LAWRENCE
LIVERMORE
NATIONAL
LABORATORY

LLNL-JRNL-418035

Quantification of absorption, retention and elimination of two different oral doses of vitamin A in Zambian boys using accelerator mass spectrometry

E. K. Aklamati, M. Mulenga, S. R. Dueker, B. A. Buchholz, J. M. Peerson, E. Kafwembe, K. H. Brown, M. J. Haskell

October 14, 2009

The Journal of Nutrition

Disclaimer

Quantification of absorption, retention and elimination of two different oral doses of vitamin A in Zambian boys using accelerator mass spectrometry.

Emmanuel K. Aklamati[1], Modest Mulenga[2], Stephen R. Dueker[3], Bruce A. Buchholz[4], Janet M. Peerson[1], Emmanuel Kafwembe[2], Kenneth H. Brown[1], Marjorie J. Haskell[1]

---

[1] Program in International and Community Nutrition and Department of Nutrition, University of California, Davis, CA 95616

[2] Tropical Diseases Research Center, Ndola Central Hospital, Ndola, Zambia

[3] Vitalea Science Inc, 2121 Second Street, B101, Davis, CA 95618

[4] Center for Accelerator Mass Spectrometry, Lawrence Livermore National Laboratories, Livermore, CA 94551

ABSTRACT

Background: A recent survey indicated that high-dose vitamin A supplements (HD-VAS) had no apparent effect on vitamin A (VA) status of Zambian children <5 y of age.

Objective: To explore possible reasons for the lack of response to HD-VAS among Zambian children, we quantified the absorption, retention, and urinary elimination of either a single HD-VAS (60 mg) or a smaller dose of stable isotope (SI)-labeled VA (5 mg), which was used to estimate VA pool size, in 3–4 y old Zambian boys (n = 4 for each VA dose).

Study design: A 25 nCi tracer dose of [$^{14}C_2$]-labeled VA was co-administered with the HD-VAS or SI-labeled VA, and 24-hr stool and urine samples were collected for 3 and 7 consecutive days, respectively, and 24-hr urine samples at 4 later time points. Accelerator Mass Spectrometry (AMS) was used to measure the cumulative excretion of ^{14}C in stool and urine 3d after dosing to estimate, respectively, absorption and retention of the VAS and SI-labeled VA. The urinary elimination rate (UER) was estimated by plotting ^{14}C in urine vs. time, and fitting an exponential equation to the data.

Results: Estimates of mean absorption, retention and the UER were 83.8 ± 7.1%, 76.3 ± 6.7%, and 1.9 ± 0.6%/d, respectively, for the HD-VAS and 76.5 ± 9.5%, 71.1 ± 9.4%, and 1.8 ± 1.2%/d, respectively for the smaller dose of SI-labeled VA. Estimates of absorption, retention and the UER did not differ by size of the VA dose administered (P=0.26, 0.40, 0.88, respectively). Estimated absorption and retention were negatively associated with reported fever (P =0.011) and malaria (P =0.010).

Conclusion: HD-VAS and SI-labeled VA were adequately absorbed, retained and utilized in apparently healthy Zambian preschool-age boys, although absorption and retention may be affected by recent infections.

Support provided by: USAID/CDC, NIH RR13461 and the Bristol Myers Squibb Freedom to Discover program, and performed in part under the auspices of the U.S. Department of Energy by the Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344.

Key words; Absorption, retention, utilization, vitamin A, accelerator mass spectrometry, children, Zambia, tracer, carbon-14

INTRODUCTION

A national survey conducted in Zambia indicated that ~54% of children 6 months to 5 years of age were marginally deficient in vitamin A (serum retinol concentration <0.70 μmol/L) one month after they received a high-dose VAS (60 mg) through the national vitamin A supplementation (VAS) program [1]. This apparent high-prevalence of deficiency persisted even after controlling for the presence of systemic inflammation, based on serum concentrations of C-reactive protein and α-1-acid glycoprotein. These findings were unexpected because the national VAS reaches ~80% of children <5 years of age [1]. Possible biological reasons for these observations are: 1) the high-dose VAS was not adequately absorbed; 2) the high-dose VAS was absorbed but rapidly excreted; 3) the high-dose VAS was well-absorbed and retained, but mobilization from the liver was impaired, possibly because of infection or inflammation; and 4) the high-dose VAS was absorbed and retained, but daily utilization of VA in the children was greater than expected, such that by one month after dosing the high-dose VAS no longer contributed to their VA status.

Limited information is available on absorption, retention and utilization of VA from high-dose supplements in children, primarily because of the lack of a safe and sensitive method for studying vitamin A metabolism. A few studies were conducted more than 30 years ago [2-4], using radiolabeled VA as a metabolic tracer in a small number of Indian children. However, radiolabeled tracers studies, particularly in children and women of child bearing age, have fallen out of favor due to concerns with administering the high levels of radioactivity necessary to be detectable by liquid scintillation spectrometry. Recently, accelerator mass spectrometry (AMS) has been proven as a powerful method for studying metabolism safely in humans. True tracer doses of radiolabeled compounds result in levels of radioactivity in biological tissues and fluids that do not differ substantially from the natural background after administration. AMS was originally developed for carbon dating, which required a highly sensitive and quantitative method for measurement of carbon-14 (and subsequently other isotopes) with high precision [5, 6]. The

method is approximately 4 to 5 orders of magnitude more sensitive than liquid scintillation counting [7], and can measure isotopic ratios of $^{14}C/C$ to parts per quadrillion or attomole levels in milligram-sized samples. AMS has been used in human nutrition research to study the bioavailability of β-carotene [8] and its bioconversion to vitamin A [9]; folate metabolism [10]; and bone calcium balance [11].

To investigate possible reasons for the apparent lack of effect of high-dose VAS on the VA status of Zambian children, we conducted a study to estimate the absorption, retention and urinary elimination of an oral high-dose VAS (60 mg), in 3-4 year-old Zambian boys using AMS methodology. This study was nested within a larger study to assess the impact of a single high-dose VA supplement on total body VA pool size, using the paired-stable isotope dilution technique. Because there is no direct information in children on absorption, retention and utilization of the oral dose of stable isotope labeled VA that is used to estimate total body VA pool size using the stable isotope dilution technique, we also used AMS methodology to estimate absorption, retention and utilization of the oral dose of stable isotope labeled VA (5 mg). This information may be useful for refining the equation that is used for predicting VA pool size in this age group using the stable isotope dilution technique [12].

METHODS

Study site

The study was carried out in children from a low-income shanty compound on the outskirts of Ndola, Zambia.

Subjects

Boys 3–4 years of age were examined by a physician to assess their general health. The study was restricted to boys to facilitate collection of 24-hr urine samples. Twenty boys who were free from chronic disease, symptoms of malaria or diarrhea within the past 2 weeks and signs or symptoms of VA deficiency were eligible for the study. Those who had received a high-dose VAS within the previous 2 months were excluded. The study protocol was approved by the Institutional Review Boards of the Tropical Diseases Research Center, Ndola, Zambia, and the University of California, Davis (UCD) and the Radioactive Drug Research Committee of UCD. Informed, written consent was obtained from a parent or legal guardian before beginning the study procedures. The amount of radioactivity the children were exposed to was 2.1 mrem. By comparison, the total effective dose of a 6-hour airline flight is 3.0 mrem and the dose of a dental X-ray examination is 20 mrem.

