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Vitamin B12 Added as a Fortificant to Flour Retains High Bioavailability when Baked in Bread

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Abstract

Vitamin B12 deficiency and depletion are common world-wide, particularly in populations that consume low amounts of animal source foods. WHO and the Food Fortification Initiative recommend that wheat flour be fortified with vitamin B12 in regions where intake of B12 is low. The purpose of this pilot study in five participants was to determine if fortification of flour with B12 produced a bread product with intact B12 still present and to determine if healthy elderly absorb sufficient B12 from bread fortified in this manner. High-purity crystalline ^{14}C-B12 was dissolved in water and added to flour (2 μg B12 /100 g flour) in a bread maker and made into rolls (average 1.17 kBq (31.5 nCi) ^{14}C-B12 in a total of 0.8 μg B12 per roll). Excess ^{14}C first appeared in plasma 4 h after ingestion of the ^{14}C fortified bread and plasma levels returned almost to background by 72 h. Measurement of ^{14}C in plasma verified that the dose was absorbed into the systemic circulation. The cumulative % dose recovered in urine was 4.8-37.0% (mean = 20.1%). Most of the ^{14}C label in the stool appeared by day 4, and the cumulative % dose recovered in stool was 24.5- 43.0% (mean = 31.8%). Bioavailability among the 5 participants, calculated by subtracting the sum of urinary and fecal ^{14}C excretion from the administered dose, was 28.4-63.7% (mean = 48.0%). This study showed that when B12 is added as a fortificant to flour it survives the fermentation and baking processes, and retains ~ 50% bioavailability when fed in small doses to healthy subjects. The Recommended Dietary Allowance of B12 for adults is 2.4 μg/d. This recommendation assumes that usual bioavailability of low doses of the vitamin in the crystalline form is 60%, while for the same amount in foods such as meat and fish it is 50%. Our pilot study shows that B12 added to bread as a fortificant in flour was absorbed as well as it is from endogenous food sources such as meat and fish.

Keywords

Vitamin B12; bioavailability; fortification; cobalamin; vitamin pharmacokinetics

Introduction

Vitamin B12 (B12; cobalamin) deficiency is very common in the US and world-wide. The risk of deficiency increases with aging, with an estimated 25% of persons over age 65 deficient or depleted in the U.S.A. [1-4]. In one study, 40% of the elderly with low serum B12 had a problem absorbing the vitamin from food [1]. Loss of gastric function (gastric atrophy) and low acid secretion by the stomach, typical of aging, are thought to be involved. However, most elderly can absorb crystalline forms of the vitamin, such as those used in supplements and in fortification [5].

Also, vitamin B-12 deficiency and depletion are highly prevalent in the many populations world-wide who consume low amounts of animal source foods, the only natural source of the vitamin. For this reason, WHO and the Flour Fortification Initiative, for example, are currently recommending that wheat flour be fortified with vitamin B-12 in regions where intake of the vitamin is low. However, because there have been no studies that quantified the bioavailability of B-12 administered in this way there is inadequate data on which to recommend an appropriate level of fortification.

This question is also particularly relevant today in the U.S.A. and the many other countries where flour is fortified with folic acid but not yet fortified with vitamin B12. There is concern that high levels of folic acid fortification, especially when combined with folic acid supplements, may worsen the neurological and cognitive deterioration that can occur with vitamin B12 deficiency [1,4], suggesting that flour fortification with vitamin B12 in addition to folic acid may be especially important. When this study was done, however, it was unclear how much vitamin B12 to add, and whether the elderly would be able to absorb a sufficient amount of the vitamin from this food source.

The purpose of this pilot study was to determine if fortification of flour with crystalline vitamin B12 produced a bread product with intact B12 still present and to determine if healthy elderly absorb sufficient vitamin B12 from bread fortified in this manner.

