Plasma kinetics of vitamin A in humans after a single oral dose of [8,9,19-^{13}C]retinyl palmitate

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Abstract The kinetics of vitamin A and its major metabolites were investigated in humans. Eleven healthy male subjects ingested 105 μmol (100,000 IU) of [8,9,19-^{13}C]retinyl palmitate in an oily solution. Twenty-seven blood samples were collected during the 1-week study. Plasma samples were analyzed for retinyl esters and for [^{12}C]- and [8,9,19-^{13}C]retinol. Retinol isotopes were quantified using a newly developed GC-MS method. Total retinyl esters peaked at about 4.45 μmol/L from 3.5 to 12 h after dosing. As a result of the perturbation of the tracee system, the plasma concentration of [^{12}C]retinol increased and then decreased as the concentration of [8,9,19-^{13}C]retinol increased, indicating rapid distribution kinetics. A broad single peak (1.16 ± 0.32 μmol/L) was observed for [8,9,19-^{13}C]retinol at about 10 to 24 h postdose; this likely reflects hepatic secretion of [8,9,19-^{13}C]retinol associated with retinol-binding protein. Then, declining levels of the tracer and increasing levels of the tracee were observed. At its peak, the ingested [8,9,19-^{13}C]retinol reached about 51% of the observed total plasma retinol concentration. This percentage dropped to 13.4% on day 7 indicating slow final elimination from plasma. Our data support the concept that the liver follows the principle 'last in/first out' in maintaining vitamin A homeostasis.–**v. Reinersdorff, D., E. Bush, and D. J. Liberato.** Plasma kinetics of vitamin A in humans after a single oral dose of [8,9,19-^{13}C]retinyl palmitate. *J. Lipid Res.* 1996. **37:** 1875–1885.

Supplementary key words mass spectrometric method • retinol • retinyl esters • stable isotopes • vitamin A metabolism

Vitamin A is an essential nutrient for humans that is involved in important physiological functions including vision, growth, reproduction, and the differentiation and maintenance of epithelial tissue (1, 2). The absorption, transport, storage, and metabolism of vitamin A are highly regulated, involving a number of specific binding proteins and enzymes (3).

In recent years, great progress has been made in the field of vitamin A and retinoid research, especially regarding receptor-mediated retinoid actions (4, 5) and retinoid-binding proteins (6, 7). In spite of this growing interest in retinoids, many questions concerning plasma retinol homeostasis (8) and whole body turnover of vitamin A remain to be answered. Extensive tracer kinetic studies on whole-body vitamin A metabolism in the rat have been conducted by Green and colleagues (9–14). These studies quantified plasma retinol turnover in the rat and demonstrated that there is multiple recycling of retinol in the body.

To date, only a few retinol kinetic and metabolic studies have been carried out in humans (15–18) and there are limited data on plasma retinol turnover in humans (8). Such studies require the use of a label in order to distinguish the endogenous plasma concentrations from those resulting from the administered dose (19).

In the present study, we administered vitamin A labeled with a stable isotope and measured the plasma kinetics of vitamin A and its major metabolites in humans for 1 week. Here we describe the contribution of the labeled retinol to the total RBP-retinol plasma pool after a single oral dose of 105 μmol of [8,9,19-^{13}C]retinyl palmitate, the study design, and a new method for the analysis of stable isotope labeled versus unlabeled plasma retinol, and we discuss the results of the observed vitamin A plasma concentration versus time profiles.

SUBJECTS AND EXPERIMENTAL DESIGN

The study was conducted at Clinical Research Associates, 50 Madison Ave., New York, NY, in compliance with the principles of the 'Declaration of Helsinki' and after approval of the study protocol by the Institutional Ethics Review Committee. The subjects were recruited

Abbreviations: C_{max}, maximal measured plasma concentration; HPLC, high performance liquid chromatography; IU, international units; RBP, retinol-binding protein; t_{max}, time to reach maximal plasma concentration; UV, ultraviolet spectroscopy.

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from an available population of healthy male volunteers at the study location. All eleven healthy male subjects, 24 to 40 years of age, gave written informed consent before they were enrolled in the study.

All subjects' body weights were within 15% of ideal body weight according to the Metropolitan Life Insurance Tables. Their health status was determined by medical history, physical examination, and laboratory tests. All volunteers were non-smokers, had no history of drug or alcohol abuse, were not using medication of any kind, discontinued intake of any multivitamin preparations 2 weeks prior to the study, and did not consume alcoholic beverages starting 3 days prior to blood collection for lab tests and baseline sampling or during the study. The subjects consumed a vitamin A restricted diet throughout the study according to a list which was provided to them. Therefore, their vitamin A intake during the remainder of the 1-week study was thought to be minor. In order to collect blood for determination of fasting plasma concentrations of vitamin A and its metabolites, subjects reported to the study site 24 h prior to ingestion of the vitamin A dose. They received a low-fat snack for supper and remained at the study site overnight. After a 10-h overnight fast, a zero-h blood sample was obtained. Then, a single oral dose of 105 μmol (100,000 IU) of [8,9,19-^{13}C]-labeled retinyl palmitate was administered with 7 parts (w/w) of coconut oil in a hard gelatin capsule. Simultaneously, each subject consumed 0.5 L of whole milk, which contained 20 g of fat. No other food or liquids were allowed until after the 4-h blood sample was taken. Low-fat meals were provided after the 4-h and 8-h blood samples. Blood samples were obtained at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 7, 8, 9, 10, 11, 12, 18, 24, 48, 72, 96, 120 and 168 h after [8,9,19-^{13}C]retinyl palmitate administration. The subjects remained at the study site until after collection of the 24-h blood sample and returned after 8-h overnight fasts for collection of the final samples. At each sampling time 15 mL of blood was collected into heparinized Vacutainers by venipuncture of a forearm vein. Blood samples were immediately centrifuged for 10 min at 2500 rpm and 10°C. After the plasma had been transferred into amber, siliconized scintillation vials, samples were immediately frozen at -70°C until analysis. All sample handling and analytical work was carried out under yellow lights.

