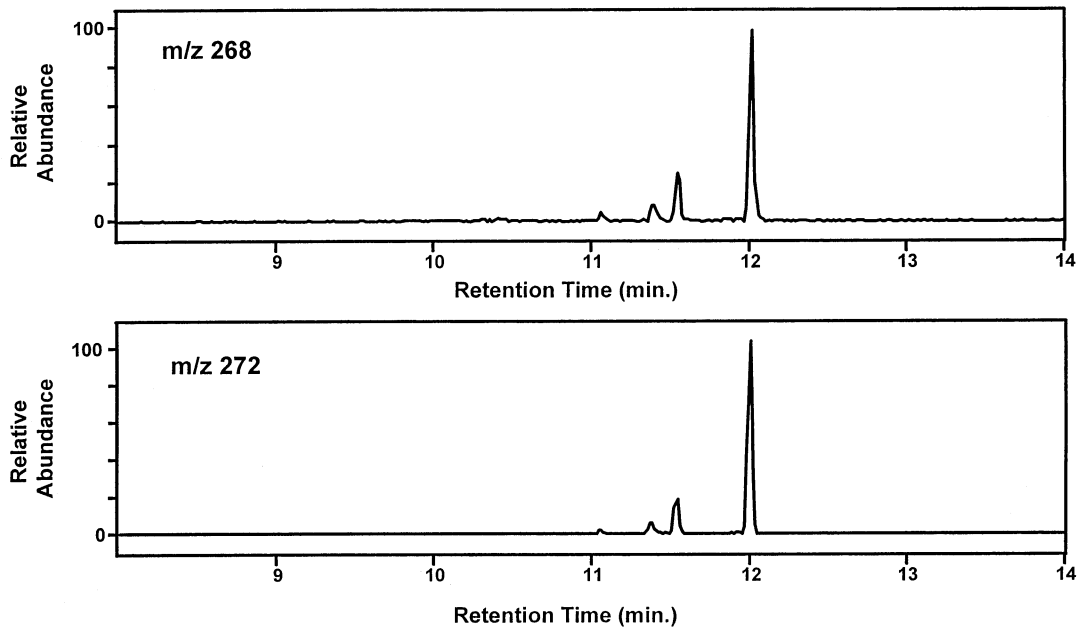

Figure 3 Mass chromatograms for unlabeled retinyl trimethyl ether (m/z 268, top panel) and tetradeuterated retinyl trimethyl ether (m/z 272, bottom panel).

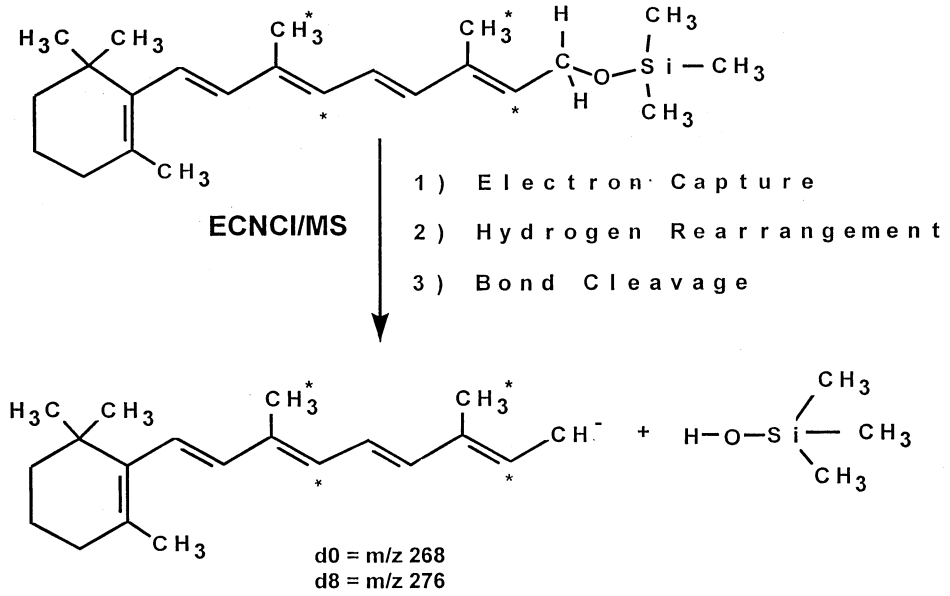

Figure 4 A possible cleavage mechanism in the formation of a major fragmentation at m/z 268 from retinyl trimethyl silyl ether. The asterisks (*) represents the deuterated position in retinol molecules (m/z 276).