Materials and Methods

Recruitment

The goal was to quantify vitamin B12 absorption from fortified bread in five healthy subjects age 60 y or older. The pilot study was limited to five subjects since this number typically provides sufficient variability to plan a larger, more comprehensive study. Subject recruitment and study procedures were approved by the Human Subjects Review Committees at the University of California, Davis and Lawrence Livermore National Laboratory (LLNL), and written informed consent was obtained from all participants. Exclusion criteria included any chronic health disorder, anemia, renal insufficiency, excessive alcohol consumption, prior gastrointestinal surgery, use of any drugs that interfere

with B12 absorption or metabolism, and use of vitamin B12 supplements. A normal serum vitamin B12 and pepsinogen I level (≥300 pg/mL and >25 ng/mL, respectively) were required for inclusion. All 5 subjects completed the study.

Preparation of ^{14}C-B12 fortified bread

The ^{14}C-B12 was prepared at the University of California Davis Medical Center in Sacramento, CA using the method described in detail by Carkeet et al., (2006) [6]. In short, the synthesis of ^{14}C-B12 was accomplished by using *Salmonella enterica* to incorporate ^{14}C-[C2]dimethylbenzimidazole (DMB) in the DMB moiety of B12. Normally *Salmonella enterica* produces B12 *de novo* only under anaerobic conditions to support growth on ethanolamine. The cells cannot grow in aerobic conditions feeding on ethanolamine because they lack B12 precursors DMB and cobinamide, although they retain the ability to produce B12. By supplying ^{14}C-DMB and cobinamide B12 is assembled without isotope dilution, producing ^{14}C-B12 of high specific activity (2.1 GBq/mmol, 58 mCi/mmol) [6] assayed at 98.6% radiopurity. The procedure utilizes a cyanation step to convert all forms of B12 to cyanocobalamin with the ^{14}C label as shown in Figure 1.

Crystalline ^{14}C-B12 was dissolved in water, which was the first ingredient placed in the bread machine, followed by flour, sugar, salt, and yeast. Dissolving the ^{14}C-B12 in water allowed homogeneous distribution and insured the labelled B12 was uniformly distributed in the bread product. The amount of B12 used to prepare the bread dough was similar to what might be used in a flour fortification program (2 μg B12/100 g flour) [7]. The bread machine utilized a program to make dough for dinner rolls, homogeneously mixing the dough and allowing it to rise initially inside the bread machine without any human intervention (Fig. 2). Dough was divided into rolls, weighed (64.8 g each), and allowed to rise (proof) a second time on a cookie sheet outside of the bread machine until double in size, and then baked in an oven at 190 °C for 12 min. For the purposes of this project, using single serving dinner rolls to deliver each dose provided best control and accounting of each dose. Each roll contained an average dose of approximately 31 nCi ^{14}C-B12, in a total of 0.8 (0.79-0.81) μg B12 (Table 1). This amount was shown in a previous study to be sufficient to achieve measurable ^{14}C enrichment in serum and urine of human subjects [6]. The ^{14}C values were confirmed by analysis following extraction of a bread roll using liquid scintillation counting. Active B12 levels were confirmed by microbiological assay done at Covance Laboratories, Inc. (Madison, WI).

Dosing and sample collection

Subjects reported to the USDA, ARS Western Human Nutrition Research Center, Davis, CA and consumed single labelled dinner rolls with coffee at 08:00 h. Blood samples were taken at baseline, one hour after ingestion of the bread, and hourly through the 12th hour after dose. Volunteers were then sent home for the night and returned 24 h after the dose for another blood draw. The subjects began collection of total urine and stool from the time of the dose and continued these collections for a total of six days.

Stool collections were homogenized in order to obtain representative samples. A stool sample ≤350 g was placed in a 3.8 L paint can and an equivalent mass of water added. Fifty

grams of 7.9 mm diameter 316 stainless steel balls were added to the can and the lid sealed with duct tape. The can was shaken on a commercial paint shaker for 30 min, then aliquots of 1.5 mL and 50 mL were retained and the remaining stool discarded. The 1.5 mL tube was sent to LLNL for graphitization and AMS analysis of ^{14}C content. The 50-mL sample was retained for possible future analysis. Each sample was processed in a new can with new stainless steel balls to prevent cross-contamination from sample to sample. This procedure was modified from previous nutrient tracer studies that used strong base and isopropyl alcohol to extract compounds with poor solubility in water [8-10]. The assay using only water was developed to avoid the generation of hazardous waste and was validated by measuring the recovery of a spike 2.5 nCi ^{14}C-B12 using various solvents to generate the homogenate.