Protocol for co-administration of [$^{14}C_2$]-retinyl acetate with a high-dose VA supplement or a dose of stable isotope-labeled VA

The boys were randomly assigned to one of two groups (10/group) to receive a tracer dose of [$^{14}C_2$]-retinyl acetate (25 nCi) with either an oral high-dose VAS (60 mg RE), or an oral dose of stable isotope-labeled VA (5 mg RE). The study protocol is shown in Figure 1. On study day 0, the children were admitted to the research facility. On study day 1, 24-hour collections of stool and urine were obtained for measurement of the baseline ^{14}C contents before the tracer dose was administered. On study day 2, children who were assigned to the high-dose VAS group (n=10) received an oral dose of 60 mg RE VA in corn oil (194 µL), followed immediately by an oral dose of [$^{14}C_2$]-retinyl acetate (25 nCi) in corn oil (98 µL). The doses were pipetted directly into each child's mouth using a positive displacement pipet and a disposable tip. The children were not given a high-fat snack to facilitate absorption of the VA, because this is not done in the national VAS program. Children who were assigned to the SI-labeled VA group

(n=10) received an oral dose of 5 mg of SI-labeled VA (5 mg RE) in corn oil (194 μL), followed immediately by an oral dose of 25 nCi of [$^{14}C_2$]-retinyl acetate in corn oil (98 μL). Both doses were administered directly into the children's mouths using a positive displacement pipet. Thereafter, the children in this group were given a high-fat snack (bread and peanut butter) to enhance absorption of the dose of SI-labeled VA, as is recommended for the stable isotope dilution technique [13]. Twenty-four hour stool samples were collected for 3 consecutive days (study days 2-4) for measurement of the cumulative amount of ^{14}C excreted in stool to estimate VA absorption. Twenty-four hour urine samples were collected for 7 consecutive days (study days 2-8), for measurement of the cumulative amount of ^{14}C excreted in urine to estimate VA retention. Additionally, 24-hour urine samples were collected on study days 14, 27, 45, and 59, for measurement of ^{14}C in urine, to estimate the daily urinary elimination rate of ^{14}C.

In addition, in the larger study on the longer-term impact of a HD VA supplement on VA pool size, blood samples were collected before and 45 days after administration of the high-dose supplement or placebo (corn oil) for measurement of plasma concentrations of retinol, C-reactive protein (CRP), α_1-acid glycoprotein (AGP) and hemoglobin concentration. The subjects in this sub-study who received [$^{14}C_2$]-retinyl acetate with the dose of SI-labeled VA received the placebo capsule (corn oil) in the larger study.

Preparation of the [$^{14}C_2$]-retinyl acetate dose

[$^{11,12\text{-}14}C_2$]-retinyl acetate was synthesized by Hoffman-La Roche (Nutley, NJ). The [$^{14}C_2$]-retinyl acetate was purified by reverse-phase high performance liquid chromatography (HPLC). Briefly, 255 μL of the stock solution of [$^{14}C_2$]-retinyl acetate in toluene, was placed in a scintillation vial containing 100 μL butyl hydroxyl toluene (BHT), dried under N_2 gas and reconstituted in 500 μL hexane. The [$^{14}C_2$]-retinyl acetate in hexane was loaded onto a NH_2 solid phase extraction column (0.8 x 4.0 cm, 3 μm; GenPore, Reading, PA), and eluted with 2 mL hexane containing 15% ethyl acetate. The eluent was dried under N_2 gas and reconstituted

in 200 µL methanol for purification by HPLC, using a Class VP HPLC (Shimadzu, Columbia, MD), equipped with a photo-diode array detector. The [$^{14}C_2$]-retinyl acetate was injected onto a C18 HS Adsorbosphere column (150 x 4.6 mm, 5 µm) (Alltech, Deerfield, Il), using a mobile phase of methanol/water (93:7). The retinyl acetate peak for each injection was collected into a vial containing BHT, between 10.0 and 12.0 minutes. The HPLC fractions (n=3) containing the [$^{14}C_2$]-retinyl acetate were combined, dried under N_2, reconstituted in methanol, and re-injected onto the HPLC column as a second purification step, and eluted with methanol/water (93:7). The ^{14}C activity in the purified fraction was measured by counting three 10 µL aliquots in a liquid scintillation counter (Wallac 1410, Shelton, CT).

To construct a radiochromatogram, 60 µL of purified [$^{14}C_2$]-retinyl acetate was injected onto the HPLC column (150 x 4.6 mm, 5 µm) (Alltech, Deerfield, Il), and eluted with methanol/water (93:7). Fractions of eluent were collected every minute for a total of 28 minutes (n = 28 fractions). ^{14}C counts in the collected fractions were measured using liquid scintillation counting, and a radiochromatogram was constructed (Figure 2). The radiochromatogram indicates that 96.9% of the ^{14}C counts were associated with the retinyl acetate peak.

The remaining purified [$^{14}C_2$]-retinyl acetate was evaporated under N_2 gas and dissolved thoroughly in corn oil. The radioactivity of five aliquots (10 µL) of [$^{14}C_2$]-retinyl acetate in corn oil were counted using liquid scintillation spectrometry to determine the volume required for a dose of 25 nCi. The mean activity of ^{14}C in corn oil was 566,340 DPM/mL ± 27,058. An aliquot of 98 µL of [$^{14}C_2$]-retinyl acetate in corn oil provided a dose 25 nCi ^{14}C (0.413 µg retinol equivalent (RE)).

Stable isotope labeled vitamin A and the high-dose vitamin A supplement: The stable isotope labeled VA [$^{10,19,19,19\text{-}2}H_4$]-retinyl acetate, was obtained from Cambridge Isotopes Inc. (Andover, MA), and transferred into corn oil, as previously described [13]. The concentration of SI-labeled VA in corn oil was determined by UV-Vis spectrophotometry to determine the volume

required for a dose of 5 mg RE [13]. Retinyl acetate in soybean oil (UNICEF, UN Essential Drugs Programme, MEDICAP, Ltd., Samutprakarn, Thailand) was used as the source material for the high-dose VAS; the VA concentration of the retinyl acetate in soybean oil was determined by UV-Vis spectrophotometry to determine the volume required for a dose of 60 mg RE [13].

Collection of 24-hour stool samples: Twenty-four hour stool samples were collected into plastic bags (0.4 mm thickness, Fisher Scientific, Tutsin, CA). At the end of the 24-hour period, the samples were weighed using a triple beam balance (Ohaus, Pine Brook, NJ). The samples were double-bagged, carefully sealed and stored in a freezer at -20°C until they were shipped to UCD for analysis.

Collection of 24-hour urine samples: Twenty-four hour urine samples were collected into 2 L polypropylene jugs. During the 24-hour collection period, the jugs were stored at 4°C. When the 24-hour collection period had ended, total urine volumes were measured. The samples were well mixed and two, 2 mL aliquots were placed in cryovials and stored at -20°C until they were shipped to UCD for analysis.

Preparation of samples for AMS analyses: Urine and stool samples were processed for AMS analysis, as previously described. The major steps in this process are homogenization (for stool samples), combustion and graphitization [7]. The ^{14}C measurements were done at the Center for Accelerator Mass Spectrometry at the Lawrence Livermore National Laboratories (Livermore, CA).

Total carbon analyses: The total carbon content of stool and urine samples was determined using a dynamic flash combustion system combined with a gas chromatography (GC)

separation system and a thermal conductivity detection (TDC) system [14]. For both urine and stool samples, 75 µL of sample (neat urine, and homogenized stool) was lyophilized in aluminum capsules for 24 hours, and analyzed for total carbon content. The total carbon measurements were done at the Agriculture and Natural Resources Analytical Lab at the UCD.

Calculation of ^{14}C concentration: AMS data are expressed as isotopic ratios of $^{14}C/C$ and more conveniently expressed in units of fraction modern (fMC). One fMC equals 6.11 fCi ^{14}C/ mg C. The isotopic ratios were corrected for the baseline values, by subtracting the ratio of the pre-dose samples from post-dose samples. The ^{14}C concentration of samples was calculated using the following equation:

$$\text{Modern} \times \frac{6.11\ \text{fCi}\ ^{14}C}{\text{mg C}} \times \frac{\text{mg C}}{\text{mL suspension}} = \frac{\text{fCi}\ ^{14}C}{\text{mL suspension}}$$

1 fCi = 0.00222 DPM: Multiplying the ^{14}C content in the sample (fCi) by 0.00222 DPM gives the ^{14}C content in the sample in DPM/mL of suspension [15].

Estimation of ^{14}C excreted in stool and urine as a percentage of dose administered: To determine the total ^{14}C content in each 24-hour collection, the ^{14}C concentration (in DPM/mL) is multiplied by the total volume of the suspension (for stool), and the total volume of urine collected in the 24-hour period (for urine). The amount of ^{14}C excreted per day is expressed as a percentage of the administered dose:

$$\frac{^{14}C\ \text{content of each 24-hour sample (DPM)}}{\text{Dose of}\ ^{14}C\ \text{administered (DPM)}} \times 100 = \text{Percent of dose excreted/d}$$

Estimation of percent absorption: To estimate absorption, the cumulative amount of ^{14}C excreted in stool during 72 hours (3d) after dosing was expressed as a percentage of dose

administered, and subtracted from 100%. Beyond 72 hours, any ^{14}C excreted in stool was assumed to be derived from biliary excretion of VA metabolites and the normal sloughing of intestinal cells [8].

