



Vitamin A concentrations in liver determined by isotope dilution assay with tetradeuterated vitamin A and by biopsy in generally healthy adult humans¹⁻³

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ABSTRACT The vitamin A status in 11 generally healthy surgical patients was estimated by measuring the dilution of a 45-mg oral dose of tetradeuterated retinyl acetate (99% pure). After purification of retinol by high-performance liquid chromatography, the ratio of ²H₄-retinol:¹H-retinol in plasma was measured by gas chromatography-mass spectrometry. On the basis of the observed ratios of [²H₄]retinol:[¹H]retinol over 19–47 d, the total body reserves and liver concentrations of vitamin A were calculated. Liver biopsy samples taken at surgery were directly analyzed for vitamin A. The correlation coefficient between calculated and measured liver vitamin A concentrations for 10 of the subjects was 0.88, and the Spearman's rank correlation coefficient was 0.95 ($p < 0.002$). Thus, total body reserves of vitamin A in humans can be estimated validly in the marginal and satisfactory ranges by a benign, relatively noninvasive procedure. *Am J Clin Nutr* 1989;49:713–6.

KEY WORDS Vitamin A, retinol, tetradeuterated retinol, isotope dilution, total body reserves of vitamin A, GC-MS analysis

Introduction

Serum retinol (vitamin A alcohol) levels are maintained nearly constant over a wide range of vitamin A intakes. The bulk of the total body vitamin A in the well-nourished individual (perhaps 90% of the total) is stored in the liver as retinyl esters (1, 2). Thus, determination of serum vitamin A levels alone is inadequate for estimation of vitamin A status in experimental animals and in humans except when values are very low ($< 0.35 \mu\text{mol/L}$) or very high ($> 3.5 \mu\text{mol/L}$). The direct measurement of liver vitamin A concentrations by biopsy, needless to say, can be carried out only in a few subjects under exceptional, justifiable circumstances.

An indirect approach to estimating total body vitamin A (namely, isotope dilution of a dose of a labeled vitamin A tracer) was suggested and experimentally verified in rats, sheep, and cattle (3–6). This approach also was employed in one human study in which both radioactive [³H]vitamin A and a nonradioactive [²H]vitamin A tracer gave similar results (6). However, in that study the results of the isotope dilution analyses were not verified by direct sampling of the liver vitamin A of the subject. Because the isotope-dilution procedure using nonradioactive label shows promise as a relatively noninvasive procedure for the estimation of human vitamin A status, we wished to establish its validity in human subjects.

Methods

Subjects

Eleven hospitalized patients (aged 22–87 y) scheduled for cholecystectomy ($n = 6$), gastroplasty ($n = 4$), and splenectomy ($n = 1$) were admitted into the study. In all except subject 7 (Table 1), who had viral hepatitis A, liver function tests as well as concentrations of serum albumin and total plasma protein were normal. As judged by dietary interview, nine subjects routinely ingested normal amounts (900–2100 mg) of carotenoids and vitamin A whereas subjects 1 and 2 ate foods containing very small quantities of carotenoids and vitamin A.

During abdominal surgery, biopsy samples (20–110 mg) were taken from the right lobe of the liver by the attending sur-

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² Supported by USDA Competitive Research Grant 84-CRCR-1-1418 and by NIH-AM/DK 32793 of the US Public Health Service. This is Journal Paper J-12776 of the Iowa Agriculture and Home Economics Experiment Station, Ames, IA, Project 2534.

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Received September 2, 1987.

Accepted for publication April 5, 1988.

TABLE 1

Calculated and measured liver concentrations of vitamin A and related variables in generally healthy surgical patients

Subject	Sex	Age	Weight	Serum retinol	H-D ratio	Calculated TLR*	Vitamin A in liver		
							Calculated	Measured	Calculated:measured
		y	kg	μmol/L		mmol		μmol/g	
1	F	23	129	1.40	5.78	0.16	0.065	0.049	1.32
2	M	52	70	1.61	5.03	0.13	0.068	0.105	0.65
3	F	22	73	1.96	5.85	0.16	0.093	0.20	0.47
4	F	38	92	1.29	19.2	0.72	0.30	0.30	1.00
5	F	54	127	2.69	17.5	0.63	0.23	0.33	0.69
6	F	25	132	1.36	25.6	1.09	0.53	0.37	1.42
7	F	29	84	2.52	24.4	0.88	0.38	0.39	0.98
8	F	87	76	2.06	4.74	0.12	0.080†	0.40	0.20
9	F	32	145	1.64	26.3	0.99	0.37	0.40	0.94
10	F	31	128	1.85	38.5	1.60	0.72	0.48	1.49
11	F	69	59	1.33	50.0	2.02	1.12	0.56	2.01

* Total liver reserve of vitamin A.