Extraction and separation of serum retinol. Two milliliters of $CHCl_3/CH_3OH$ (2:1 in volume) was added to a 200 μL serum sample. The mixture was vortexed and centrifuged for 10 minutes at 4°C and 800 g. The $CHCl_3$ layer was collected. Two milliliters of hexane were added to the aqueous layer to re-extract fat soluble nutrients. The hexane layer was combined with the $CHCl_3$ layer and evaporated under N_2 on a N-EVAP evaporator (Organomation Associates, Inc., South Berlin, MA USA). The residue was dissolved in 80 μL of ethanol and 50 μL was injected onto an HPLC equipped with a C18 column (Perkin-Elmer Inc., Norwalk, CT USA).[8] A gradient solvent procedure was as follows: 100% solvent A (acetonitrile/tetrahydrofuran/water, 50/20/30, v/v/v, 1% ammonium acetate in water) is used for 6 minutes followed by an 8-minute linear gradient to 50% solvent B (acetonitrile/tetrahydrofuran/water, 50/44/6, v/v/v, 1% ammonium acetate in water), a 5-minute hold at 50% solvent B, a 2-minute gradient to 100% solvent B, an 8-minute hold at 100% B, and a 1-minute gradient to 100% A.[9] In this system, retinol eluted at 10 minutes and was collected from 9.5 minutes to 11.0 minutes by a Gilson FC 203 fraction collector (Middletown, WI USA).

Derivatization of retinol. The retinol HPLC fraction from the serum extract was extracted first by 2 mL of $CHCl_3$ and then by 2 mL of hexane. The $CHCl_3$ and hexane extracts were combined and dried under N_2. Forty microliters of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 10% trimethylchlorosilane (TMCS; both from Pierce, Rockford, IL USA) was added to the residue in the test tube. The test tube was capped with a ground glass stopper and was heated at 70°C for 30 minutes. The reaction mixture of retinyl trimethylsilyl (TMS) ether and the unreacted BSTFA and TMCS was transferred by a glass pipette to a brown vial with a conical shaped glass insert and kept in a desiccator at 4°C until GC/MS analysis.

Sample analysis

HPLC analysis of serum samples. Serum concentrations of retinol in a 100 μL aliquot were measured by an HPLC equipped with a Pecosphere-3C C18 column (Perkin-Elmer, CT USA) and a Waters 994 Programmable Photodiode Array Detector. The detector wavelength was set at 450 nm for carotenoids and 340 nm for retinoids.[9] Retinyl acetate and echinenone (Hoffman-LaRoche, Nutley, NJ, USA) were added as internal standards to monitor the extraction of retinol and β-carotene, respectively. The concentration of retinol in serum as measured by HPLC and the percent isotopic enrichment of retinol-d_8 as measured by GC/MS was used to calculate the molar enrichment of deuterated retinol in human serum.

GC/MS analysis of retinol in human serum. One microliter of derivatized retinol was injected by an HP 7673A autosampler into an HP 5890 GC. The GC employed a cool on-column injector. The on-column injector was fitted with a 0.5 m deactivated fused silica retention gap (HP19095-60610). The retention gap was connected with a zero dead volume connector (HP 5061-5801) inside a PEEK ferrule (HP 5061-5804) to a 15 m × 0.25 mm i.d. fused silica capillary column coated with a DB-1 stationary phase of 0.25 μm film thickness from J & W Scientific (Folsom, CA USA). The temperature of the column oven and the on-column injector was programmed from 50°C to 285°C at 15°C/min. The GC/MS interface temperature was set at 285°C. Helium was used as the carrier gas. The trimethylsilyl derivative of retinol eluted at approximately 12 minutes. The GC eluate was detected by an HP 5988A quadrupole mass spectrometer, using 0.5 torr methane negative ion chemical ionization. The temperature of the ion source was 150°C. The mass spectrometer was scanned repetitively between 260 and 280 daltons. The data were collected and analyzed with an HP Chemstation data system.