Estimation of percent retention: To estimate retention, the cumulative amount of ^{14}C in stool 72 hours (3d) after dosing, and the cumulative amount of ^{14}C excreted in urine 72 hours (3d) after dosing, were expressed as percentages of dose administered, and subtracted from 100%. ^{14}C excreted in urine within 72 hours after dosing was assumed to represent [$^{14}C_2$]-labeled VA that was absorbed, but rapidly excreted. Beyond 72 hours, ^{14}C excreted in urine was assumed to be derived from end-products of VA metabolism and was assumed to have been retained initially.

Estimation of the urinary elimination rate: The urinary elimination rate (UER) of ^{14}C was estimated by plotting ^{14}C in urine as a function of time, and fitting an exponential equation, derived from a log-linear plot to the observed data. The second coefficient (b_2) of the final exponential term in the equation was used as an estimate of the urinary elimination rate [16]:

$$-a_0\exp(-b_0t) + a_1\exp(-b_1t) + a_2\exp(-\mathbf{b_2}t)$$

Biochemical assessments: Plasma retinol concentrations were measured by using HPLC [17]. Marginal VA deficiency was defined as plasma retinol concentration <0.70 μmol/L. Plasma concentrations of C-reactive protein (CRP) and α-1-acid glycoprotein (AGP) were measured by using commercial radial immunodiffusion kits (Nanorid, The Binding Site, United Kingdom). Cut-off values of >10.0 mg/L, and >1.2 g/L were used for plasma CRP and AGP, respectively, to identify children with infection and/or inflammation.

Hemoglobin (Hb) concentrations were measured using the cyanmethhemoglobin method with a Hemocue (Rankin Biomedical Corporation, Holly MI). Anemia was defined as Hb concentration <110.0 g/L.

Malaria parasites: The presence of malaria parasites in blood was assessed by thick blood smears (slides) stained with Giemsa [18].

Morbidity: Questionnaires were administered to mothers to obtain information on the number of days on which children had selected signs or symptoms of illnesses during the previous week.

Anthropometry: Anthropometric assessments were done on study day 0. Weight was measured to the nearest 0.1 kg, using a digital scale (Seca, Hanover, MD); height was measured to the nearest 0.1 cm, using a stadiometer (Salter, Midlands, United Kingdom). Standard weights were used to check the accuracy of the scale each day that children were weighed. Mid-upper arm circumference (MUAC) of the children was measured to the nearest 0.1 cm, using a non-stretchable tape.

Statistical analysis

Descriptive statistics were calculated for all variables. Mean estimates of absorption, retention and urinary elimination rates of ^{14}C were compared between the group of children who received the high-dose VAS and those who received the SI-labeled oral dose of VA by using the Student's t test. The Student's t test was also used for comparing mean absorption, retention and urinary elimination rate between children with or without elevated plasma AGP (initial plasma AGP was used for the comparisons of estimated absorption and retention; and initial and final plasma AGP were used for the comparison of UER). Estimated absorption of ^{14}C was examined in relation to reported symptoms and illnesses that occurred one week before and 3

days after administration of the high-dose VA supplement or the smaller dose of SI-labeled VA using correlation analysis. Estimated retention of ^{14}C was examined in relation to reported symptoms and illnesses that occurred one week before and one week after administration of the high-dose VA supplement or the smaller dose of SI-labeled VA. Similarly, the estimated urinary elimination of ^{14}C was examined in relation to reported symptoms and illnesses one week before administration of the test doses of VA, and throughout the remaining weeks of the study period, using correlation analysis.

RESULTS

Subjects: A total of 20 boys completed the study protocol, however only samples from 8 children, 4 of whom received the high-dose VAS and 4 of whom received the dose of SI-labeled VA were analyzed using AMS. Nevertheless, we are presenting these preliminary results because very little direct, quantitative information exists on VA metabolism in children in the scientific literature. The data reported herein are the first quantitative estimates of VA metabolism in preschool age children based on the AMS tracer method, and they demonstrate the feasibility of applying this technique in this age group.

Initial characteristics of the 8 children for whom we have AMS data are presented in Table 1. Absorption, retention and daily utilization of a high-dose VAS (60 mg) was estimated in four boys, and absorption, retention and daily utilization of a dose of SI-labeled VA (5 mg) was estimated in the other 4 boys. Children from the two groups did not differ in age, weight, height, MUAC or anthropometric indices ($P>0.50$).

Absorption of the high-dose vitamin A supplement and the smaller dose of stable isotope labeled VA: The cumulative excretion of ^{14}C in stool of children who received the high-dose VAS is shown in Figure 3. Excretion of ^{14}C appeared to reach a plateau by 3d after dosing for two of the four children. The mean (± SD) cumulative amount of ^{14}C recovered in stool, 3d after

dosing, expressed as a percentage of the dose administered was 16.2 ± 7.1%. The estimated mean absorption was 83.8 ± 7.1% (range: 73.3 to 88.8%; n=4).

The cumulative excretion of ^{14}C in stool of children who received the smaller dose of SI-labeled VA is also shown in Figure 3. Excretion of ^{14}C appeared to reach a plateau by 3d after dosing in two of the 4 children. The mean (± SD) cumulative amount of ^{14}C recovered in stool, 3d after dosing, as a percentage of the dose administered was 23.5 ± 9.5%. The estimated mean absorption was 76.5 ± 9.5% (range: 62.4 to 82.9%; n=4).

Mean estimates of absorption did not differ in children who received the high-dose VAS or the dose of SI-labeled VA (P = 0.26, n=8, Table 2).

Retention of the high-dose VA supplement and the smaller dose of SI-labeled VA. The cumulative excretion of ^{14}C in urine of children who received the high-dose VAS is shown in Figure 4. Urinary excretion of ^{14}C appeared to reach a plateau for all children by 3d after dosing. The mean (± SD) cumulative amount of ^{14}C recovered in urine by 3d after dosing, as a percentage of dose administered was 7.4 ± 2.4%. The estimated mean retention was 76.3 ± 6.7% (range: 67.4 to 81.8%; n=4).

The cumulative urinary ^{14}C excretion by children who received the dose of SI-labeled VA is also shown in Figure 4. Urinary excretion of ^{14}C appeared to reach a plateau for all children by 3d after dosing. The mean (± SD) cumulative amount of ^{14}C recovered in urine by 3d after dosing, as a percentage of dose administered was 5.4 ± 1.6%. The estimated mean retention was 71.1 ± 9.4% (range: 57.1 to 76.7%; n=4). Estimates of mean retention did not differ in children who received the high-dose VAS or the SI-labeled VA (P = 0.40; Table 2).

Urinary elimination of ^{14}C by children who received the high-dose VA supplement or the dose of stable isotope labeled VA: The UER of ^{14}C in children who received the high-dose VA supplement was 1.9 ± 0.6%/d (range: 1.5 to 2.7%/d; n=4, Figure 5). The UER appeared to

stabilize at 8.4d after dosing (this represented the time at which the second coefficient of the final exponential term of the equation accounted for >95% of the fraction of ^{14}C in urine), and continued to decline until 59d after dosing for all children.

The mean UER of ^{14}C of the children who received the dose of SI-labeled VA was 1.8 ± 1.2%/d (range: 0.9 to 3.4%/d; n=4, Figure 5). Mean estimates of the UER of VA did not differ between children who received the high-dose VAS or the dose of SI-labeled VA (P=0.88, Table 2).

Biochemical assessments

Hemoglobin concentration: The initial mean (±SD) hemoglobin concentration was 119.4 ± 5.8 g/L, and none of the children had anemia (Hb concentration <110.0 g/L).

Plasma CRP and AGP: None of the children had elevated plasma CRP (>10 mg/L) at either time point. Three of the eight boys (37.5%) had elevated plasma AGP (>1.2 g/L) initially, and 4 of the boys had elevated plasma AGP on study day 75. In children with or without elevated initial AGP concentrations, estimates of mean absorption (82.7% vs. 78.6%), retention (78.0% vs. 71.2%), and the UER of VA (1.5%/d vs. 2.1%/d) did not differ significantly (P=0.56, P=0.28, P=0.15, respectively); however, the sample size (n=8) only permitted detection of a difference of 22.3%, 19.1% and 1.9%/d for estimated absorption, retention, and UER, respectively, given the observed levels of variation.