† Excluded from analysis.

geon. Informed consent was obtained in all cases. The procedures followed were formally approved by the Human Use Committee of Mary Greeley Medical Center and by the Iowa State University Committee on the Use of Human Subjects in Research. In all cases the structure of the liver was normal by histological examination of the biopsy specimen. The remainder of the biopsy sample was frozen at -20°C and stored frozen in the dark in a hermetically-sealed vial until analyzed for vitamin A by extraction and high-performance liquid chromatography (HPLC) (7).

Laboratory procedures

Four to 6 d after surgery, a relative dose response (RDR) test was conducted in the conventional manner with an oral dose of $450\text{ }\mu\text{g}$ retinyl acetate in corn oil. The results of these studies already have been reported (8). Shortly thereafter each subject was given 45 mg all-*trans* 10,19,19,19- $[\text{}^2\text{H}_4]$ retinol as retinyl acetate in corn oil. The $[\text{}^2\text{H}_4]$ retinyl acetate (99% isotopic purity) was synthesized, characterized, and purified as described previously (9).

Two or three blood samples (5 mL each) were taken from each subject at times ranging from 10 to 47 d after administration of the labeled vitamin A dose; plasma was obtained and frozen at -20°C . Two-milliliter portions of plasma from each sample were extracted with hexane after precipitation of plasma proteins with ethanol; the combined hexane extracts of each plasma sample were evaporated gently under argon and redissolved in 2-propanol. Each sample was analyzed by gradient reversed-phase HPLC (10) and the retinol fraction from each was collected. Retinol was analyzed for its ratio of ^1H to $^2\text{H}_4$ (H-D ratio) by capillary gas chromatography-mass spectrometry (GC-MS) as recently described (11). Underivatized retinol was chromatographed by on-column injection on a $15\text{ m} \times 0.25\text{ mm}$ DB-1 open tubular capillary column (J&W Scientific, Rancho Cordova, CA) with direct connection to a VG ZAB-HS mass spectrometer (VG Instruments, Stamford, CT). $[\text{}^2\text{H}_4]$ Retinol standard was prepared by saponification of $[\text{}^2\text{H}_4]$ -retinyl acetate and HPLC purification; $[\text{}^1\text{H}]$ retinol standard was prepared by reduction of retinal (Sigma, St Louis, MO) with NaBH_4 and purified by HPLC. Selected ion monitoring

in the mass spectrometer was performed on m/z 286 $[\text{M}^+]$ and m/z 268 $[\text{M}-\text{H}_2\text{O}]^+$ for $[\text{}^1\text{H}]$ retinol and on m/z 290 and m/z 286 for $[\text{}^2\text{H}_4]$ retinol. A calibration curve was determined by analyzing known ratios of $[\text{}^2\text{H}_4]$ retinol to $[\text{}^1\text{H}]$ retinol. H-D ratios for samples were determined by using the mass spectrometer responses for the $[\text{M}-\text{H}_2\text{O}]^+$ ion ratios (m/z 268: m/z 272) because of their greater sensitivity (11).

Calculation of total body reserves and liver concentrations of vitamin A

In using the isotope-dilution procedure in animals, Bausch and Rietz (6) employed the formula

$$\text{TLR} = F \times \text{dose} \times [(\text{H:D}) - 1] \quad (1)$$

where TLR is the pretreatment total liver reserve of vitamin A in millimoles retinol, F is a factor that expresses the efficiency of storage of an orally administered dose (taken as 0.5 in their extensive studies), dose is the oral dose of labeled retinol in millimoles, H:D is the ratio of ^1H to deuterated retinol in the plasma after an equilibration period (eg, 4 d in rats and 14 d (6) or 26 d (12) in humans), and -1 corrects TLR for the contribution of the administered dose to the total body pool. Because experimental animals in those studies were fed a vitamin A-free diet during the whole experiment, a true equilibrated state was reached.

When vitamin A is ingested in normal dietary amounts (300–1500 mg/d) during the experimental period, as in the current studies, the H-D ratio in plasma is affected in two ways: 1) plasma retinol is derived both from dietary vitamin A and from endogenous liver reserves, and 2) labeled retinol in the total body reserves is replaced in part by unlabeled vitamin A of dietary origin. As to the first, the ratio of the specific activities of retinol in the plasma vs liver over a significant range (0.02–0.12 $\mu\text{mol/g}$) of liver concentrations of vitamin A in rats was found to be 0.65 ± 0.05 ($\bar{x} \pm \text{SEM}$) (13). As to the second, the H-D ratio should be corrected for the half-life of vitamin A turnover in the liver, eg, 140 d (12, 14). The formula then becomes

$$\text{TLR} = F \times \text{dose} \times (S \times a \times [(\text{H:D}) - 1]) \quad (2)$$