MATERIAL AND METHODS

Chemicals and solvents

Retinyl palmitate, all-*trans* retinol, oleoyl chloride, stearoyl chloride, and butylated hydroxytoluene (BHT) were purchased from Sigma Chemical Co., St. Louis, MO, and nonanoyl chloride was from Aldrich Chemical Company, Milwaukee, WI. [8,9,19-^{13}C]-all-*trans* retinol and [2,3,6,7,10,11-^{13}C]-all-*trans* retinol were synthesized by A. A. Liebmann and W. Burger, Department of Isotope Synthesis, Hoffmann-La Roche Inc., Nutley, NJ. Retinyl oleate, retinyl stearate, and retinyl nonanoate were synthesized in our laboratory using the procedure of Huang and Goodman (20). All solvents (HLPC-grade), Dulbecco's phosphate-buffered saline (PBS) 1×, sterile, without calcium and magnesium were obtained from Fisher Scientific Co., Springfield, NJ, and N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) was from Pierce, Rockford, IL.

Plasma analyses

Plasma samples were analyzed for retinyl esters, [^{12}C]- and [8,9,19-^{13}C]retinol, [^{12}C]- and [8,9,19-^{13}C]-all-*trans*-retinoic acid, [^{12}C]- and [8,9,19-^{13}C]-13-*cis*-retinoic acid, [^{12}C]- and [8,9,19-^{13}C]-all-*trans*-4-oxo-retinoic acid, and [^{12}C]- and [8,9,19-^{13}C]-13-*cis*-4-oxo-retinoic acid. The data on vitamin A metabolites will be presented elsewhere.

For the reversed-phase HPLC analysis of individual plasma retinyl esters, plasma aliquots of 0.25 to 1.0 mL were used. The retinyl ester calibration standards (1 to 1000 ng/mL) were prepared in a 5% dilution of a retinyl ester-poor plasma lot with PBS. Retinyl nonanoate was added as internal standard prior to extraction of samples and standards with 1 mL ethanol containing 1 ppm BHT and 1 mL of n-hexane. The hexane was removed and the aqueous phase was reextracted with 2 mL of n-hexane (21). The combined upper phases were dried under reduced atmospheric pressure at 8°C and samples were redissolved in 50 μL of 20% ethyl acetate in isopropanol containing 0.02% BHT. Ten-μL aliquots were injected onto a Zorbax R_xC_{18} (150 by 46 mm, 5 μm) analytical column with C_{18} Brownlee guard cartridges (15 by 32 mm). The HPLC unit consisted of a Waters Model 590 pump with an automated gradient controller, a Hewlett-Packard Autosampler Series 1050, and an ABI 783 programmable absorbance detector (Applied Biosystems).

Retinyl nonanoate, oleate, palmitate, and stearate (respective retention times of 3.8, 8.8, 9.4, and 12.2 min) were separated using a modification of the solvent system described by Furr, Cooper, and Olson (22). The mobile phase of 20% methylene chloride in acetonitrile was used at flow gradient starting with a flow rate of 1.2 mL/min for the first 6 min and then 2.0 mL/min for the next 8 min. As the HPLC method was optimized for fast separation of the retinyl esters, the retinol peak elutes very close to the void volume. For simultaneous quantification of retinol with this method, the solvent conditions would need to be adjusted to ensure separa-

tion from BHT. The UV-signal was monitored at 325 nm and automatically transferred via a Nelson Series 900 Interface into a data base. Peak integration and quantification was performed based on the peak height ratios of each of the measured esters to the internal standard using linear regression analysis. Each tray was internally validated by analysis of plasma quality control samples containing known concentrations of retinyl esters. The correlation coefficient for all of the regression lines was >0.990. The interassay variation coefficient as determined from spiked control plasma samples was 5.2%, and the limit of detection for the individual retinyl esters in plasma was 5 pmol.

A GC/MS method was developed for analysis of plasma [^{12}C]- and [8,9,19-^{13}C]-all-*trans* retinol. [2,3,6,7,10,11-^{13}C]-all-*trans* retinol (internal standard) was added to plasma aliquots (1 mL) prior to liquid–liquid extraction with 1 mL of methanol containing 1 ppm BHT and 1 mL of n-hexane. The hexane was removed and the aqueous phase was reextracted with 2 mL of n-hexane. After the hexane from the combined upper layers had been brought to dryness under a stream of nitrogen, the samples were reconstituted in 50 µL of acetonitrile. The samples were vortexed, sonicated for 10 min, and briefly centrifuged. The supernatant containing retinol was removed again into a clean tube and brought to dryness. Then, the solubilization in acetonitrile was repeated in another clean tube and the supernatant was removed and brought to dryness. Using this procedure a substantial fraction of the extracted plasma lipids remained at the bottom of each tube and were eliminated due to their poor solubility in small volumes of acetonitrile. The sample purification was necessary in order to avoid peak broadening on the GC column. The samples were then dissolved in 50 µL BSTFA to derivatize retinol to trimethysilylretinol in an instant reaction. TMS-retinol proved to be a stable derivative that could be kept up to 4 weeks in the refrigerator. Retinol analysis was performed on a Hewlett-Packard mass spectrometer HP5989A MS-Engine interfaced to a HP5890 gas chromatograph. Two-µL aliquots of the samples in BSTFA were injected via cool on-column injection onto a 1-m piece of 0.53 mm deactivated fused silica tubing (SGE, Austin Texas) connected to the stationary phase of 15-m DB-1 capillary GC column with 0.25 mm ID and 0.1 µm film thickness (J&W Scientific, Folsom, CA). Helium was used as carrier gas at a constant linear velocity of 2 mL/min. The gradient program started at an initial oven temperature of 35°C with an initial injector temperature of 38°C. The oven and the injector temperatures were then ballistically brought up to 300°C and held for 5 min. The interface temperature was kept constant at 275°C, the ion source at 250°C, and the quadrupole analyzer at 100°C. Derivatized all-*trans* retinol eluted at 4.6 min from the GC column directly into the ion source. Ions were produced by negative chemical ionization using methane/reagent gas at a gas pressure of about 1.5 Torr. Fragment ions corresponding to anhydroretinol (16, 23, 24) (**Fig. 1**) were measured in the selected ion monitoring mode with a dwell time of 20 msec/ion at the mass units of *m/z* 268, 271 and 274 (**Fig. 2**). Quantification of the samples was based on the peak height ratios of [^{12}C]- and [8,9,19-^{13}C]anhydroretinol to the internal standard [2,3,6,7,10,11-^{13}C]anhydroretinol. The reproducibility was found to be higher using the peak height ratios rather than the ratios of the integrated peak areas.