When the labeled retinol enrichment was below 1%, it was difficult to obtain accurate isotope ratios due to dynamic range limitations of the GC/MS detection system (see *Figure 4*, the arrow on the m/z 268). In these cases, we measured the ratio of the labeled retinyl ion at m/z 276 to the natural abundance ^{13}C-retinol ion at m/z 269. The measurement of the m/z 268 to m/z 269 ratio at lower concentrations allowed us to calculate the m/z 268 to m/z 276 ratio. By using the reconstructed mass response, we were able to measure percent enrichments to 0.01%.

Results and discussion

We measured the retinyl TMS ether by GC/ECNCI-MS. The GC/ECNCI-MS of retinyl TMS ether showed no molecular ion, but a major fragment ion at m/z 268 (*Figure*

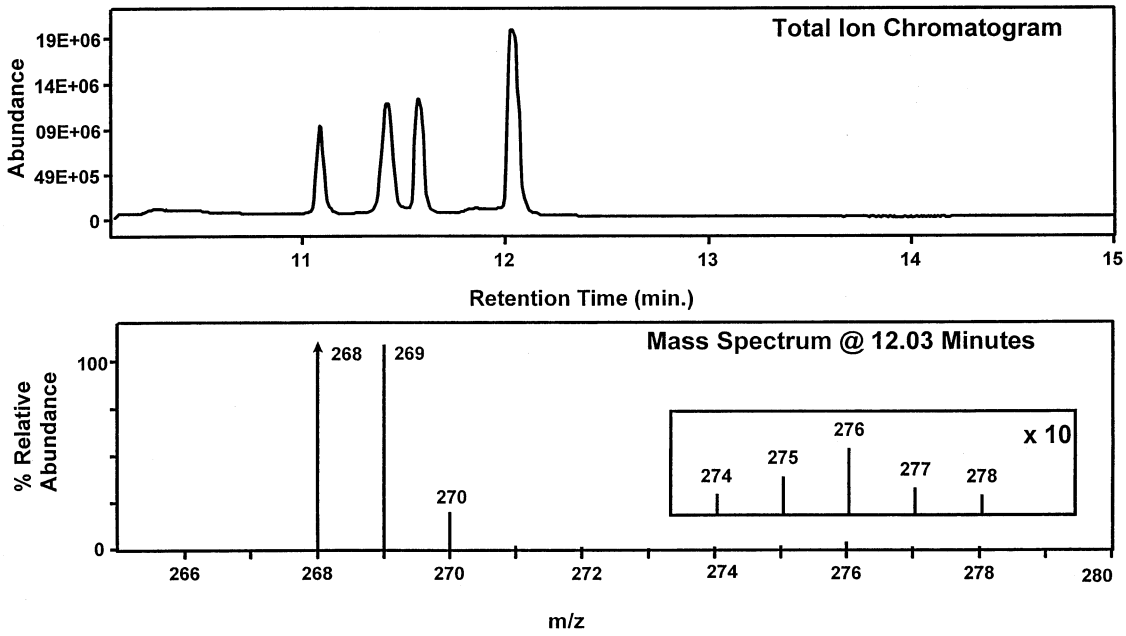

Figure 5 A representative gas chromatograph/methane electron capture negative chemical ionization-mass spectrometry chromatogram (top panel) from the analysis of derivatized retinol in human serum collected 1 day after 1.5 mg of retinyl acetate-d_8. Bottom panel is a representative mass spectrum that shows m/z 268 of retinol-d_0, m/z 269 of ^{13}C-retinol-d_0, m/z 270 of $^{13}C_2$-retinol-d_0, m/z 274 of retinol-d_6, m/z 275 of retinol-d_7 and ^{13}C-retinol-d_6, m/z 276 of retinol-d_8 and ^{13}C-retinol-d_7, m/z 277 of ^{13}C-retinol-d_8, and m/z 278 of $^{13}C_2$-retinol-d_8. The peak at m/z 268 is off scale due to the dynamic range limitation of the mass spectrometry instrument.