Plasma retinol concentration: The initial and final mean plasma retinol concentrations did not differ in children assigned to the high-dose VAS group or the SI-labeled VA group (p>0.68; Table 4). No child had a concentration <0.70 μmol/L at either time point. Plasma retinol concentration was not associated with absorption, retention or urinary elimination of VA, either before or after controlling for elevated initial AGP.

Morbidity: None of the children had positive malaria smears; however one caretaker reported that her child had malaria for one day, 3 days prior to administration of ^{14}C-labeled VA. The mean (±SD) period prevalences of selected illnesses for children (n=8) during the week prior to administration of ^{14}C-labeled VA and the 3 days during which absorption was estimated, and the 7 days during which retention was estimated, are presented in Table 5.

A significant negative association was observed between estimated VA absorption and reported fever (r= -0.83, P=0.01) and malaria (r= -0.83, P=0.01), and between estimated VA retention and reported fever (r= -0.84, P=0.01) and malaria (r= -0.84, P=0.01); There were no associations between estimated urinary elimination of VA and reported morbidity.

DISCUSSION

Using recently available AMS techniques for measuring very low doses of radio-labeled VA tracers, we found adequate absorption and retention of both a high oral dose (60 mg) and a lower oral dose (5 mg) VA supplement among preschool-age Zambian boys. Their urinary elimination of VA was consistent with earlier estimates in children, and somewhat greater than that reported for adults [19]. This is the first time that parameters of VA metabolism have been estimated directly in preschool age children using a very low physiologic tracer dose of radiolabeled VA and AMS methodology. Importantly, this study demonstrates that the AMS methodology can be carried out successfully in a pediatric population in a low-income community setting. The main purpose of the study was to assess whether oral high-dose VAS are adequately absorbed and retained in Zambian preschool age children. A secondary objective was to assess the absorption, retention and daily utilization of a smaller dose of SI-labeled VA that was used to estimate total body VA pool size in this group of children using the VA SI-dilution technique.

Estimated absorption of vitamin A: The estimated mean absorption of VA was adequate for both the high-dose supplement and the smaller dose of SI-labeled VA in Zambian preschool age boys (~84% and ~77%, respectively). In this study, estimated absorption of the high-dose supplement tended to be higher than previously reported values. In 4-5 year old Indian children (n=6), absorption of a high dose VA supplement (30 mg RE) was estimated to range from 68.2% to 79.4% [3], and in a group of 3-6 year old Indian children, absorption of a high-dose VA supplement (60 mg RE) was estimated to be 67.2% (n=3) [4]. However, absorption of a smaller dose of retinyl acetate (1000 µg) was estimated to be 99.2%, in apparently healthy 2-10 year old Indian children [2]. There are methodological differences across these studies that may account for the differences in estimates of absorption. The Indian children received either 4-5 µCi [$^{3}H_2$]-retinyl acetate [2, 4], or 14.4 µCi [$^{3}H_2$]-retinyl acetate [3] as a metabolic tracer. Absorption was estimated based on cumulative excretion of $^{3}H_2$ in 24-hour stool samples for 4d [2, 4] and 8d [3] after dosing, respectively, and radioactivity was measured using liquid scintillation spectrometry. In the present study, a tracer dose of [$^{14}C_2$]-retinyl acetate (25 nCi) was administered to children. Absorption was estimated based on cumulative excretion of ^{14}C in 24-hour stools for 3 days after dosing, and radioactivity in stool and urine samples was quantified using accelerator mass spectrometry, which is a far more precise and sensitive analytical method. The differences in analytical methodologies, and in the number of days after dosing that radioactivity was measured in stool to estimate absorption, may account for the differences in estimates of percent absorption across studies.

The number of 24-hour stool samples that are required after dosing to obtain an accurate estimate of cumulative excretion of unabsorbed ^{14}C is not certain. We expected that all of the unabsorbed ^{14}C would be excreted in stool by 3 days after dosing, but that was probably not the case for all children, because fecal excretion did not reach a plateau in all of them. In a US man, excretion of [^{14}C]-labeled β-carotene in stool was biphasic, with an initial spike from 0 to 48 hours representing unabsorbed label, and a slower output after 48 hours [8], which was

attributed to biliary excretion of metabolites or normal sloughing of intestinal cells that had accumulated β-carotene and/or its metabolites. In Indian children, the excretion of ^{3}H in stool appeared to reach a plateau by 2-4 days after oral dosing with [$^{3}H_2$]-retinyl acetate [2-4]. In our study, we observed an initial spike in fecal excretion of ^{14}C, but did not observe an obvious plateau by 3 days after dosing in all children. In contrast, urinary excretion of ^{14}C appeared to stabilize by 3 days after dosing in all children, suggesting that fecal excretion of ^{14}C beyond 3 days after dosing represents metabolites of VA that are excreted into feces in bile. However, it is possible that some of the ^{14}C in feces beyond 3 days was derived from unabsorbed VA that had accumulated in sloughed intestinal cells, and that absorption was over-estimated in children who did not reach an obvious plateau by 3 days after dosing. The magnitude of over-estimation would probably be small because, as stated above, urinary excretion of ^{14}C stabilized by 3 days after dosing suggesting that the ^{14}C in fecal samples beyond 3 days is probably derived from VA metabolites. In the earlier study in Indian children in which the estimate of absorption was based on fecal excretion of ^{3}H for 8 days after dosing [3] absorption was likely under-estimated because fecal excretion of label during the later days (~d4-d8) probably represented metabolites of absorbed labeled VA.

It is also possible that differences in VA status may be related to the differences in estimates of absorption across studies. The Indian children in two of the earlier studies [2, 3] had marginal to low VA status based on reported serum retinol concentrations (ranging from 0.38–0.94 μmol/L; serum retinol concentrations were not reported for the other study [4]), whereas children in the present study had adequate plasma retinol concentrations (1.07 ± 0.23 μmol/L). However, a study in rats [20] indicates that absorption efficiency of VA does not differ among animals with low or marginal VA status, so it seems less likely that vitamin A status might explain the observed differences in vitamin A absorption.

Estimated retention of VA: The estimated mean retention of VA was adequate for both the high-dose VA supplement and the smaller dose of SI-labeled VA in Zambian preschool age boys (~76% (n=4), and 71% (n=4), respectively). It appears that retention of VA was driven largely by VA absorption. VA retention was estimated by subtracting the cumulative amount of ^{14}C in urine during the first 3 days after dosing, from the amount of VA absorbed (which was estimated by subtracting total cumulative ^{14}C in feces during the first 3 days after dosing). The cumulative amounts of ^{14}C lost in urine during the first 3 days after dosing do not differ from the amounts of ^{14}C lost in urine in the first 3 days after dosing based on estimates of each individual's estimated UER for the 59-d period (Table 3). This suggests that VA excreted in urine during the first 3 days after dosing is similar to the usual daily VA elimination rate, and that retention is driven largely by absorption. The estimated retention of the high-dose VA supplement tended to be higher than previously reported values. In 4-5 y old Indian children, retention of a high-dose VA supplement (30 mg) was estimated to range from 23%-54% (n=6) [3]; and in 3-6 year old Indian children, retention of a high-dose supplement (60 mg) was estimated to be 47.9% (n=3) [4]. In contrast, retention was higher, 82.2% (n=5), in 2-10 year old Indian children who received a smaller dose of vitamin A (1000 μg) [2]. As stated above, it is likely that the reasons for the discrepancies across studies are related to differences in analytical methodologies, and in the number of days after dosing that radioactivity was measured in 24-hr stool and 24-hr urine samples to estimate retention. In the Indian studies, estimates of absorption and retention were based on cumulative excretion of radioactivity for 4d in both stool and urine [2, 4], and 8d in stool and 5d in urine [3], compared to 3d in stool and urine in the present study. Retention may have been under-estimated in the earlier studies because of prolonged sampling times.

Urinary elimination rate: The estimated mean UER of the high-dose VA supplement and of the smaller dose of stable isotope-labeled VA was 1.9 ± 0.6%/d and 1.8 ± 1.2%/d, respectively.