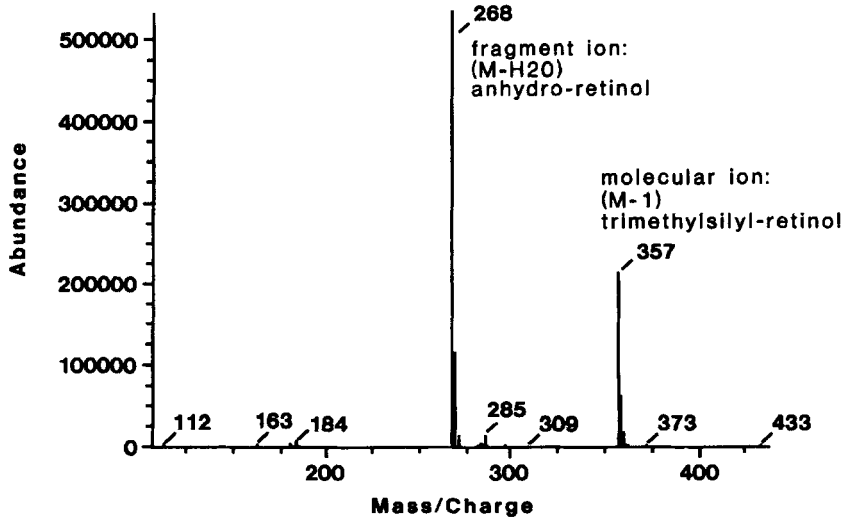

Fig. 1. Negative chemical ion mass spectrum of trimethylsilylretinol. The molecular ion (M-1) appears at *m/z* 357. The fragment ion, anhydroretinol, that is monitored for quantitative analysis appears at *m/z* 268.

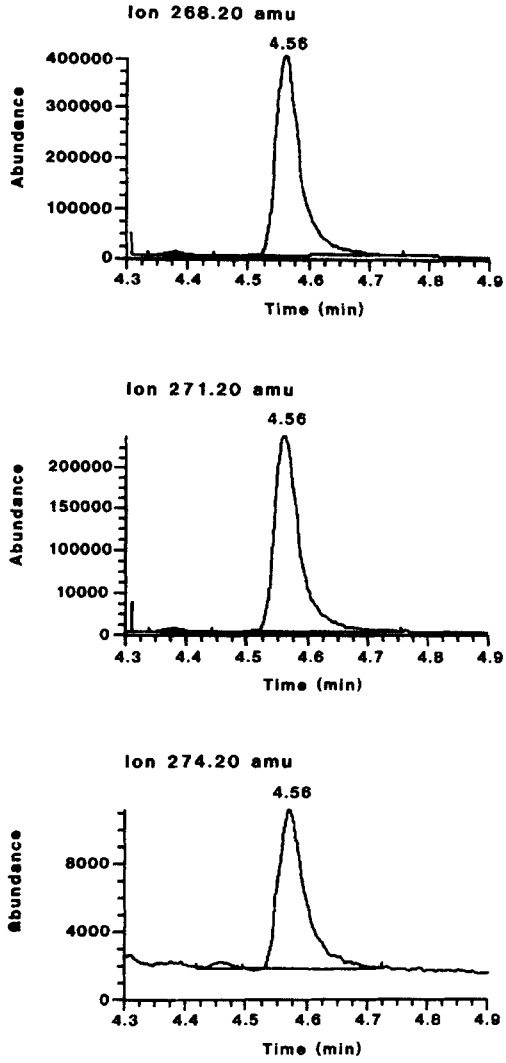

Fig. 2. GC–MS chromatograms obtained from a plasma sample 24 h after oral administration of [8,9,19-^{13}C]retinyl palmitate. The mass spectrometric detection was carried out in the single-ion monitoring mode at *m/z* 268, 271, and 274 for the unlabeled analyte, labeled analyte, and internal standard, respectively.

In order to correct for natural abundance signal overlaps, a multivariant regression analysis was performed using matrix notation (25). The peak height ratios and the corresponding concentrations of a series of standards containing varying amounts of both [^{12}C]- and [8,9,19-^{13}C]retinol and a constant amount of [2,3,6,7,10,11-^{13}C]retinol (10 ng/mL) served as reference for the unknowns. Because a deconvolution technique was applied to separate the label from natural abundance, the use of the more enriched [2,3,6,7,10,11-^{13}C]-label would not have significantly lowered the sensitivity limits of the tracer. The calibration range for [8,9,19-^{13}C]retinol ranged from 1 to 625 ng/mL and for [^{12}C]retinol from 250 to 850 ng/mL. Accuracy was within 6%, and the interassay precision as determined from spiked control plasma samples were 6.2% (unlabeled analyte) and 3.6% (labeled analyte). Spiked control plasma samples were also used in each tray for internal validation of the assay. The purity of the standards was confirmed on the same analytical system by individual injection of the three derivatized retinol isomers and simultaneous monitoring of all relevant masses. The contamination with the respective other analyte mass was found to be less than 0.25%.

