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Ion Trap Mass Spectrometry for Kinetic Studies of Stable Isotope Labeled Vitamin A at Low Enrichments

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The role of β-carotene in chemoprevention of cancers and other chronic diseases generated controversy when subpopulations taking β-carotene supplements showed increased mortality in clinical trails. Determination of the dynamics of β-carotene in individual human subjects has emerged as a high priority. Stable isotope labeled β-carotene tracers can be employed to determine rates of conversion to retinol (vitamin A), but tracer doses must be small to minimize perturbation of endogenous retinoid and carotenoid pools. In such cases, ratios of labeled tracer/endogenous retinol are often low, and quantitative analysis at enrichments of <1 mol % are unreliable owing to ion−molecule reactions that generate ions at the same mass as the labeled tracer even when no tracer is present. The current study demonstrates improved gas chromatography/mass spectrometry quantification of retinol-d_4 and unlabeled retinol, as their *tert*-butyldimethylsilyl ethers, at low enrichments using an ion trap mass spectrometer operated in selected ion storage mode. Electron ionization of analyte takes place in the ion trap using conditions that eject ions outside the range m/z 390−420, and molecular ions at m/z 400 and 404 from retinol and retinol-d_4 are quantified. Using this approach, unlabeled retinol yields a signal close to values calculated from natural isotopic abundances (∼0.13%), whereas several quadrupole instruments operated using selected ion monitoring yielded 2−5 times greater signal when no labeled retinol was present.

β-Carotene is a phytochemical polyene and essential micronutrient that is cleaved enzymatically to vitamin A (retinol) and other metabolites including retinoic acid. Many of these metabolites are potent modulators of cellular differentiation and proliferation. Lower incidences of some diseases including cancers have been associated with consumption of diets rich in carotenoid-rich vegetables.[1−3] As a result, carotenoids have attracted attention as potential chemopreventive agents and antioxidants. However, in recent clinical trials, cigarette smokers adminstered β-carotene supplements showed increased incidences of cancer and mortality.[4] Other studies suggested β-carotene supplements were neither beneficial nor harmful.[5] These conflicting observations highlight a need for an improved understanding of the conversion of β-carotene to its metabolites and delivery of bioactive carotenoids and retinoids to various tissues and organs in humans.

To establish the role of dietary β-carotene and vitamin A in human health, stable isotope labeled β-carotene is administered orally and the resulting time-dependent concentrations of plasma metabolites such as retinol are determined using gas chromatography/mass spectrometry (GC/MS). Such investigations are facilitated by use of β-carotene-d_8, which is metabolized to retinol-d_4.[6,7] The use of multiple heavy isotopes minimizes interferences from isotopomers containing naturally occurring ^{13}C, ^{2}H, ^{18}O, and ^{29}Si and ^{30}Si (in silyl derivatives) and improves measurements at low enrichments.

In retinol-d_4/retinol GC/MS analyses, the deuterated tracer (retinol-d_4) must be measured in the presence of substantial excess of endogenous unlabeled retinol. Thus, the critical analytical performance characteristics are instrument dynamic range and measurement precision at low enrichments. For retinol measurements, the sensitivity of the method is rarely limiting because blood concentrations of retinol are significant and relatively constant (∼1 μM), and sufficiently large blood samples may be drawn and processed. Even in the case of low retinol-d_4 enrichment, precision and accuracy are more limited by interference from unlabeled retinol.

The ability to accurately measure vitamin A isotopomer ratios at low enrichments has been hampered using linear-beam quadrupole instruments by high and variable artifactual signal at the mass of the deuterated isotopomer when nonlabeled retinol is measured.[8−10] Analyses performed on unlabeled retinol showed more abundant signal at the mass of the deuterated isotopomer than predicted by natural isotope abundances. Deuterio/protio

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signal ratios of 0.4−1.0% are typical for unlabeled retinol. This phenomenon has been documented with several linear-beam single-quadrupole instruments and has been attributed to ion−molecule reactions occurring in the mass spectrometer ion source.[8,9]