3, top panel). This is confirmed by the result obtained for a retinol-d_4 standard with a major fragment ion at m/z 272 (*Figure 3*, bottom panel). Even though the ECNCI-MS for both retinol and derivatized retinol showed the identical negative ion, at m/z 268 derivatization improved the detection limit remarkably due to the sharp peak of retinyl TMS ether. That is, the derivatized retinol showed a peak of 0.1 minute peak width (*Figure 3*) while underivatized retinol showed a peak of 1 minute peak width with a shorter retention time (data not shown). A possible mechanism of electron capture, hydrogen rearrangement, and bond cleavage of derivatized retinol is shown in *Figure 4.* This type of fragmentation is typical of silyl ethers under ECNCI conditions.[10] In *Figure 3*, three minor peaks, assumed to be *cis*-isomers of retinol, are observed in standards of both retinol-d_0 and retinol-d_4 analysis. Because the HPLC method does not separate the isomers of retinol, we do not know whether these *cis*-isomers of retinol are naturally occurring or are formed during the GC process.

The retinol in the serum extract was collected from a gradient HPLC procedure.[8,9] This HPLC procedure is designed to separate retinol from other fat soluble components to avoid interference from biological impurities (such as lutein) in further derivatization. This gradient HPLC procedure can be used to chromatograph hundreds of samples without a need for column flushing.

Retinol collected from the HPLC chromatogram of a serum extract was derivatized with BSTFA at 70°C for 30 minutes. Compared with the derivatization conditions using N-methyl-N-(tert-butyldimethylsilyl)trifluoroacetamide (MTBSTFA),[5,6,11] this is a very mild and efficient derivatization process for retinol derivatization. We found that the peak of derivatized retinol is very sharp and has no tailing or background peaks in the scanned mass range. A similar derivatization reagent was reported by von Reinersdorf et al.[12] The derivatized retinol in a sealed vial can be kept in a desiccator at 4°C for 1 month without degradation.

In the analysis of retinol-d_8, the retinol-d_6 and retinol-d_7 peaks were observed in MS analysis for the dose (*Figure 2*) and the blood extracts (*Figure 5*). We did not see this kind of pattern associated with the analysis of retinol-d_0 (*Figure 5*). Therefore, peaks of retinol-d_6 and retinol-d_7 are not formed in the mass spectrometer due to fragmentation but are already preformed in the given dose of retinyl acetate-d_8. The total enrichment of labeled retinol was determined by integrating the peak area under the reconstructed mass chromatograms of the negative ions at m/z 274 (d_6), 275 (d_7 + ^{13}C-d_6), 276 (d_8 + ^{13}C-d_7 + $^{13}C_2$-d_6), 277 (^{13}C-d_8 + $^{13}C_2$-d_7), and 278 ($^{13}C_2$-d_8). The natural abundance of retinol was determined by integrating the peak area under the reconstructed mass chromatograms of the negative ions at m/z 268 (d_0), m/z 269 (^{13}C-d_0), and m/z 270 ($^{13}C_2$-d_0) (*Figure 5*). Therefore,

$$\% \text{ enrichment} = \{S_{\text{labeled}}/(S_{\text{labeled}} + S_{\text{unlabeled}})\} \times 100,$$

where

$$S_{\text{unlabeled}} = 268\ (d_0) + 269\ (^{13}\text{C-}d_0) + {}^{13}C_2\text{-}d_0$$

and

$$S_{\text{labeled}} = 274\ (d_6) + 275\ (d_7 + {}^{13}\text{C-}d_6) + 276 \cdot (d_8 + {}^{13}\text{C-}d_7 + {}^{13}C_2\text{-}d_6) + 277\ (^{13}\text{C-}d_8 + {}^{13}C_2\text{-}d_7) + 278\ (^{13}C_2\text{-}d_8).$$

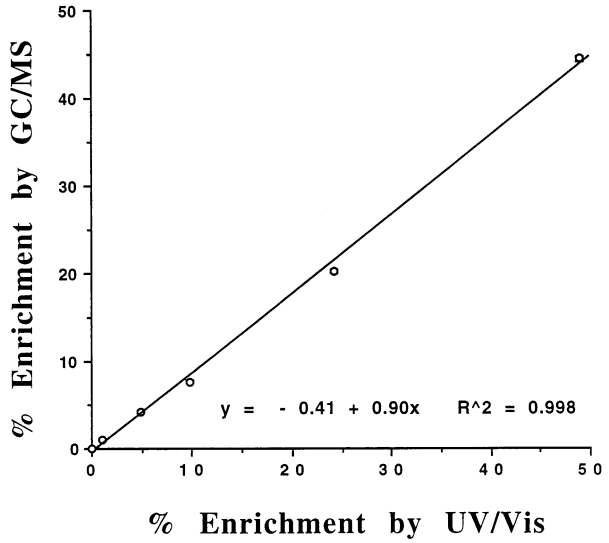

Figure 6 The correlation of the enrichment of labeled retinol measured by ultraviolet/vis spectrometer and the signal area response obtained by the gas chromatograph/mass spectrometry.