Statistics and kinetic calculations

The Student's *t*-test at the significance level of 5% was used to test the difference between two groups of values. The data are presented as mean ± SD. The plasma concentration versus time profiles are presented for one representative subject, in order to demonstrate the characteristic plasma features.

For labeled and unlabeled retinol, the data were transformed as described by Cobelli et al. (26) in order to correct for natural abundance associated with the tracer mass.

The data were analyzed by non-compartmental methods. Plasma peak concentrations (C_{max}) and times to reach maximal plasma concentrations (t_{max}) were determined directly from the data points. The areas under the concentration versus time profiles from time t_0 to t_n ($AUC_{t0\text{-}tn\ h}$) were calculated using the linear trapezoidal rule. Half-times were estimated by linear regression analysis of the final observed slope of the concentration versus time data (27).

RESULTS

The endogenous concentrations for total plasma retinol (1.88 ± 0.39 μmol/L) and for plasma retinyl esters (0.022 ± 0.013 μmol/L) were consistent with values for healthy male adults reported in the literature (2, 28–30). As the subjects were recruited from a healthy population of volunteers and the results of the laboratory tests demonstrated that they were healthy, it is reasonable to assume that their liver vitamin A levels were in the normal sufficient range. The concentration versus time profiles for plasma retinyl esters and retinol are shown in **Fig. 3** during the first 24 h after the ingestion of 105 μmol of [8,9,19-^{13}C]retinyl palmitate and in **Fig. 4** for the entire study time. We assume that the retinyl ester peak between 3 and 9 h corresponds to absorption of the vitamin A dose and clearance of the resultant absorptive lipoproteins, whereas the profiles for retinol species reflect basically metabolized retinol carried by RBP.

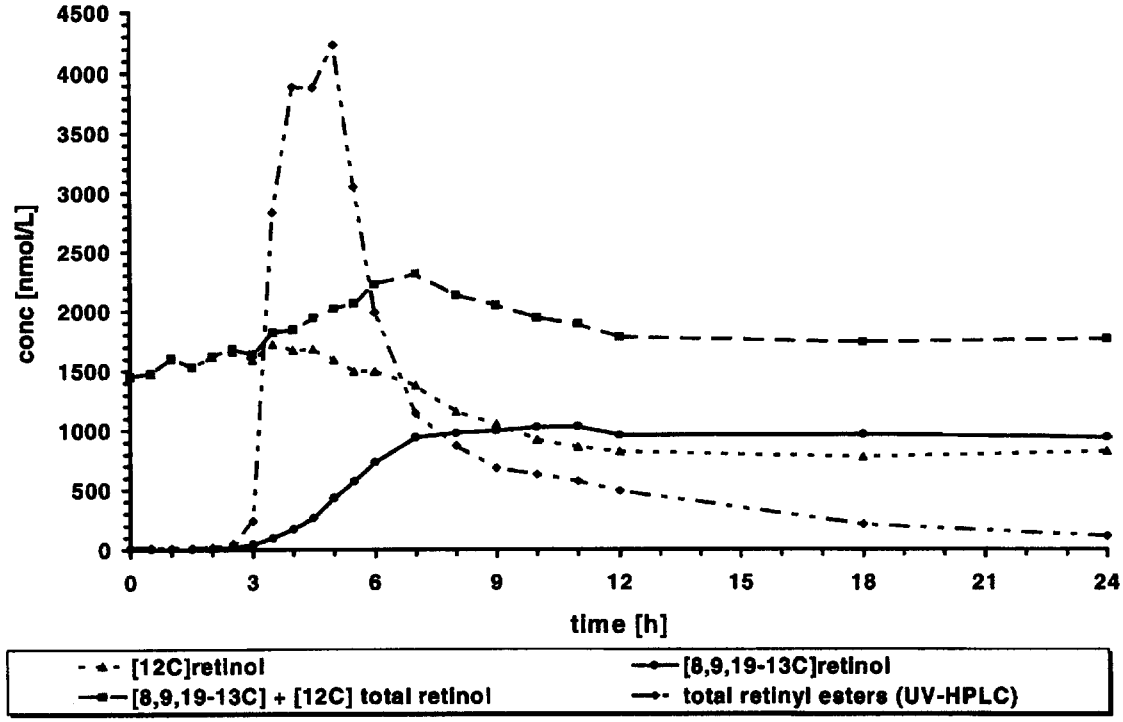

Fig. 3. Plasma concentration versus time profiles of total retinyl esters, [8,9,19-^{13}C]retinol, [^{12}C]retinol, and total retinol (sum of [8,9,19-^{13}C]- and [^{12}C]retinol) during the first 24 h after oral administration of 105 μmol [8,9,19-^{13}C]retinyl palmitate in subject 5. Data from subject 5 were chosen to illustrate, because these data appeared to be representative for most of the subjects (Figs. 3 to 5).