While many studies employing labeled vitamin A and β-carotene tracers have used pharmacological doses of retinyl-d_4 acetate (20−45 mg),[8−11] [8,9,19-^{13}C]retinyl palmitate (55 mg),[12] or β-carotene-d_8 (30−120 mg),[6,7,13] smaller doses more typical of daily dietary intake are desirable for quantitating rate processes and evaluating nutritional status by isotope dilution techniques; physiological-sized doses will minimize perturbations of endogenous retinoid and carotenoid pools and limit departure from steady-state kinetics during the metabolic course of the tracer. Furthermore, administration of smaller doses reduces experimental costs and improves the feasibility of epidemiological studies of larger populations. Smaller doses, however, require increased instrument dynamic range and precision at lower enrichments. The modeling of β-carotene is further complicated by a low rate of bioconversion where as little as one-sixth of a β-carotene dose is converted to retinol when taken orally.[14]

The search for improved precision at low enrichments led to our examination of ion trap mass spectrometry (ITMS). Ion trap mass spectrometers have the ability to selectively accumulate ions and then eject them in a rapid burst to a detector. This concentrating effect creates higher effective concentrations seen by the detector, leading to high sensitivity. Traditionally, they have had a limited dynamic range because of the requirement that total ion density in the trap does not exceed $\sim 10^5$ ions.[15−17] Without other developments, ITMS instrumentation would probably not offer many advantages over linear-beam instruments for retinol isotopomer analyses. However, two developments, the selected ion storage (SIS) scan functions and automatic gain control (AGC) have significantly improved the versatility of these instruments for quantitative analysis.[17,18] The SIS scan function enables the selective storage and/or ejection of single ion species using multifrequency wave forms. This feature can increase instrument sensitivity through the removal of unwanted ions from the sample matrix and column bleed (gas chromatography applications). Automatic gain control prevents the buildup of excessive space charge by maintaining the correct number of ions in the trap through the adjustment of ionization time, leading to a greatly improved dynamic range.

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It was our hypothesis that by sweeping out nontarget molecules from the ionization chamber using a complex broad-band wave form, ion−molecule reactions would be minimized and undesirable chemical noise eliminated. This reduction in noise would expand the analytic range of the method to isotopic enrichment levels lower than those previously obtainable on linear-beam instruments. We applied this technique to measure retinol-d_4/retinol ratios in human plasma from an individual administered an oral dose of 30 mg of β-carotene-d_8.

EXPERIMENTAL SECTION

Chemicals. *trans*-β-Carotene-10,10′,19,19,19,19′,19′,19′-d_8 (β-carotene-d_8) and retinyl-10,19,19,19-d_4 acetate were purchased from Cambridge Isotope Laboratories (Woburn, MA). Isotopic purity for the deuterated retinol was 83% retinol-d_4, 17% retinol-d_3; β-carotene-d_8 was 80% β-carotene-d_8, 16% β-carotene-d_7, and 4% β-carotene-d_6. *N*-Methyl-*N*-(*tert*-butyldimethylsilyl)trifluoracetamide (MTBSTFA) was purchased from Regis (Morton Grove, IL) in glass ampules.

Sample Preparation. Serial blood specimens were drawn over a 16-day period from a healthy 22-year-old female volunteer (55 kg) who ingested 30 mg of β-carotene-d_8 followed by a low carotenoid breakfast consisting of a bagel with cream cheese and a glass of low fat milk. Blood was collected in glass tubes containing K_3EDTA and the plasma separated by centrifugation and stored at −20 °C prior to analysis. Retinol isotopomers were extracted using a single-column solid-phase extraction (SPE) method previously described,[7] converted to their *tert*-butyldimethylsilyl ethers (tBDMS-retinol and -retinol-d_4) with MTBSTFA,[8] and stored at −20 °C until analysis by GC/MS.

GC/MS Conditions. The GC/MS experiments were carried out using a Varian Saturn 4D quadrupole ion trap mass spectrometer (Varian Associates, Walnut Creek, CA) running Saturn Revision 5.2 software. SIS experiments were created using SIS Version 1.0 software. The system was equipped with a Varian Star 3400CX gas chromatograph and a 1078 universal capillary injector. The GC was fitted with a DB-23 column (50% cyanopropylpoly(dimethylsiloxane); J&W Scientific, Folsom, CA) 15 m in length, with an internal diameter of 0.25 mm and a phase thickness of 0.15 μm. A helium head pressure of 12 psi was used; the carrier gas also acted as the buffer gas within the ion trap. The injector and MS transfer line were maintained at 250 °C. The GC oven temperature was 140 °C (initial hold 1 min), then 25 °C/min to 195 °C, then 12 °C/min to 218 °C, and then 25 °C/min to 255 °C (final hold 2 min). The ion trap manifold was held at 200 °C.