By including all of the isotopes instead of retinol-d_8 alone in the analysis, the accuracy of the isotope ratio analysis is increased by up to 8.6% based on the analysis of standards of known isotopic composition. To determine the MS responses to labeled and unlabeled retinol derivatives, the standard retinol and retinol-d_4 were first purified by HPLC. The solutions of retinol-d_0 and retinol-d_4 were measured individually by ultraviolet/visible (UV/VIS) spectrometer and then mixed and measured by GC/MS. UV/VIS and GC/MS determinations at different percent enrichment of retinol-d_4 are shown in *Figure 6*. The response curve for retinol-d_4 enrichment is linear but retinol-d_4 enrichment has slightly less response in GC/MS than in UV/VIS. The slope of the line is 0.9. However, the difference between the UV/VIS spectrum absorbance and the GC/MS measurement is within measurement error in the determination of the enrichment of labeled retinol in human samples (below 10%). Therefore, we have made no mathematical correction to the percent enrichment determined by GC/MS.

The enrichment of deuterated retinol in serum after administration of 1.5 mg of retinyl acetate-d_8 showed an increase starting at 7 hours, which reached a peak at 24 hours with 2.26% enrichment and an absolute concentration of 32.9 nM (*Figure 7*). The absolute concentrations of deuterated retinol were determined by multiplying the percent enrichment of retinol by the concentration of retinol determined by HPLC analysis.[8]

In 200 μL of human serum, the minimum detectable percent enrichment of retinol was 0.01%. Thus, the method is sensitive enough to analyze the serum samples collected from subjects in a wide range of vitamin A status. A