These UERs are similar to the estimated system fractional catabolic rate (2.2%/d; 95%CI: 1.4, 3.0%/d) for Peruvian preschool age children, that was estimated based on plasma retinol kinetic data using SI-labeled VA [16]. An estimated daily VA elimination rate of ~2%/d in preschool age children is higher than the reported value of 0.5%/d for adults [19]. However, this is not unexpected. It is likely that the utilization rate for VA is greater in children than in adults because children require VA for growth in addition to other physiologic functions.

Effect of infection on estimates of absorption, retention and urinary elimination of VA: In the present study, the prevalence of symptoms of illness was low; however, VA absorption and retention were negatively associated with reported fever, cough and malaria. These associations were driven by one of the eight children whose caretaker reported that her child was sick for one day, 3 days prior to receiving his dose of [$^{14}C_2$]-retinyl acetate and SI-labeled VA. Estimates of absorption and retention of VA for that child were 62.4% and 57.1%, respectively. If the values for this child are omitted from the data set, estimates of mean absorption and retention of the smaller dose of SI-labeled VA are 81.2 ± 1.4% and 75.8 ± 0.8%, respectively. The UER of VA was not associated with the selected symptoms or illnesses. Similarly, estimates of mean absorption, retention and urinary elimination of VA did not differ between children with or without subclinical infection (elevated plasma AGP). However, because of the small sample size (n=8), we cannot not be certain that there are no associations among these variables. In the earlier studies in Indian children, severe infection at the time of dosing was associated with a reduction in absorption and retention of VA [2].

The results of the present study on absorption, retention and urinary elimination of the dose of SI-labeled VA are useful for refining the 'Olson equation' that is used for estimating total body VA stores in this age group using SI-dilution methodology [13]. In the 'Olson' equation, a factor of 0.5 is used for the efficiency of storage (retention) of a dose SI-labeled VA. This factor is based on experimental data in rats [12], and represents the proportion of a test dose of

radiolabeled VA that was retained in the liver, following intravenous infusion, at the time when the dose had fully mixed with the total body VA pool. In the present study, retention of an oral dose of SI-labeled VA (5 mg) was estimated as ~75% (omitting the child with reported malaria), 3d after administration of the dose. In preschool age children, an oral dose of labeled VA takes between 8 and 12d to fully mix with the endogenous VA pool. Using the estimated retention (75%) at 3 days after dosing, and the urinary elimination rate of VA of 1.9%/d from the present study, it can be estimated that ~64.4% of the dose of SI-labeled VA would have been retained at 10 d after dosing. This value is higher than the value of 50% that is used to estimate retention in the 'Olson' equation. Improved estimates of VA pool size may be obtained for this age group if a value of ~64% is used in the 'Olson equation' as the estimate for retention of the dose of stable isotope-labeled VA. Also, the data in the present study suggest that the stable isotope dilution methodology should not be carried out in children whose caretakers report symptoms of fever or malaria within the previous week.

Considerations regarding the study population: The main purpose of this study was to assess whether high-dose VA supplements are well-absorbed and retained in Zambian preschool age children in low income communities who are likely to participate in the national high-dose VAS program. Our results indicate that the high-dose VAS are adequately absorbed and retained. However, the children who participated in our study had better VA status and a lower prevalence of morbidity and subclinical infection than children who participated in the national VA status survey in Zambia in 2003 [1]. We might have obtained different estimates of absorption, retention and urinary elimination of VA from children with low initial VA status and/or a higher prevalence of inflammation/infection, as suggested by the results for the child with reported malaria. In the larger study, we estimated the impact of a high-dose VA supplement on total body VA stores using the VA stable isotope dilution method. For that methodology, it is recommended that the test be carried out in children who are initially free from symptoms of

illness, to maximize the likelihood that they will adequately absorb and retain the oral dose of SI-labeled VA. Thus, we selected children for the larger study who were initially apparently healthy. In the present study, children in the high-dose VA group received the VA supplement and the dose of [$^{14}C_2$]-retinyl acetate 15 days after they received their first dose of SI-labeled VA for estimation of their initial VA pool size, and the children were followed for a total of 59 days. During that 59-day period, the children remained relatively healthy; morbidity rates and rates of subclinical infection were low. Thus, our results for estimated absorption, retention and urinary elimination of VA following administration of the high-dose VA supplement reflect the responses of a small group of relatively healthy Zambian preschool age boys from a low-income community; it is uncertain whether these findings would apply to the larger population of Zambian children who may experience more illnesses.

In summary, estimates of absorption and retention of VA were adequate in response to an oral high-dose VAS, or a smaller dose of SI-labeled VA in apparently healthy preschool age Zambian boys from a low-income community. Absorption and retention of VA did not differ for a single VA supplement of either 5 or 60 mg. The data suggest that recent fever and malaria may adversely affect absorption and retention of VA. The UER of VA in Zambian boys was similar to the estimated system catabolic rate for VA in preschool-age Peruvian children, and did not differ by size of the VA dose administered. The estimated retention of the oral dose of stable isotope labeled VA was higher (~64%) than the value of 50% that is currently used in the 'Olson equation' for estimation of total body VA pool size using stable isotope dilution methodology. This study demonstrates that AMS methodology can be carried out in a pediatric population in a low-income community setting, and suggests that this methodology could be used to obtain direct, quantitative estimates of VA metabolism for other population sub-groups for which data do not exist.

ACKNOWLEDGEMENTS

We thank the children and their caretakers for their participation in the study. We are grateful to Dr. Michael Green for providing the [$^{14}C_2$]-labeled VA, and to Mrs. Betty Adams (Department of Animal Science, UC Davis), and Tracy Francis (DANR, UC Davis) for their assistance in completing the carbon measurements. This work performed under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344.

Figure Legend

Figure 1

Diagram of study protocol

Figure 2

Radiochromatogram of [$^{11,12\text{-}14}C_2$]-retinyl acetate

Figure 3

Cumulative excretion of ^{14}C in stool expressed as percentage of dose of ^{14}C administered. Panel A: 4 children who received a single, oral high-dose vitamin A supplement (60 mg); and Panel B: 4 children who received a single, oral dose of stable isotope-labeled VA (5 mg).

Figure 4

Cumulative excretion of ^{14}C in urine expressed as percentage of dose of ^{14}C administered. Panel A: 4 children who received a single, oral high-dose VA supplement (60 mg); and Panel B: 4 children who received a single oral dose of stable isotope-labeled VA (5 mg).

Figure 5

Urinary elimination of ^{14}C expressed as fraction of dose of ^{14}C administered: Panel A: 4 children who received a single, oral high-dose VA supplement (60 mg); and Panel B: 4 children who received a single oral dose of stable isotope-labeled VA (5 mg).

References

1. Clewes C: **Report of the national survey to evaluate the impact of vitamin A interventions in Zambia in July and November 2003.** United States Agency for International Development, Centers for Disease Control, Atlanta, 2003.

2. Sivakumar B, Reddy V: **Absorption of labelled vitamin A in children during infection**. *Br J Nutr* 1972, **27**(2):299-304.

3. Pereira SM, Begum A: **Retention of a single oral massive dose of vitamin A**. *Clinical science and molecular medicine* 1973, **42**(2):233-237.

4. Kusin JA, Reddy V, Sivakumar B: **Vitamin E supplements and the absorption of a massive dose of vitamin A**. *Am J Clin Nutr* 1974, **27**(8):774-776.

5. Brown K, Dingley KH, Turteltaub KW: **Accelerator mass spectrometry for biomedical research**. *Methods Enzymol* 2005, **402**:423-443.

6. Le Vuong T, Buchholz BA, Lame MW, Dueker SR: **Phytochemical research using accelerator mass spectrometry**. *Nutr Rev* 2004, **62** (10):375-388.

7. Ognibene TJ, Bench G, Vogel JS, Peaslee GF, Murov S: **A high-throughput method for the conversion of CO2 obtained from biochemical samples to graphite in septa-sealed vials for quantification of 14C via accelerator mass spectrometry**. *Anal Chem* 2003, **75**(9):2192-2196.

8. Dueker SR, Lin Y, Buchholz BA, Schneider PD, Lame MW, Segall HJ, Vogel JS, Clifford AJ: **Long-term kinetic study of beta-carotene, using accelerator mass spectrometry in an adult volunteer**. *J Lipid Res* 2000, **41**(11):1790-1800.