The molecular masses of tBDMS-retinol and -retinol-d_4 are 400 and 404 D, respectively. Analyses were performed using electron impact ionization in the SIS mode to store ions between m/z 390 and 420. The voltage adjustment factor (VAF) was set at 175%. This factor controls the amplitude of the applied rf (radio frequency) wave form. The manufacturer does not document the actual value of the voltage controlled by this factor. The background mass was set to m/z 48 during the SIS experiments; this corresponds to a q_z value of ∼0.11 for the molecular ions. Spectra were recorded using an m/z range of 240−420 using the AGC feature and an ion current target value set at 8000.

Instrument Calibration. The normal physiological concentration of retinol ranges from 1 to 1.5 μM (286−429 ng/mL) in

human plasma.[14] Calibration standards were prepared by adding 8670 ng of retinol from a spectrophotometrically calibrated solution to 0.00, 19.7, 42.3, 84.6, 141.0, 423.0, 1692.0, and 8670.0 ng of retinol-d_4. These solutions were concentrated to dryness under argon and derivatized in 100 μL of MTBSTFA to produce molar ratios of 0.000, 0.0023, 0.0049, 0.0098, 0.0163, and 0.0488 of retinol-d_4/retinol. Molar ratio standards of 0.1952 and 1.0000 of retinol-d_4/retinol were also prepared though it was necessary to dilute these standards by a factor of 10 with isooctane for MS analysis (see Results section). Typically, 0.5 μL of standard was injected onto the chromatograph. The best-fit line was determined by least-squares linear regression of the peak area retinol-d_4/retinol ratios and measured ratios.

Other Instrumentation. For comparative purposes, the following linear quadrupole instruments were also tested using the same standards and samples: VG Trio-2 mass spectrometer (VG Masslab, Altrincham, U.K.), Hewlett-Packard MSD model 6890 mass spectrometer (Hewlett-Packard, Palo Alto, CA), and Shimadzu QP-5000 mass spectrometer (Kyoto, Japan). Data were acquired using 70-eV electron ionization and selected ion monitoring (SIM) of fragments at m/z 255 and 259, which were chosen because molecular ion abundances were lower with the quadrupole instruments than the ion trap.

RESULTS

Ion trap mass spectra of tBDMS-retinol and tBDMS-retinol-d_4 from m/z 200−420 are presented in Figure 1. Molecular ions are present at m/z 400 and 404 for tBDMS-retinol and -retinol-d_4 respectively. Major fragment ions are generated from the loss of C_4H_9 $[M - 57]^+$on the silyl group and cleavage between the C-14 and C-15 $[M - 145]^+$ carbons on the side chain, producing fragment ions of m/z 343 and 347, and 255 and 259, from tBDMS-retinol and tBDMS-retinol-d_4, respectively.

The molecular and main fragment $[M - 145]^+$ ions are present at approximately equal magnitudes in the ITMS-generated spectra (Figure 1), and both were tested for their ability to serve as quantitative analytes. The utility of the analytes was assessed by the magnitude and reproducibility of the signal ratio (A_{404}/A_{400}, A_{259}/A_{255}) obtained from unlabeled retinol. The ratio (A_{259}/A_{255}) obtained from analysis of the tBDMS-retinol fragment (SIS mass window from m/z 250 to 265, VAF 150%) was 0.33 ± 0.038%, relative standard deviation (RSD) 11.5% ($n = 5$), with a limit of quantification of 0.44 mol % enrichment (mean + 3 SD). Quantitation at the molecular ion provided superior analytical results owing to reduced background signal and improved precision. Replicate ITMS analysis of tBDMS-retinol generated an observed ratio (A_{404}/A_{400}) of 0.13 ± 0.0095%, RSD 7.4% ($n = 5$). This is below the value predicted from natural abundances (0.21%). This small discrepancy is attributed to truncation of weak signal during signal digitization. The limit of quantification was determined to be 0.16 mol % enrichment.