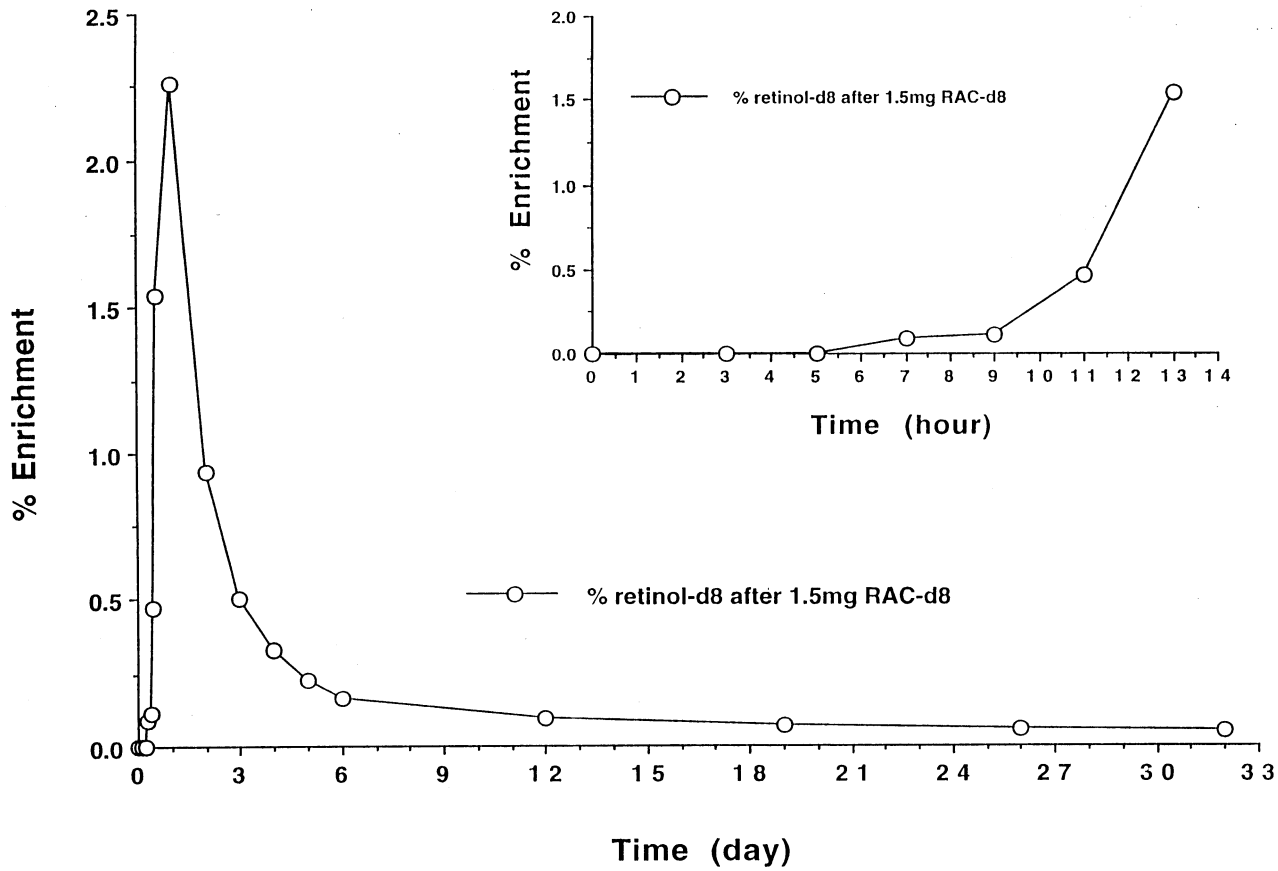

Figure 7 Percent enrichment of retinol-d_8 in the serum collected at 0, 3, 5, 7, 9, 11, and 13 hours (inserted panel) and at 1, 2, 3, 4, 5, 6, 12, 19, 26, and 32 days following a dose of 1.5 mg retinyl acetate-d_8.

physiologic dose of retinyl acetate-d_8 has been chosen for use as an isotope reference to determine vitamin A activity of β-carotene-d_8 in humans because β-carotene-d_8 will be converted to retinol-d_4. Therefore, both retinol-d_8 (from retinyl acetate-d_8) and retinol-d_4 (from β-carotene-d_8) can be monitored simultaneously in human blood samples.

Isotope effects have been observed with the association with the steric effect, the inductive effect, and the hyperconjugative effect in physical chemistry studies.[13] We do not expect to see any detectable isotope effects in the absorption and metabolism of retinyl acetate-d_8 and retinol-d_8 in biological system because the labeling is at the C19 and C30 (three deuteriums) and the C10 and C14 (one deuterium) positions of the retinol molecule. Deuterium atoms in these positions are not expected to change the structure or affinity of the molecule substantially or to change the strength of the conjugated double bonds in the retinol molecule to influence its biological activity.

Unlike positive ion chemical ionization, ECNCI does not use protons in the ionization process that might cause deuterium-hydrogen exchange but uses low energy electrons that are captured by compounds with high electron affinities, such as derivatized retinol, to form negative ions. To confirm this, we have measured standards of known deuterium-hydrogen isotope ratio. We have observed no deuterium-hydrogen exchange and no unusual hydrogen radical/molecule reactions[14] in the MS analysis.

Total liver stores of a person can be calculated using the modified Bausch and Rietz equation,[3,4] that is,

$$\text{TLR (total liver reserve)} = \text{F} \times \text{Dose} \times [\text{s} \times \text{a} \times (\text{H} : \text{D} - 1)]$$

where dose is deuterated retinyl acetate in mmole; H : D is the ratio of retinol-d_0 to retinol-d_4 in serum from MS analysis; F is 0.5, the fraction of the dose to be stored in liver; S is 0.68, the correction for H : D ratio in serum; and a is exp (−kt) (k is 1/140 day and t is the number of days since dose, the correction to the H : D ratio due to dietary unlabeled vitamin A over time).

Based on the modified Bausch and Rietz equation and the isotope dilution (0.07% enrichment) observed 32 days after a 1.5 mg retinyl acetate-d_8 dose (4.5 μmol), the total liver stores of vitamin A in the volunteer can be calculated to be 1.7 mmol of retinol.

Having developed methods for the derivatization of retinol, gradient HPLC, and GC/ECNCI-MS, we can learn more about the kinetics of the blood response after a labeled vitamin A dose when it is given at a physiologic level. We can also evaluate the vitamin A status in humans and determine the vitamin A activity of provitamin A carotenoids in humans. We intend to use this sensitive and accurate method in populations of vitamin A deficient children to evaluate the outcomes from different strategies for improving and sustaining vitamin A status.

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