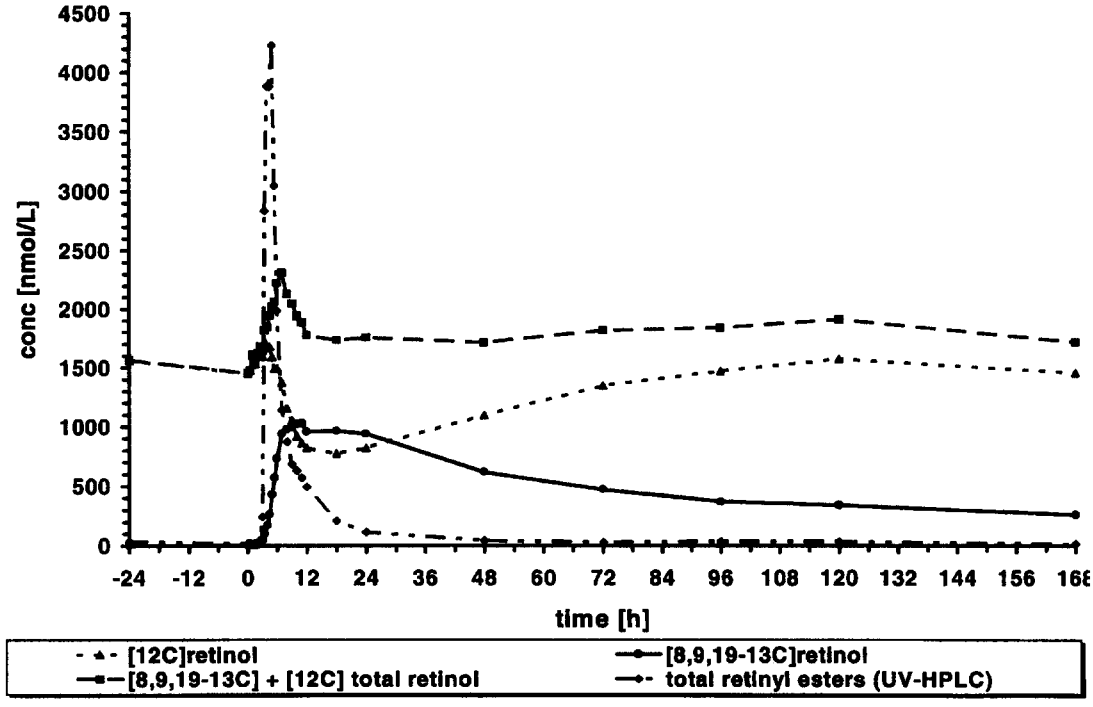

Fig. 4. Plasma concentration versus time profiles of total retinyl esters, [8,9,19-^{13}C]retinol, [^{12}C]retinol, and total retinol (sum of [8,9,19-^{13}C]- and [^{12}C]retinol) during the 1-week study period after oral administration of 105 μmol [8,9,19-^{13}C]retinyl palmitate in subject 5.

TABLE 1. Pattern of plasma retinyl esters at C_{max} of total retinyl esters (sum of individual retinyl esters). Calculations based on concentrations normalized to retinol

Subject	Retinyl Palmitate	Retinyl Stearate	Retinyl Oleate
		%	
1	74.6	18.7	6.7
2	69.2	25.6	5.2
3	76.3	19.0	6.7
4	67.4	28.0	4.6
5	68.0	26.8	5.2
6	67.6	25.3	7.1
7	68.1	27.3	4.6
8	70.4	24.9	4.7
9	67.9	26.6	5.5
10	74.3	19.1	6.6
11	63.1	31.9	5.0
Mean	69.7	24.8	5.6
SD	3.9	4.2	1.0
C.V.%	5.6	17.0	17.0

Retinyl esters

When samples were analyzed for individual retinyl esters, only three moieties showed significant absorption maxima at 325 nm: retinyl palmitate, retinyl stearate, and retinyl oleate. This was confirmed by running an absorbance spectrum of a pooled plasma sample at the concentration maxima of the retinyl esters (data not shown). The distribution of total plasma retinyl esters among these three fractions was remarkably similar among subjects and averaged 69.7% retinyl palmitate, 24.8% retinyl stearate, and 5.6% retinyl oleate (**Table 1**). The dynamic behavior of the three esters was similar as evident from the concentration versus time profiles (**Fig. 5**). Thus, as expected, these data indicate that the individual ester species are metabolized in parallel. Therefore, we refer to the sum of these three individual esters as plasma retinyl esters.

The observed maximal plasma concentrations of the retinyl esters ranged from 1.91 to 7.74 μmol/L (**Table 2**). The peak occurred 3.5–12 h after vitamin A administration. Thus, there was a substantial interindividual variation in both the time and the magnitude of the plasma peak. Typically, there was a pronounced lag time before the rapid increase in plasma concentration. As the plasma concentration approached the maximum, the slope of the increase was reduced, creating almost a plateau in four of the eleven subjects. In two of the eleven subjects the maximum occurred as late as 12 h after dosing and these curves displayed multiple peaks before the main maximum. The semi-logarithmic plots of the concentration versus time profiles of the plasma retinyl esters revealed for all subjects at least two distinct phases in the decay within the first 24 h after dosing.

Retinol

Plasma concentration versus time profiles for [8,9,19-^{13}C]retinol, [^{12}C]retinol, and total retinol (sum of labeled and unlabeled retinol) after administration of the retinyl palmitate are shown in Figs. 3 and 4. Plasma