Selected ion storage chromatograms showing the dilution of the retinol-d_4 isotope in serial human plasma collected over 16 days following a 30-mg oral dose of β-carotene-d_8 are presented in Figure 2. The broad-band wave form was constructed so that all ions between m/z 390 and 420 were retained, while matrix and fragment ions outside this m/z range were ejected. The observed signal ratio (A_{404}/A_{400}) is 0.64%. Triplicate analyses showed excellent precision with an RSD < 4% ($n = 3$). The

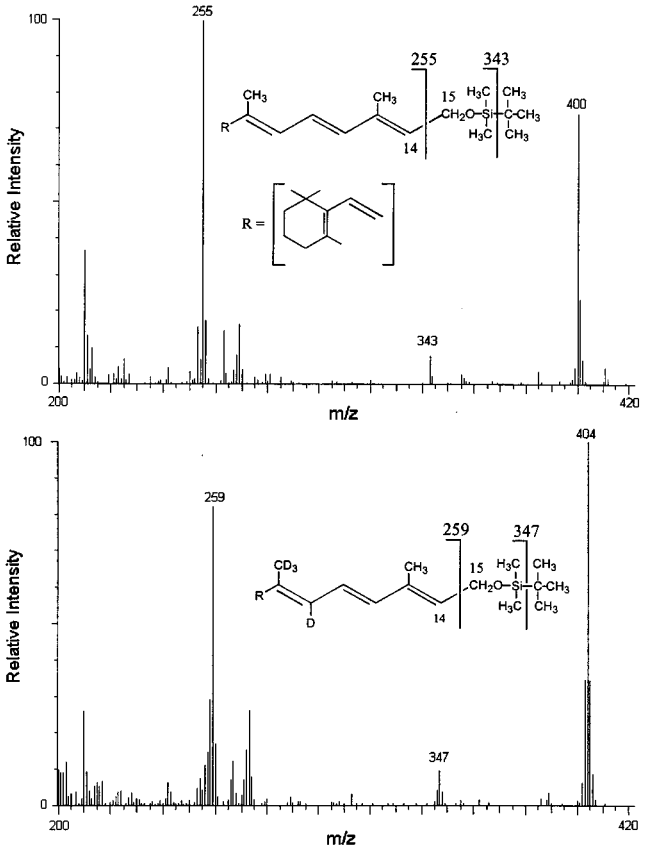

Figure 1. Electron ionization impact spectra from retinol and retinol-d_4 tBDMS silyl ethers. Molecular ions appear at m/z 400 and 404 and are sufficiently abundant for quantitative analysis. Major fragment ions appear from mass losses of $[M - 57]^+$and $[M - 145]^+$. The mole fraction of the deuterated compound was 83% retinol-d_4, 17% retinol-d_3.

selectivity provided by the SIS method is apparent from the minor abundance of ions outside the specified mass storage range in the spectra (bottom panel) and the absence of closely coeluting interferences in the chromatograms as evidenced by the symmetrical peak shape of the ion channel chromatograms.

Selected ion chromatograms of tBDMS-retinol from a VG Trio-2 quadrupole mass spectrometer are shown in Figure 3. The observed integrated ratio (A_{259}/A_{255}) was 0.5% instead of the theoretical value of 0.006% (based on natural abundances). The observed ratio varied from 0.4 to 1.0% among the quadrupole instruments tested (VG Trio-2, Hewlett-Packard MSD model 6890 and Shimadzu QP-5000 mass spectrometers) under conditions of optimal tuning and mass resolution. Molecular ions were insufficiently abundant for reliable quantitative analysis though substantial signal at m/z 404 was detectable during tBDMS-retinol analysis.