9. Ho CC, de Moura FF, Kim SH, Burri BJ, Clifford AJ: **A minute dose of 14C-{beta}-carotene is absorbed and converted to retinoids in humans**. *J Nutr* 2009, **139**(8):1480-1486.

10. Clifford AJ, Arjomand A, Dueker SR, Schneider PD, Buchholz BA, Vogel JS: **The dynamics of folic acid metabolism in an adult given a small tracer dose of 14C-folic acid**. *Adv Exp Med Biol* 1998, **445**:239-251.

11. Denk E, Hillegonds D, Hurrell RF, Vogel J, Fattinger K, Hauselmann HJ, Kraenzlin M, Walczyk T: **Evaluation of (41)calcium as a new approach to assess changes in bone metabolism: effect of a bisphosphonate intervention in postmenopausal women with low bone mass**. *J Bone Miner Res* 2007, **22**(10):1518-1525.

12. Furr HC, Amedee-Manesme O, Clifford AJ, Bergen HR, 3rd, Jones AD, Anderson DP, Olson JA: **Vitamin A concentrations in liver determined by isotope dilution assay with tetradeuterated vitamin A and by biopsy in generally healthy adult humans**. *Am J Clin Nutr* 1989, **49**(4):713-716.

13. Haskell MJ, Ribaya-Mercado JD: **Handbook on Vitamin A Tracer Dilution Methods to Assess Status and Evaluate Intervention. Harvest-Plus Technical Monograph Series** Washington, DC; Cali, Colombia; 2005.

14. AOAC: **Official Methods of Analysis; Method 972 43**, 16th Ed. Arlington, VA; 1997.

15. Vogel JS, Love AH: **Quantitating isotopic molecular labels with accelerator mass spectrometry**. *Methods Enzymol* 2005, **402**:402-422.

16. Haskell MJ, Lembcke JL, Salazar M, Green MH, Peerson JM, Brown KH: **Population-based plasma kinetics of an oral dose of [2H4]-retinyl acetate among preschool-aged, Peruvian children**. *Am J Clin Nutr* 2003, **77**(3):681-686.

17. Bieri JG, Tolliver TJ, Catignani GL: **Simultaneous determination of alpha-tocopherol and retinol in plasma or red cells by high pressure liquid chromatography**. *Am J Clin Nutr* 1979, **32**(10):2143-2149.

18. Iqbal J, Hira PR, Al-Ali F, Khalid N, Sher A: **Modified Giemsa staining for rapid diagnosis of malaria infection**. *Med Princ Pract* 2003, **12**(3):156-159.

19. Sauberlich HE, Hodges RE, Wallace DL, Kolder H, Canham JE, Hood J, Raica N, Jr., Raica N, Jr.: **Vitamin A metabolism and requirements in the human studied with the use of labeled retinol**. *Vitam Horm* 1974, **32**:251-275.

20. Green MH, Green JB: **Vitamin A intake and status influence retinol balance, utilization and dynamics in rats**. *J Nutr* 1994, **124**(12):2477-2485.

Table 1. Initial characteristics of the subjects

Parameters	60 mg VA (n = 4)	5 mg VA (n = 4)
Age (y)	3.9 (0.3)	3.6 (0.3)
Weight (kg)	13.2 (1.7)	14.6 (0.8)
Height (cm)	94.2 (8.0)	96.1 (3.2)
MUAC (cm)	16.7 (0.4)	16.7 (0.3)
Ht-for-age z-score	-1.4 (1.3)	-0.9 (0.8)
Wt-for-age z-score	-1.8 (0.7)	-0.9 (0.7)
Wt-for-ht z-score	-1.1 (1.2)	-0.2 (0.3)

Mean (SD); Mean values do not differ between groups, Student's t-test ($P > 0.50$); VA=vitamin A; MUAC=mid-upper arm circumference

Table 2: Comparison of estimated absorption, retention and urinary elimination of vitamin A by study group

::

Indicator	60 mg VA (n = 4)	5 mg VA (n = 4)	P-value
Absorption, %	83.8 (7.1)	76.5 (9.5)	0.26
Retention, %	76.3 (6.7)	71.1 (9.4)	0.40
Urinary Elimination, %/d	1.9 (0.6)	1.8 (1.2)	0.88

Mean (SD); mean values do not differ by group; Student's t test;
VA=vitamin A

Table 3: Comparison of estimated amount of cumulative vitamin A excreted in urine during the first 3 days after dosing and estimated amount of vitamin A excreted in urine based on the UER during the first 3 days after dosing

::

Group	Estimated vitamin A excreted in urine, mg (based on cumulative amount at 3 days after dosing)	Estimated vitamin A excreted in urine, mg (based on UER at 3 days after dosing)	P-value
60 mg VA (n=4)	3.24 ± 0.97	2.60 ± 0.92	0.15
5 mg VA (n=4)	0.38 ± 0.12	0.22 ± 0.11	0.13

Means (SD); mean values do not differ within groups; Student's t test;
VA=vitamin A; UER=urinary elimination rate

Table 4: Summary of initial and final plasma retinol concentration for the boys who received the high-dose VA supplement (60 mg) and those received the stable-isotope labeled VA (5 mg)

	60 mg VA (n = 4)	5 mg VA (n = 4)	P-value
Initial plasma retinol (μmol/L)	1.10 (0.28)	1.09 (0.28)	0.68
Final plasma retinol (μmol/L)	1.03 (0.20)	1.09 (0.26)	1.00

Mean (SD); Mean values do not differ between groups at either time point; Student's t test; VA=vitamin A

Table 5: Period prevalence of various illnesses for all children during the periods of estimation of absorption and retention of vitamin A.

	Period prevalence of illness (%) during the period for estimation of absorption[1]	Period prevalence of illness (%) during the period for estimation of retention [2]
Cough	25.0 (25.6)	20.2 (17.8)
Malaria	1.1 (3.2)	0.7 (2.0)
Skin rashes	4.6 (12.9)	8.3 (23.6)
Diarrhea	1.1 (3.2)	0.7 (2.0)
Fever	1.1 (3.2)	0.7 (2.0)

Mean (SD); n = 8; [1]one week prior to and 3 days after dosing; [2] one week prior to and one week after dosing

Vitamin A supplemented group: n=10

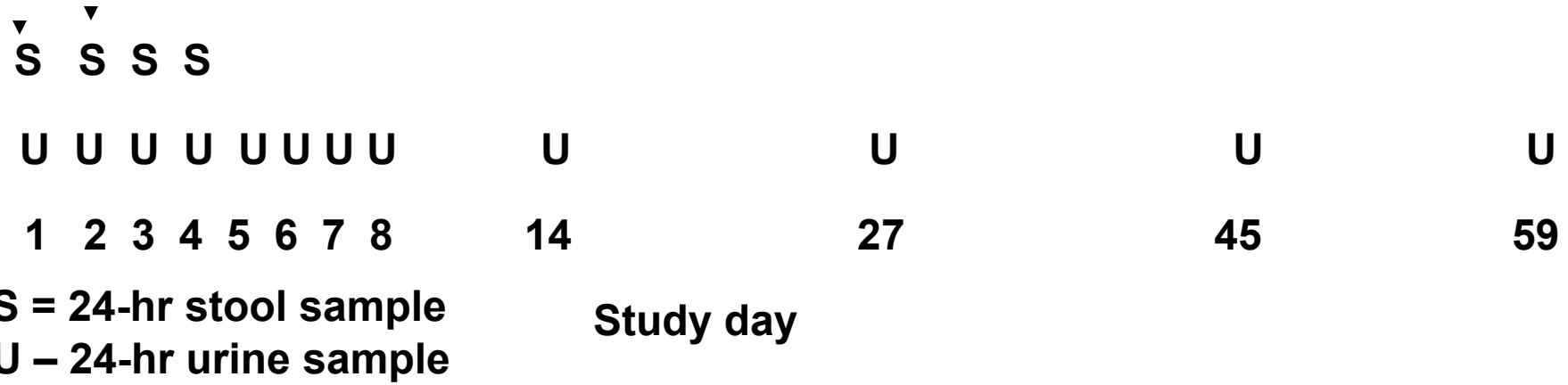
High-dose VA (60 mg) + ^{14}C-RA (25 nCi)