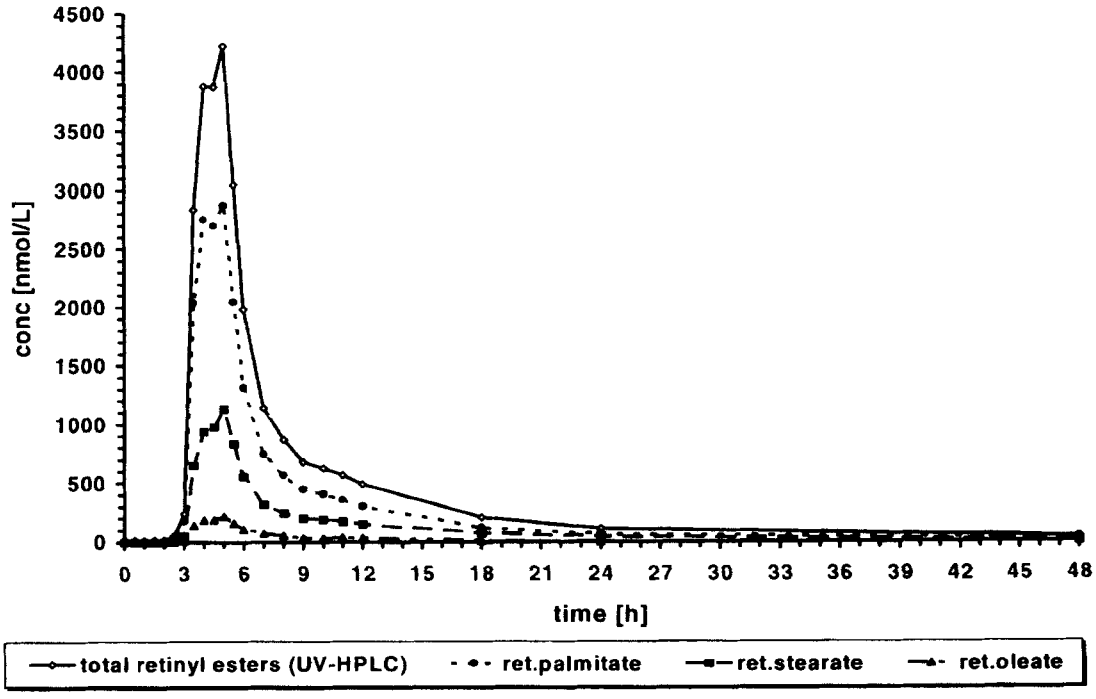

Fig. 5. The plasma concentration versus time profiles of individual retinyl esters (retinyl palmitate, -oleate, and -stearate) in subject 5.

TABLE 2. Maximal plasma concentrations (C_{max}) and corresponding times (t_{max}) of retinyl esters and retinol isotopes after oral administration of 100,000 IU of [8,9,19-^{13}C]retinyl palmitate

Subject	Retinyl Esters		[8,9,19-^{13}C]Retinol		[^{12}C]Retinol		Total Retinol	
	C_{max}	t_{max}	C_{max}	t_{max}	C_{max}	t_{max}	C_{max}	t_{max}
	μmol/L	*h*	*μmol/L*	*h*	*μmol/L*	*h*	*μmol/L*	*h*
1	1.91	5.5	0.98	12.0	1.60	4.5	2.05	10.0
2	5.48	6.0	1.23	24.0	2.17	4.5	2.51	9.0
3	2.20	12.0	2.05	24.0	2.48	3.0	3.23	18.0
4	7.74	5.5	1.13	12.0	1.71	3.0	2.15	8.0
5	4.22	5.0	1.03	11.0	1.72	3.5	2.31	7.0
6	2.26	3.5	1.31	10.0	2.36	1.5	2.96	7.0
7	5.96	5.0	1.04	10.0	1.89	3.5	2.32	8.0
8	2.99	3.5	1.12	10.0	2.65	1.5	2.81	7.0
9	4.70	5.0	0.89	12.0	2.23	3.5	2.28	3.5
10	5.96	12.0	1.15	24.0	1.91	5.0	2.00	24.0
11	5.52	5.0	0.89	10.0	1.58	2.5	1.77	6.0
Mean	4.45	6.2	1.16	14.5	2.03	3.3	2.40	9.8
SD	1.90	3.0	0.32	6.2	0.37	1.2	0.44	5.9
Median	4.70	5.0	1.12	12.0	1.91	3.5	2.31	8.0
C.V.%	42.7	48.2	27.6	42.8	18.4	35.1	18.5	60.9

kinetics for the three moieties showed distinct characteristics.

For labeled retinol, plasma concentrations increased after a lag phase of 1.5–5.5 h. This is similar to the delay observed for the retinyl esters. Also, there was a positive correlation between the t_{max} for the retinyl esters versus the t_{max} for [8,9,19-^{13}C]retinol ($r^2 = 0.837$). On average, [8,9,19-^{13}C]retinol concentrations peaked 14.5 h after dosing, and 8.3 ± 4.0 h later than the peak of the retinyl esters. The maximal plasma concentration of [8,9,19-^{13}C]retinol was 1.16 ± 0.32 μmol/L (Table 2). The slope of the increase was much smoother than the retinyl esters and the curves displayed a broad single peak in all subjects.

A perturbation in the plasma levels of endogenous unlabeled retinol was observed as early as 3.3 ± 1.2 h after dosing. This rise occurred before the increase in retinyl esters was observed. On average, these retinol peak concentrations of 2.03 ± 0.37 μmol/L were 0.15 ± 0.06 μmol/L higher than their respective predose values. After this maximum, declining concentrations of [^{12}C]retinol were observed, as the concentrations of [8,9,19-^{13}C]retinol increased in plasma. While [8,9,19-^{13}C]retinol levels peaked, the [^{12}C]retinol values reached a minimum. This was followed by an asymptotic increase towards endogenous concentrations for [^{12}C]retinol, while [8,9,19-^{13}C]retinol levels declined.

In all subjects, the total plasma retinol levels were perturbed and elevated at 9.8 ± 5.9 h and 0.52 ± 0.23 μmol/L above baseline levels. As expected, the magnitude of the perturbation was not correlated to the initial retinol concentration prior to dosing ($r^2 = -0.31$). On average, the initial baseline concentration of total plasma retinol was restored to 102 ± 16% at 168 h after the vitamin A dose.

Thus, overall, there was a consistent time course for appearance of the peak concentrations of retinyl esters and the retinol isotopes in plasma after retinyl palmitate administration. First, unlabeled retinol concentrations peaked, followed by retinyl esters, and total plasma retinol. Finally, [8,9,19-^{13}C]retinol reached maximal plasma concentration after a pronounced lag phase (Figs. 3 and 4).

At its peak (10–24 h after dosing), the ingested labeled retinol accounted for 51.4 ± 6.6% of the observed plasma [^{12}C]- + [8,9,19-^{13}C]retinol concentration (Figs. 3, 4 and **Table 3**). By the end of the study period (168 h after dosing), the plasma concentrations of labeled retinol were still elevated considerably (0.27 ± 0.21 μmol/L) and contributed on average 13.4% to the plasma retinol concentration. Based on our data, we predict that the kinetic half-time ($t_{1/2}$) for elimination of labeled retinol from plasma (estimated from the final observed slopes) is at least 5.0 ± 2.3 days.