The ITMS method was tested for linearity over molar concentration ratios ranging from 0.0023 to 1.0000 (A_{404}/A_{400}). Plots generated over a 4-day period showed good linearity with $r^2 > 0.99$ ($n = 5$) though there was difficulty with the 0.1952 and 1.0000 standards. These samples required a 10-fold dilution to provide accurate and consistent values. The dilution minimized mass shifts that are attributed to ion-coupling interactions in the trap, a concentration-dependent phenomenon that is observed when ions of close secular frequencies are stored (see Discussion). In

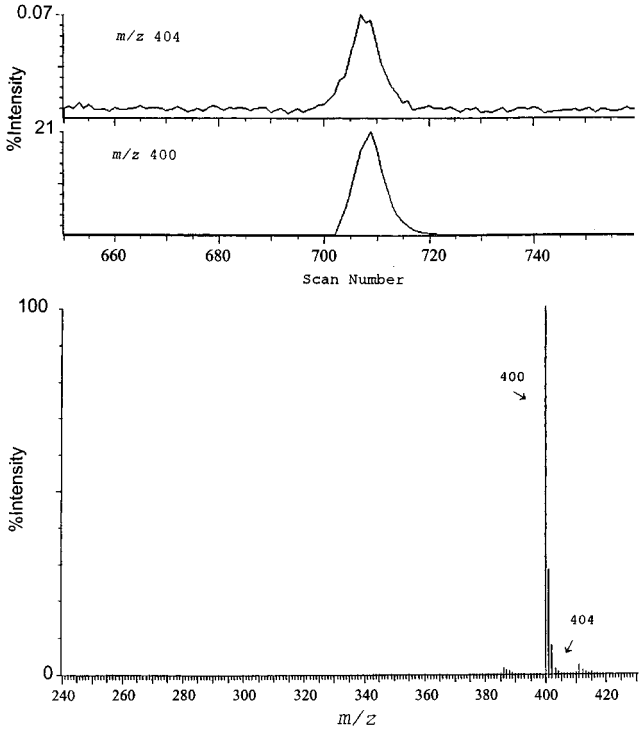

Figure 2. Selected ion storage chromatograms showing dilution of the retinol-d_4 isotope in human plasma drawn 16 days following a 30-mg oral dose of β-carotene-d_8. The calculated ratio (A_{404}/A_{400}) shows the plasma sample to contain 0.64% isotope (top panel). Triplicate analysis showed excellent precision with an RSD < 4% (n = 3). The efficiency of unwanted ion removal is apparent from the minor abundance of ions outside the mass storage range (bottom panel).

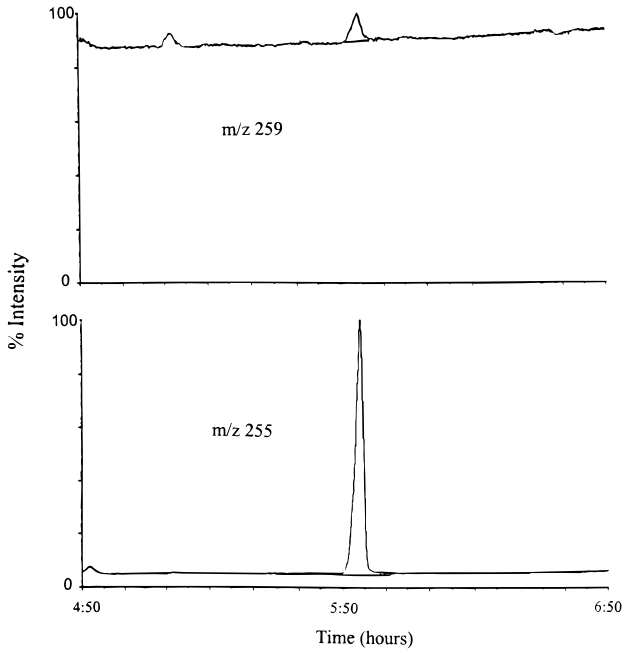

Figure 3. Selected ion chromatograms of a *t*BDMS-retinol standard (*m*/*z* 255) acquired on a VG Trio-2 quadrupole mass spectrometer illustrating the unexpectedly high background signal at the mass of the deuterated isotopomer (*m*/*z* 259). The observed ratio of 0.5% (A_{259}/A_{255}) exceeds the value of 0.02% predicted by natural abundances. Background values of 0.4−1% were observed in the spectra generated on a VG Trio-2, Hewlett-Packard 5972, and Shimadzu QP-5000 quadrupole instrument.