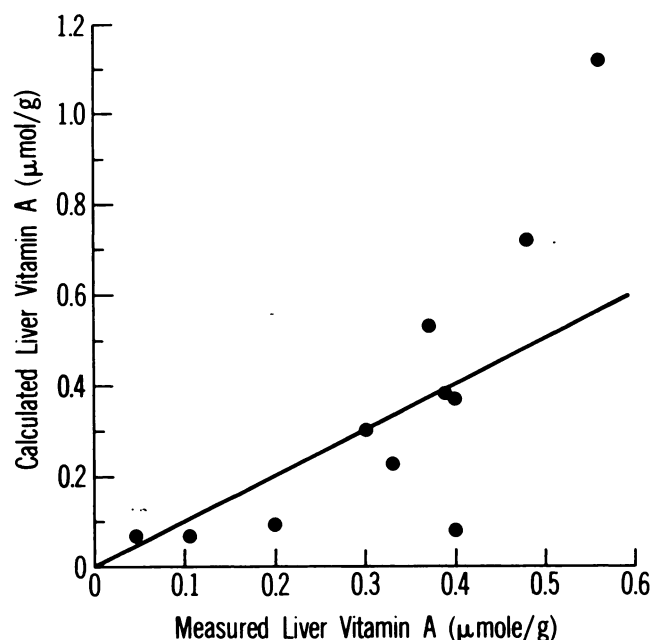


FIG 1. Relationship between calculated and measured amounts of vitamin A ($\mu\text{mol/g}$ wet wt) in the livers of 11 generally healthy human adults. The solid line is the line of identity (1:1 correspondence).

where S is the ratio of specific activities of retinol in serum to that in liver, taken as 0.65. The fraction of the absorbed dose of deuterated retinol remaining in the liver reserves at the time of blood sampling is designated a ; it is introduced into the equation as a correction to the H-D ratio because of the loss of labeled vitamin A by catabolism and because of replacement by absorbed unlabeled dietary vitamin A over time ($a = e^{-kt}$, where $k = 1/140$ d and t is time [days] since dose). For simplicity, it was assumed that a is independent of the size of liver stores and that it is time-invariant. For the various samples taken (ie, from 11 to 47 d), the estimated value of a ranged between 0.92 and 0.71.

The liver vitamin A concentration, expressed as μmol retinol/g liver, was calculated by assuming that liver weight is 0.024 of total body weight in nonobese adult subjects. In all clearly obese subjects (> 92 kg) the liver weight was presumed to be 2.2 kg.

Statistical analysis

Correlation coefficients and the Spearman's rank correlation coefficient were calculated by use of the Stat Pac program for the HP-41C calculator (Hewlett-Packard, Corvallis, OR).

Results

Information about the subjects, the H-D ratio of plasma retinol after extensive equilibration, and the calculated (means of two or three determinations) and measured liver vitamin A levels are presented in Table 1, and the latter as well in Figure 1. The correlation coefficient between calculated and measured vitamin A values, except for subject 8, is 0.88. The Spearman rank correlation coefficient, except for subject 8, is 0.95 ($p < 0.002$).

The H-D ratios alone reflect relative total body reserves with values < 6 at the lower end, 17–26 in the middle, and > 38 at the higher end under these conditions of measurement. In the two to three plasma samples taken from each patient over the course of the experiment, the H-D ratio of plasma retinol expectedly increased with time (data not shown). As indicated earlier (8) plasma values showed no relationship with liver reserves over the range studied (0.05 – 0.56 $\mu\text{mol/g}$). Similarly, liver reserves were not age-related in this small sample of adults (Table 1).

Discussion

Vitamin A status can best be expressed in terms of total body reserves of vitamin A, or alternatively, of liver concentrations of the vitamin (14). Five states of vitamin A nutriture might be defined; namely, deficient, marginal, satisfactory, excessive, and toxic. Liver concentrations of vitamin A characteristic of the deficient, marginal, and satisfactory states are < 0.017 $\mu\text{mol/g}$, 0.017 – 0.070 $\mu\text{mol/g}$, and > 0.07 $\mu\text{mol/g}$, respectively (15). Liver reserves are less appropriate indicators, however, for defining excessive and toxic states.

In the current study, we present for the first time a generally applicable method for determining total body reserves of vitamin A from the marginal through an appreciable part of the satisfactory (adequate) range. The method has been validated by comparing measured and calculated liver vitamin A concentrations, which gave a correlation coefficient of 0.88. Although subject to future refinement, the procedure currently is applicable to specific groups in the population. Its advantages are 1) a sizable but nontoxic dose of retinol labeled with a benign heavy isotope in a nonexchangeable position is given, 2) few, and possibly only one, blood sample need be taken, and 3) no other method exists for determining the total body stores of vitamin A.