In order to estimate the relative contribution of labeled retinol to the retinol plasma pool, we calculated the area under the plasma versus time curves (AUC) between 0 and 168 h (**Table 4**). During the observed time period, an AUC of 86.2 ± 38.5 μmol/L × h was estimated for [8,9,19-^{13}C]retinol and 242.6 ± 38.7 μmol/L × h for [^{12}C]retinol. Thus, the relative average contribution of [8,9,19-^{13}C]retinol to the total retinol $AUC_{0-168\,h}$ was 25.8 ± 7.6%. The $AUC_{0-48\,h}$ of the retinyl esters (22.0 ± 11.2 μmol/L × h) was not correlated with the $AUC_{0-168\,h}$ of the labeled retinol ($r^2 = 0.13$).

DISCUSSION

The present study was performed to qualitatively and quantitatively determine the kinetic behavior of plasma

TABLE 3. Percent distribution of labeled [8,9,19-^{13}C]retinol and unlabeled [^{12}C]retinol retinol in total plasma-retinol [8,9,19-^{13}C]- + [^{12}C]retinol at C_{max} of [8,9,19-^{13}C]retinol

Subject	[8,9,19-^{13}C]Retinol C_{max}	[^{12}C]Retinol C(t) at C_{max} of [8,9,19-^{13}C]Retinol	Total Retinol [8,9,19-^{13}C]- + [^{12}C] Retinol	[8,9,19-^{13}C]Retinol as % of Total Retinol
		μmol/L		%
1	0.98	0.96	1.94	50.28
2	1.23	1.11	2.34	52.69
3	2.05	1.16	3.21	63.77
4	1.13	0.86	1.99	56.69
5	1.03	0.86	1.88	54.48
6	1.31	1.48	2.78	47.00
7	1.04	1.16	2.20	47.29
8	1.12	1.67	2.79	40.22
9	0.89	1.10	1.99	44.79
10	1.15	0.85	2.00	57.55
11	0.89	0.88	1.76	50.24
Mean	1.16	1.10	2.26	51.36
SD	0.32	0.27	0.47	6.61
C.V.%	27.6	24.4	20.6	12.9

vitamin A and its metabolites after an oral dose of 105 μmol of vitamin A to healthy male subjects. The concentration versus time profiles of the retinyl esters and of labeled, unlabeled, and total retinol showed very similar characteristics among the eleven subjects.

Retinyl esters

The labeled and unlabeled isotopes of retinyl esters were not distinguished in this study, because an appropriate mass-spectrometric method was not available. However, it is reasonable to assume that the majority of the retinyl esters were newly absorbed labeled species. This assumption is based on current knowledge about vitamin A absorption (3, 6). In humans of normal vitamin A status, the plasma concentrations of retinyl esters are below 0.1 μmol/L in the fasting state and only increase transiently as a result of dietary vitamin A absorption (31, 32). The large interindividual variation in the lag time between the ingestion of the vitamin A dose and the increase in the plasma retinyl ester concentrations might be related to the dose formulation in which the vitamin A was administered (in coconut oil). Other possible explanations are influences on fat metabolism due to the low-fat supper and the 10-h fasting period prior to the vitamin A administration.

In all cases, retinyl ester levels in plasma peaked before labeled retinol. Also, a positive correlation was found between the t_{max} for retinyl esters and the t_{max} for labeled retinol. This observation is consistent with what is known about vitamin A metabolism and likely reflects hepatic secretion of [8,9,19-^{13}C]retinol associated with RBP (33). The apparent biexponential decay of the plasma retinyl esters was similar to what Berr (31) found in a study after intravenous infusion of retinyl palmitate-loaded autologus plasma. After separating the plasma into different lipoprotein fractions, he found that during the initial rapid decay retinyl esters were associated with chylomicron remnants and, thus, this process likely reflected hepatic uptake. The second slower phase was related to the clearance of retinyl esters associated with very low density lipoproteins (VLDL), mostly of hepatic origin.

Retinol

The earliest increases in plasma retinol concentrations after an oral dose of 105 μmol vitamin A were observed in the unlabeled [^{12}C]retinol fraction. In most subjects, [^{12}C]retinol concentrations increased even before the retinyl ester and [8,9,19-^{13}C]retinol fractions started to rise and reached a maximum before the other fractions did.

As plasma levels are a result of the influx of mass into the plasma compartment relative to the efflux of mass from the plasma into tissue compartments, an increase in a compound's plasma concentration can only be measured if mass accumulates transiently in plasma (27). This would be the case when the rate of influx into plasma from intestinal absorption is high relative to the rate of clearance from plasma.

During the initial absorption phase, the turnover of the plasma retinyl esters was probably already increased, due to starting influx of absorbed retinyl esters into plasma on the one hand and efficient plasma clearance on the other hand. This hypothesis is based on the rapid hepatic clearance rate of the retinyl esters contained in chylomicron remnants (half-time 19 ± 11 min; (31)). As a result, no measurable increase in plasma retinyl esters would be expected during the initial absorption phase.

In order to further interpret the observed kinetic profiles for plasma vitamin A we used some information from a whole-body model for vitamin A dynamics in rats (9, 13, 14). According to this model, two vitamin A pools can be distinguished in the liver. The perisinusoidal stellate cells behave kinetically as a large slowly turning-

TABLE 4. Area under the plasma concentration versus time curves ($AUC_{(t0-tn)}$ of retinyl esters and retinol isotopes. Percent distribution of [8,9,19-^{13}C]retinol-AUC in total plasma retinol-AUC