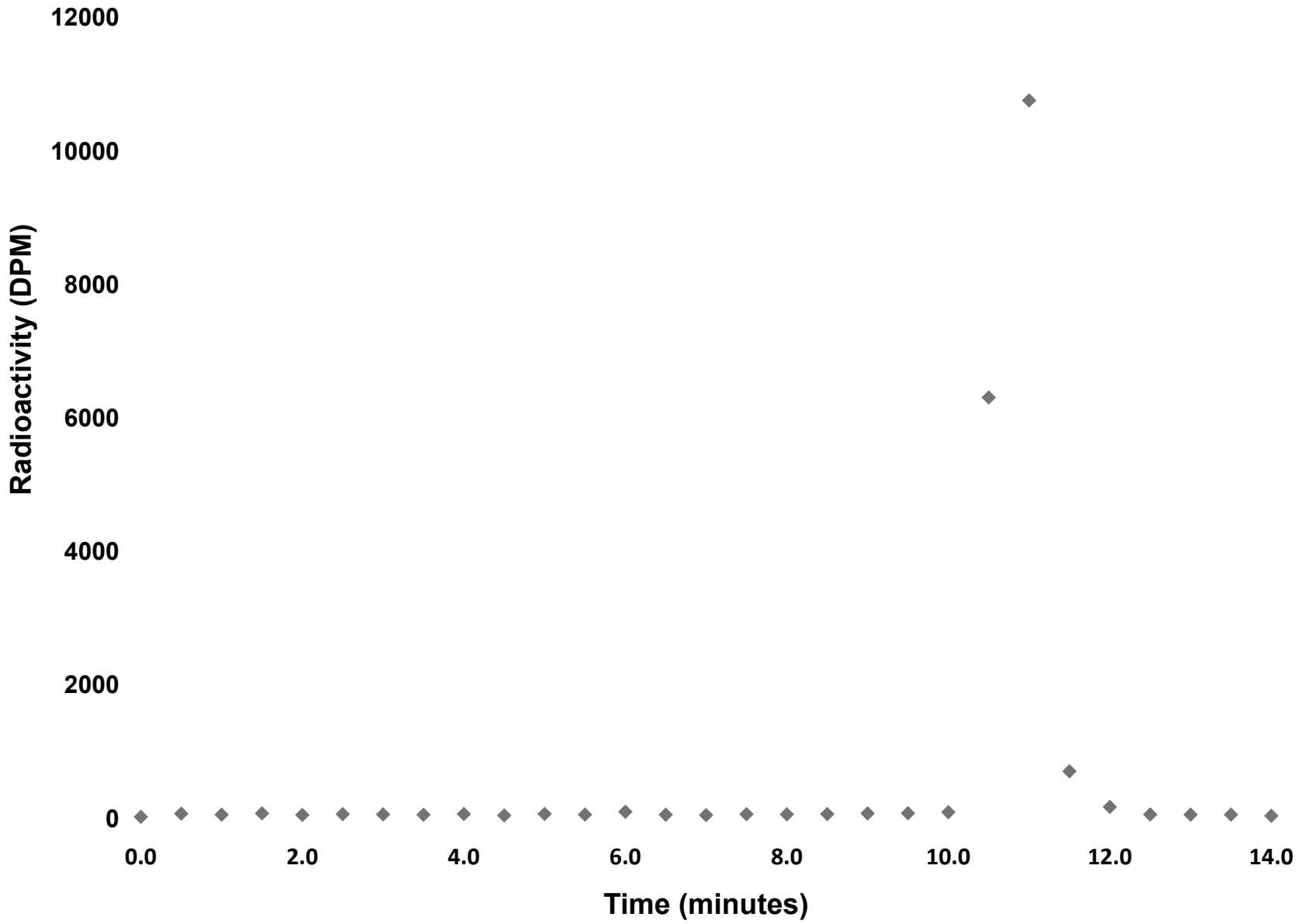
OR

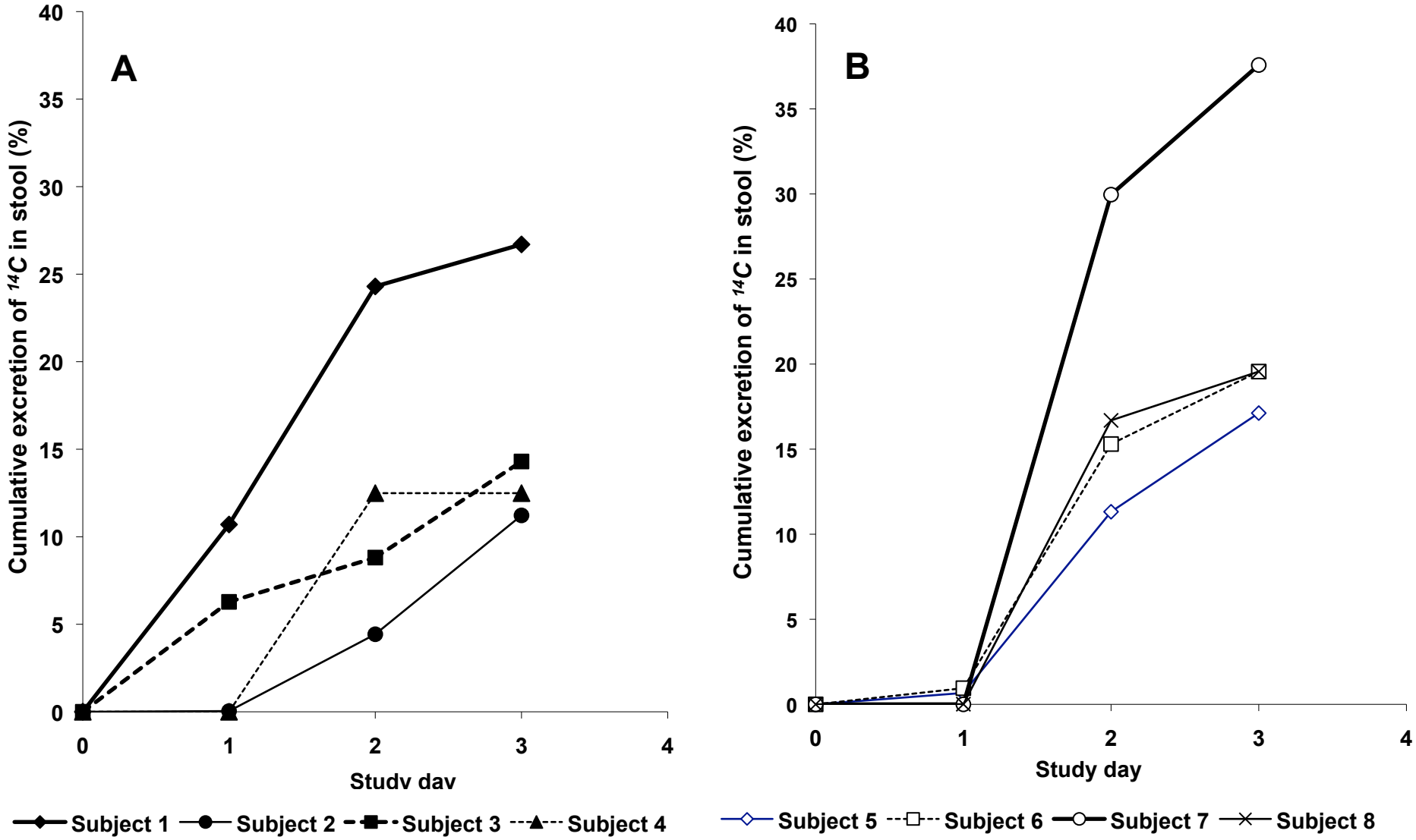
Placebo group: n=10

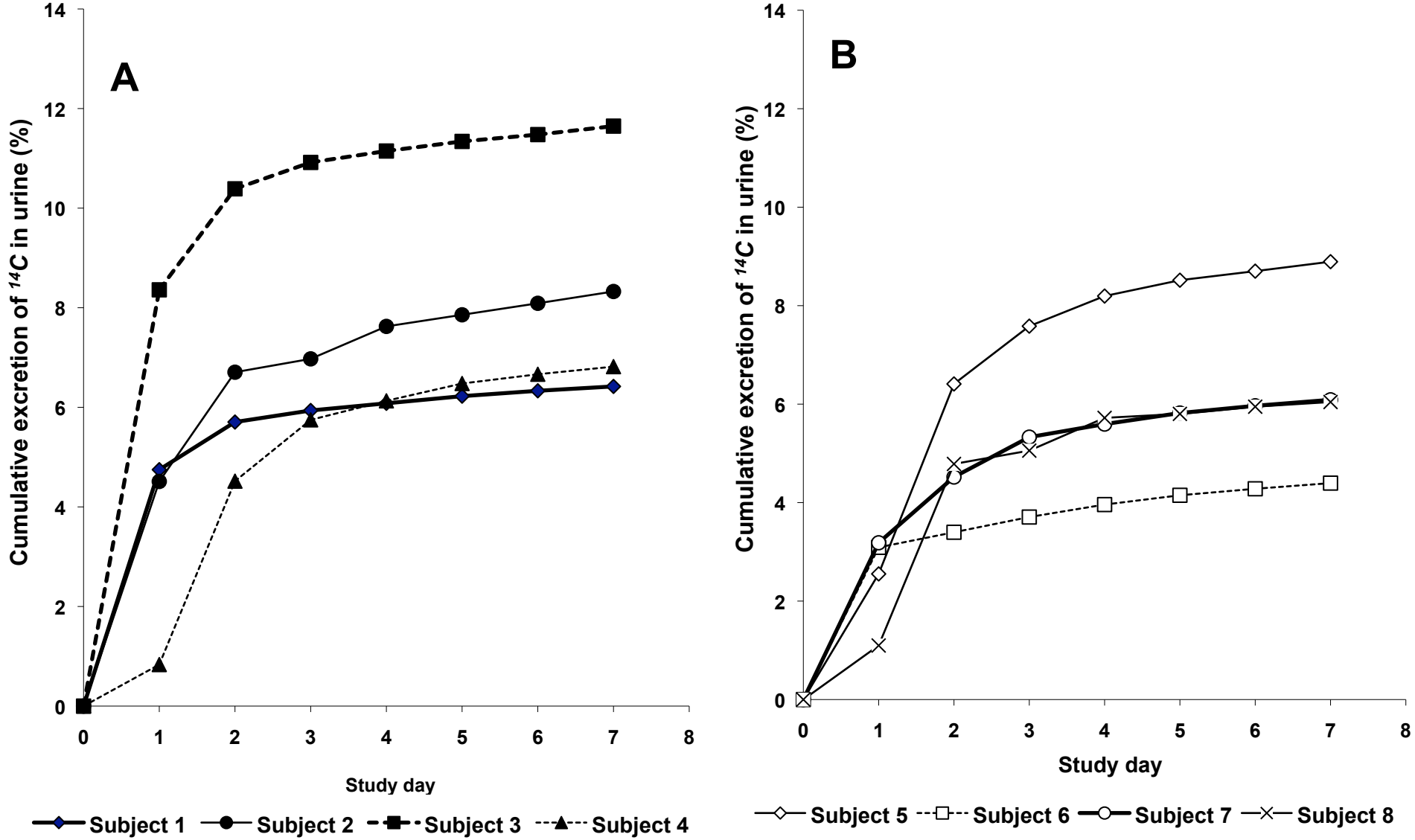
Stable isotope VA (5 mg) + ^{14}C-RA (25 nCi)

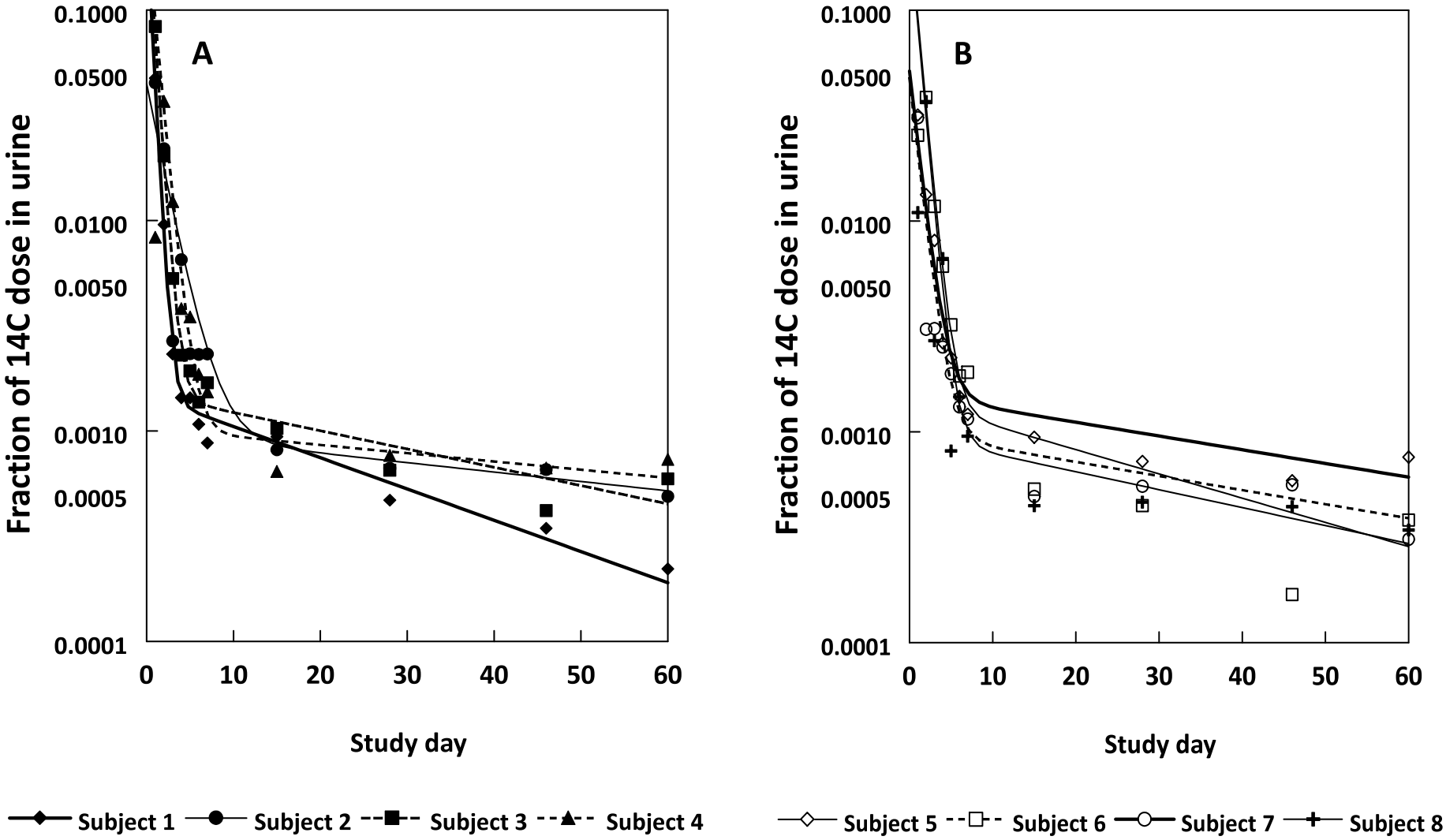
Baseline samples




12000
10000
8000
6000
4000
2000
0
Radioactivity (DPM)
0.0
2.0
4.0
6.0
8.0
10.0
12.0
14.0
Time (minutes)


A
B
Cumulative excretion of ¹⁴C in stool (%)
Studv dav
Study day
0
5
10
15
20
25
30
35
40
0
1
2
3
4
Subject 1
Subject 2
Subject 3
Subject 4
Subject 5
Subject 6
Subject 7
Subject 8


A
B
Cumulative excretion of ^{14}C in urine (%)
Study day
0
1
2
3
4
5
6
7
8
0
2
4
6
8
10
12
14
Subject 1
Subject 2
Subject 3
Subject 4
Subject 5
Subject 6
Subject 7
Subject 8


A
B
Fraction of 14C dose in urine
0.1000
0.0500
0.0100
0.0050
0.0010
0.0005
0.0001
0
10
20
30
40
50
60
Study day
Subject 1
Subject 2
Subject 3
Subject 4
Subject 5
Subject 6
Subject 7
Subject 8