The assumptions used in this calculation might be briefly explored: F , the storage efficiency of an administered oral dose, is taken as 0.5 on the basis of extensive studies in animals (6) and in man (12). Storage efficiency decreases, however, when initial liver stores are < 0.06 $\mu\text{mol/g}$ (6). Only subject 1 in our sample showed a lower liver concentration ie, 0.049 $\mu\text{mol/g}$. If the storage efficiency is only 0.4 in this case, in keeping with animal data (6), the calculated (0.052 $\mu\text{mol/g}$) and measured (0.049 $\mu\text{mol/g}$) values would be even more concordant. S , the ratio of specific activities of retinol in the plasma and liver of subjects ingesting moderate amounts of vitamin A during the equilibration period, is taken as 0.65 on the basis of rat studies (13). By using the measured liver values of vitamin A in the cited equations, a mean empirical value for S , based on data from all subjects except subject 8, is calculated to be 0.68 ± 0.08 , an empirical agreement that supports continued use of the 0.65 value. Factor a , the correction for replacement of deuterated vitamin A by dietary unlabeled vitamin A during the equilibration period, is based on a half-life of vitamin

A in human liver of 140 d (12, 14), ie, a fractional catabolic rate (k) of 0.005 d^{-1} . In keeping with this value, we recently found longest and next-longest half-life values of 241 and 33 d, ie, k values of 0.0029 and 0.021 d^{-1} , respectively, in a single subject studied with tetradeuterated vitamin A for 28 d (16). With an equilibration period of 47 d, the longest used here, the correction in the H-D ratio is only 29%. Although the fractional catabolic rate may not be truly time-invariant and may well depend to some degree on the individual's vitamin A status (as has been shown in rats at very low liver reserves (17)), the simplifying assumption of a single value for generally well-nourished adults seems justified here. Calculated values of liver vitamin A for each individual as determined at two or three time points tended to increase with time (implying an underestimate of a) but not markedly (data not shown). Thus, significant differences in the half-life values in individuals should not markedly affect the calculated TBR.

Subjects 8, 10, and 11 in our sample deserve special comment. Subject 8 was an 87-y-old woman who underwent a partial splenectomy. The measured vitamin A concentration in her liver was $0.40\text{ }\mu\text{mol/g}$ whereas the calculated value was $0.056\text{ }\mu\text{mol/g}$. Her RDR value was 12% (8), indicative of satisfactory ($> 0.07\text{ }\mu\text{mol/g}$) liver reserves. On the other hand, H-D ratios in samples taken at 11 and 41 d were both low ie, 4.10 and 4.74, respectively. Other than technical error, which is possible but seems unlikely, the only physiological explanation for this discrepancy is that for some reason the rate of equilibration of vitamin A between the plasma and liver in this very elderly patient was very slow. Because the distribution of vitamin A in the liver is heterogeneous (18), the biopsy sample in this case might not have been fully representative of her liver reserves. However, no other difference of this magnitude was noted in other subjects in this study, or in others (8). Because the ratio between measured and calculated liver values in this case was five, approximately twice as great as the next most aberrant value, it was excluded from statistical analysis.

In subjects 10 and 11, who had higher liver reserves, the calculated values are one and one-half- and two-fold higher than those measured. This discrepancy is partly due to the difficulty of accurately measuring the size of the deuterated retinol peak and of appropriately correcting for noise when the deuterated peak is very small, ie, when H-D ratios are high. The use of higher doses of deuterated retinol or a further increase in the assay sensitivity should increase the accuracy of measurements in this range of liver reserves. The seeming hyperbolic nature of the ratio of calculated to measured (calculated:measured) vitamin A status may also reflect the dependence of vitamin A metabolism on the individual's vitamin A status (17). However, in the application of most interest, ie, detection of deficient or marginal vitamin A status, the predictive nature of this simplified approach is satisfactory. 

We thank the following members of the medical community of Ames, IA, without whose help and cooperation this study would not have been possible: Drs Bruce Hardy, LeRoy Johnson, William Barnett, and William Bliss, surgeons at the McFarland Clinic who performed the liver biopsies; and Drs Donald Powers, C Paul Gryte, and Curtis Hoskins, pathologists who interpreted the histopathology of the biopsy specimens. We are also grateful for the sympathetic support of the administration and medical staff of the Mary Greeley Medical Center. Finally, we thank all the patients who participated in this study for their time and cooperation.

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