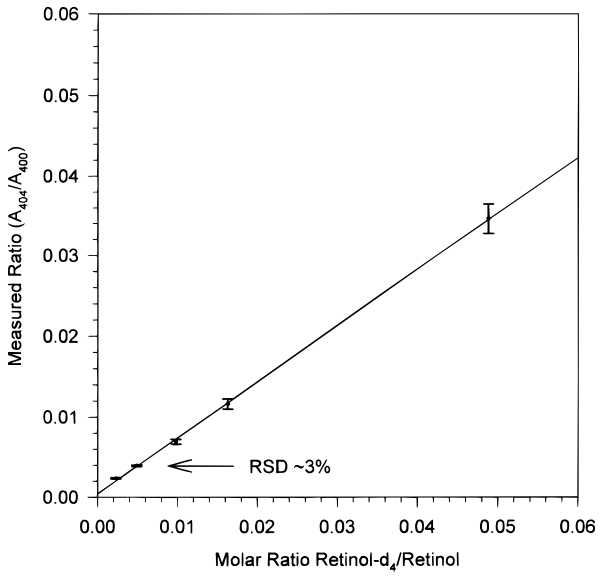

Figure 4. Expanded view of the instrument response plots for determination of retinol-d_4/retinol generated using standard mixtures of the isotopomers 0.0023−0.0488 (A_{404}/A_{400}). Mixtures were prepared by adding varying amounts of retinol-d_4 to fixed amounts of retinol as described in experimental Section.

practice, these calibration points were not important in our analyses since levels of enrichment of >5% were not exceeded. Maximum labeling at day 1 after tracer administration was <5% and declined rapidly thereafter. For this reason, the standard curve is presented in Figure 4 over the range of 0.0023−0.0488 ($y = 0.679x + 5.374 \times 10^{-4}$). As determined from the integrated areas (A_{404}/A_{400}), the observed relative molar response of retinol-d_4 centered on 0.68. The magnitude of this value can only partially be explained by the isotopic profile of the standard (83% retinol-d_4). The RSD at retinol-d_4/retinol ratio 0.00488 was ~3% (n = 5). Approximately 0.5 μL aliquots of the samples and standards were injected onto the GC/MS. Analysis of 197 pg of *t*BDMS-retinol standard (injected) yielded a signal-to-noise ratio of 20 from the chromatogram for *m*/*z* 400, suggesting detection limits in the low-picogram range.

The isotopic purity of the plasma retinol (that originates from β-carotene-d_8) and the synthesized retinol standard are expected to differ since the retinol standard does not originate from the β-carotene-d_8 molecule. This difference is adjusted for as follows: The β-carotene-d_8 (β-carotene-d_8 was 80% β-carotene-d_8, 16% β-carotene-d_7, and 4% β-carotene-d_6) is predicted to yield retinol of 88% mole fraction retinol-d_4 based upon central cleavage in the intestine (80% β-carotene-d_8 → 80% retinol-d_4; 16% β-carotene-d_7 → 8% retinol-d_4 and 8% retinol-d_3; the other β-carotene isotopomers will not produce a tetradeuterated retinol); the standard is 83% retinol-d_4. Thus, the calculated value for the plasma ratio is overestimated by 6% when determined from the calibration curve (constructed with synthesized retinol-d_4) and all values must be multiplied by 0.94 to obtain a truer estimate of the actual ratio.

Application of the SIS−ITMS method to plasma specimens from an adult female who had ingested 30 mg of β-carotene-d_8 is shown in Figure 5. Following the point of maximum label concentration at 4.6%, the ratio declines exponentially until a point of maximum concavity, whereafter, the label concentration de-

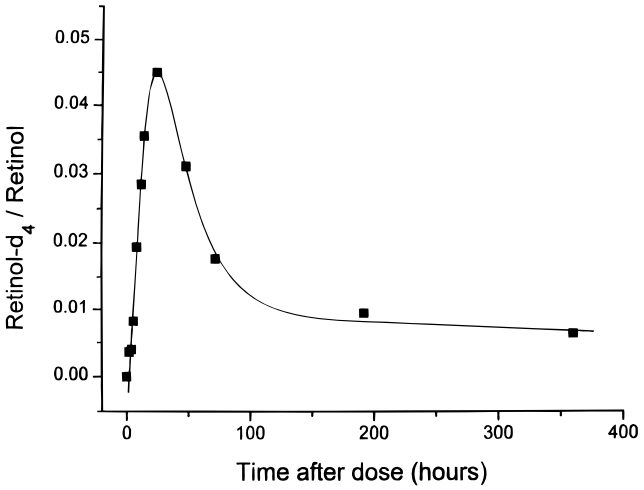

Figure 5. Changes in the ratio of retinol-d_4/retinol in human plasma following a 30-mg oral dose of β-carotene-d_8. Dramatic differences among individuals were observed in their relative abilities to metabolize β-carotene to retinol (vitamin A).