Subject	Retinyl Esters $AUC_{(0-168\ h)}$	[8,9,19-^{13}C]Retinol $AUC_{(0-48\ h)}$	[^{12}C]Retinol $AUC_{(0-168\ h)}$	Total Retinol [8,9,19-^{13}C]- + [^{12}C]- $AUC_{(0-168\ h)}$	[8,9,19-^{13}C]Retinol-AUC as % of Total Retinol-AUC
		$\mu mol/L \times h$			%
1	9.41	58.44	214.41	272.86	21.4
2	28.55	116.55	235.12	351.67	33.1
3	16.90	189.07	273.44	462.51	41.9
4	35.39	88.09	236.01	324.10	27.2
5	18.38	82.20	220.57	302.77	27.1
6	11.23	72.31	242.53	314.83	23.0
7	21.40	56.37	253.68	310.05	18.2
8	16.41	64.20	306.57	370.77	17.3
9	17.17	56.55	298.89	355.44	15.9
10	47.61	88.03	209.49	297.52	29.6
11	19.80	76.35	177.95	254.30	30.0
Mean	22.0	86.2	242.6	328.8	25.8
SD	11.2	38.5	38.7	56.4	7.6
C.V.%	50.8	44.7	16.0	17.1	29.3

over pool and the hepatocytes as a small faster turning-over pool. Both pools contribute to the liver output of RBP-bound retinol into plasma. The model predicts a contribution of dietary vitamin A to the liver output of about 9% in rats with marginally adequate vitamin A status. In the model, dietary vitamin A is taken up as retinyl esters into the hepatocyte pool and subsequently secreted as RBP-bound retinol into the plasma. If no dietary vitamin A is available, the liver output to plasma must consequently be constituted from recirculating RBP-retinol and, to a larger extent, from stellate cells. The turnover rates from these pools adjust depending upon dietary vitamin A input in order to maintain plasma retinol homeostasis (9).

In our study, the uptake of retinyl esters by the liver might have resulted in an enhanced turnover rate of retinol in the hepatocyte pool. This process could, in turn, have increased the secretion rate of RBP-retinol into the plasma. These changes in the turnover rates are thought to be induced by a mass competition process in the hepatocyte pool initiated by the incoming mass of absorbed labeled retinol. As the hepatocytes initially contained unlabeled retinol of endogenous origin, the initial output into plasma was consequently constituted from this material. This was reflected in plasma by the early increase in the [^{12}C]retinol concentration prior to the rise of the [8,9,19-^{13}C]retinol concentration. As the absorption process continued, larger quantities of labeled retinyl esters were taken up by the hepatocytes and the endogenous unlabeled vitamin A was more and more diluted. Consequently, the relative contribution of labeled retinol to the liver RBP-retinol output into the plasma was transiently increased. This was reflected in the plasma by increasing concentrations of the [8,9,19-^{13}C]retinol, while [^{12}C]retinol concentrations declined (see Fig. 4). Our data support the hypothesis that the liver follows the principle of 'last in/first out' in maintaining the plasma retinol homeostasis and keeping up the vitamin A mass balance in the hepatocyte pool. In doing so, the liver would first use the vitamin A provided by the recirculation and dietary sources to the hepatocyte pool and recruit any additional amount from the stellate cell storage pool. On the other hand, the fraction of dietary vitamin A not needed for maintaining homeostasis would be transferred to the stellate cells. As a rule of thumb, this fraction is about 50% of the dose absorbed in the normal physiological dosing range and normal vitamin A liver status (34).

During the observed study period, 25.8 ± 7.6% of the plasma RBP-retinol was contained as labeled retinol species and at its peak (10–24 h after dosing), the ingested labeled retinol reached about 51% of the total retinol plasma concentration (see Fig. 4 and Table 3). This is most likely due to the production of retinol from retinyl esters in the liver and subsequent output of this newly produced RBP-bound retinol into the plasma (35). However, our initial results from compartmental modeling indicate that there was a small fraction of labeled retinol transferred to RBP from enterocytes or transported in chylomicrons and then transferred to RBP that contributed to the early rise of the [8,9,19-^{13}C]retinol concentration versus time profile. The majority of the labeled retinol mass in plasma was, according to our model, derived from the normal pathway involving hepatic uptake of retinyl ester-containing chylomicrons and subsequent RBP-retinol release. This is consistent with data reported by Goodman and colleagues (36) where they found a small fraction of unesterified retinol associated with lymph after oral ingestion of labeled vitamin A.

After the administration of 105 μmol of vitamin A, a plasma perturbation of total retinol was observed. By

the end of the 1-week study period, these plasma levels returned back to baseline. This observation is consistent with the concept of the homeostatic regulation of plasma retinol when the vitamin A status is adequate. Also due to the homeostasis, the magnitudes of the perturbations were not correlated with the initial retinol concentrations before dosing.

The final elimination of [8,9,19-^{13}C]retinol from plasma appeared to be very slow, as indicated by still considerably elevated plasma levels 168 h after the dose. Also, the curves did not display a monoexponential terminal decay when plotted on a semi-logarithmic scale, suggesting that [8,9,19-^{13}C]retinol was not yet equilibrated among all tissues. This observation is consistent with the concept of multiple recycling of each retinol molecule among plasma, liver, and extrahepatic tissues in the body as established by the work of Green et al. (9–14). Keep in mind that the subjects were on a low vitamin A diet during the study to minimize interference of the equilibration process with additional unlabeled vitamin A. A retrospective compartmental analysis of tracer kinetic data obtained in humans by Goodman et al. (8) indicate that the sampling time in future experiments should be at least 200 days. This would allow mixing of the tracer with the exchangeable tracee pools, so that the curves reach a terminal slope, permitting an accurate calculation of the final elimination rate. Model-based compartmental analysis can accommodate standardized dietary vitamin A intakes in such long-term studies.

Overall, the absorbed vitamin A dose was traced into the plasma pools of the retinyl esters and the RBP-retinol. The incorporation of the dietary labeled vitamin A into the plasma RBP-retinol pool was characterized by fast distribution kinetics which were reflected in the transient large increases in labeled retinol and in the rapid declines of the unlabeled retinol plasma fraction. Whereas the rate of increase of labeled retinol probably reflected the secretion rate of liver RBP-retinol, the decay of the unlabeled retinol might give an estimate of the turnover rate of retinol in plasma. Currently, we are using model-based compartmental analysis to quantify these processes in more detail.

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