clines linearly. Because of the low rate of bioconversion of β-carotene to retinol, retinol-d_4/retinol ratios rarely exceed 5%. Significantly higher ratios are seen in retinyl-d_4 acetate experiments due to the efficient absorption of this compound. The large range encountered between the points of maximum labeling and latter time points illustrates the importance of instrument linearity over wide range of concentrations.

DISCUSSION

This study shows that ion trap mass spectrometry operated in the selected ion storage mode can expand the dynamic range of retinol isotopomer analyses to enrichments lower than previously obtainable using linear-beam quadrupole instruments. The improvement comes by minmizing the background signal at the mass of the deuterated isotopomer which arises from proton- and hydrogen-transfer reaction (ion−molecule reactions) to unlabeled retinol following ionization. A similar phenomenon was previously observed in a study of fatty acid methyl esters where the ratios of signals for labeled isotopomers were shown to be concentration-dependent;[19] this phenomenon was also attributed to ion−molecule reactions in the ion source. The significance of ion−molecule reactions can be expected to depend on sample amount and ion residence times in the source, and with the Saturn ion trap, ionization occurs within the source. This configuration leads to reduced ion residence times (compared to external ionization) and limits the magnitude of facile ion−molecule reaction.

Retinoids are known to be easily protonated as shown from the reactions of anhydroretinol with anhydrous hydrogen chloride[20] and among several retinyl derivatives with hydrogen ions released radiolytically in solution.[21] Similar behavior has been observed with other highly conjugated polyenes.[22] The EI mass spectra of retinol derivatives contains an abundance of odd-electron fragment ions that can serve as reactive donors of protons or hydrogens. Preliminary evidence in our laboratory showed extensive hydrogenation of retinol when hydrogen was used as a carrier gas during GC/MS analysis (unpublished observations). These findings illustrate the unusual, but not unexpected, susceptibility of retinol to undergo addition reactions when hydrogen atom concentrations become significant.

The molecular and main fragment (m/z 400 and 255, respectively, for the endogenous compounds) ions were tested for their ability to serve as quantitative analytes. Natural abundances predict a substantially lower background signal at the mass of the deuterated isotopomer for the main fragment relative to the molecular ion due to the absence of the silyl group (A_{259}/A_{255} 0.006%; A_{404}/A_{400} 0.21% for unlabeled tBDMS-retinol) though, experimentally, the molecular ion showed lower background and provided superior analytical results. The background ratio for the fragment (A_{259}/A_{255}) ion was 0.33 ± 0.038% enrichment vs 0.13 ± 0.0095% for the molecular ion (A_{404}/A_{400}). The high value associated with the fragment ion is attributed to two processes: (1) the SIS mode does not precisely isolate single mass, but rather a mass range; this inability to precisely retain only the analyte ions increases the potential for background signal due to the coisolation of reactive fragments in the lower mass regions; (2) intramolecular hydrogen transfers from the silyl group to the polyene prior to fragmentation and similar intermolecular processes. Even though a higher than expected signal (A_{259}/A_{255}) was obtained with ITMS analysis of the fragment ion, the signal was more reproducible than values ranging from 0.4 to 1% obtained on linear-beam instruments.

Improved signal-to-noise ratio for the molecular ions was associated with decreasing the frequency notch for the storage conditions and increasing the modulation amplitude of the rf storage setting using the VAF. It was observed that higher VAF settings (range 50−200%) required wider mass windows to avoid partial ejection of the molecular ions: a VAF setting of 75% with a m/z 398−408 mass window produced reproducible calibration curves; this same window at 175% VAF produced nonlinearity in the curves and a notable reduction in signal in the 404 channel, leading to a plateau in the measured ratios at increasing retinol-d_4 concentrations. Large amplitudes are known to distort the frequency domain cutoffs, causing the pulse to have a wider frequency range than desired.[23] Some loss of total signal was associated with increasing VAF settings due to increased collision-induced dissociation (CID) in addition to ion instability. A 175% setting was selected with the 390−420 mass storage window because it provided better precision, particularly with biological samples, due to superior peak shape and an improved signal-to-noise ratio.

Success with SIS depends on the avoidance of excess space charge within the trap which appears when the total ion density in the trap exceeds ~10^5 ions. Above this ion concentration, space charge effects negatively affect mass resolution and ion−molecule reactions lead to degradation of mass spectral quality.[15] Total ion concentration is controlled though adjustment of ionization times using the AGC. This feature is critical for GC/MS applications where the concentration of the analyte can vary by several orders of magnitude across the eluting peak. In our analyses of biologic samples, setting the target value above 14 000 occasionally produced a loss of mass resolution and peak shape, presumably

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from space charge effects. Standards could be analyzed at higher AGC settings before space charge effects appeared. To provide an adequate margin of safety, the method used a target value of 8000 for both standards and biologic samples.

Calibrating standards were prepared with varying retinol-d_4 concentrations in the presence of constant retinol concentrations. Aliquots of approximately 0.5 μL were injected onto the instrument. For the 0.1952 and 1.0 ratio standards, a 10-fold dilution with isooctane was necessary to obtain accurate mass assignments. At higher concentration, a 1 unit mass shift was observed for the protio species, inaccurately producing a m/z of 401 for this species, particularly at the apex of the GC peak (highest concentration). This shift was not observed at lower levels of enrichment. Such mass shifts have been described by Cox et al.[24] as arising from 'local space charge effects' and further explained in an ion-coupling model proposed by Mo and Todd.[25]

The ion-coupling phenomenon arises when more than one species of ion is trapped and is most pronounced when ions of close secular frequencies are stored. The frequency shift of one species of ion is directly proportional to the concentration of neighboring ion species and the inverse of their mass difference. The net field formed by the sum of the ion−ion interactions produces delayed ion ejection, and a higher apparent mass is observed. When experiments are performed with forward rf amplitude scans, the mass shift is only observed in the lower m/z ion as ejection of the high-massed ion would have been preceded by ejection of the lower massed ion. In our experiments, the magnitude of the shift was concentration-dependent, and at low ion concentrations, the ion−ion net field is minimized, reducing the magnitude of the effect. For this reason, a 10-fold dilution of the 0.1952 and 1.0 ratio standards was prepared. While none of our biological samples approached this level of enrichment, in such circumstances a similar level of dilution is recommended.

Samples were prepared as previously described using aminopropyl solid-phase fractionation of the hexane extracts of deproteinized plasma.[7] This method permits the separation of intact β-carotene-d_8 from its retinol-d_4 metabolite and provides retinol fractions that are sufficiently free from interferences for GC/MS analysis using a DB-23 (50% cyanopropyl methylpolysiloxane) phase. A DB-1 (100% dimethylpolysiloxane) column phase is suitable following further sample purification of the retinol containing fraction, such as the reversed-phase high-performance liquid chromatographic (RP-HPLC) technique described by Handleman et al.[8] In cases where very low retinol-d_4 incorporation into endogenous pools is expected, RP-HPLC purification will increase the accuracy of the measurement at lower levels of enrichment (~1/200) due to reduced chemical noise in the deuterated channel. Chromatographic background signal appears to originate from lipophilic compounds present at high concentrations in biological extracts. Our experience is that the DB-23 phase delivers better selectivity from interferences than the DB-1 even following RP-HPLC purification. The complete removal of lipophilic contaminants from HPLC systems is difficult and requires rigorous cleaning of the injector, system tubing, and column. The presence of lipophilic interferences necessitates the use of highly selective ion monitoring modes to minimize signal from nontarget analyte ions.

In conclusion, ITMS offers the advantage of removing reactive ions when ionization occurs inside the ion trap. Using this approach, unlabeled retinol yields a background signal at the mass of the labeled tracer close to values calculated from natural isotopic abundances. This will expand the scope of vitamin A/β-carotene kinetic studies by permitting the reliable quantitation of labeled vitamin A at low enrichments.

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