Aliquots of stool homogenate, plasma, and urine were shipped frozen by overnight courier to LLNL for graphitization using Ognibene's Method [11]. The carbon concentrations of all urine samples and stool homogenates were measured at LLNL using an Exeter Analytical CE440 carbon analyzer (North Chelmsford, MA USA). Five aliquots of each urine and stool homogenate were measured with the carbon analyzer and the average was used in calculations needing the carbon concentration of the samples. Typical carbon concentration ranges and standard deviations were 0.30-1.00±0.05% and 4.0-10.0±0.5% for urine and stool homogenates, respectively. Individual subjects had relatively little variation between timepoints over the course of the study, differences between subjects due to diet and water consumption were greater.

Analysis of ^{14}C

General lab procedures and sample prep was completed using procedures for tracer ^{14}C as described previously [11,12]. All graphite was packed into individual Al targets and analyzed on the compact 1 MV National Electrostatics Corporation (Middleton, WI) Accelerator Mass Spectrometry (AMS) system at LLNL using standard procedures [6,13-15]. Samples were normalized to 4 identically prepared IAEA C-6 standards, measured similarly to the procedure described and the data were reported in units of "fraction modern" (FM) as described by Stuiver and Polach (1977) [16]. The isotopic fractionation correction of δ13C = −25 per mil was used for all samples.

Results and Discussion

A summary of the subject characteristics, the doses of radioactivity and vitamin B-12 given, total nCi excreted, and % bioavailability is provided in Table 1. The excess ^{14}C measured in plasma, urine and stool homogenates was not assayed for chemical identity. Since excess ^{14}C in urine has previously been shown to be something other than intact B12 [6], we express ^{14}C in terms of moles of ^{14}C rather than units of B12.

Elevated ^{14}C first appeared in plasma at the 4 h time point after ingestion of the ^{14}C fortified dinner rolls and reached its peak 7-9 h after the dose. Plasma ^{14}C levels reached equilibrium that was slightly elevated above background by 72 h after dose (see Fig. 3). This was probably primarily due to B12 bound to haptocorrin, which has a longer circulating plasma

half-life than transcobalamin, approximately 10 d compared to 2 h [17]. The measurement of ^{14}C in plasma served to verify that the dose was absorbed into the systemic circulation.

The percentages of the dose radioactivity recovered in urine and stool homogenates are shown in Figure 4. The majority of ^{14}C label recovered in urine appeared in the first two 24-h urine collections. However, the amount of label recovered in the first 48 h varied widely between subjects. The cumulative % dose recovered in urine ranged from 4.8 to 37.0% with a mean of 20.1%. ^{14}C levels in urine returned to background values by the fifth 24-h urine collection (120 h). The majority of ^{14}C label in stool appeared in the stool by day 4, however, as with the urine, the amount of label recovered varied widely among the participants. ^{14}C concentration in stool returned to background levels by the fifth 24-h stool collection (120 h). The cumulative % dose recovered in stool ranged from 24.5 to 43.0% with a mean of 31.8%. See Table 1 for total ^{14}C activity excreted.

Calculation of bioavailability

While it is accepted that the isotope in feces represents the unabsorbed vitamin from bread, there was a relatively large amount of urinary ^{14}C excreted in this study. Previous studies using radiocobalt have shown that urinary excretion of an oral dose of vitamin B12 is far less than 1% of the dose given, ranging from 0.10–0.41% of ^{56}Co, ^{57}Co, ^{58}Co, ^{60}Co labelled doses in normal subjects [17-21]. In contrast, we found 5 to 37% of the ^{14}C dose in urine (Table 1). In a study by Carkeet et al. [6] who used an aqueous dose with a similar level of ^{14}C-B12, it was determined that the majority (>99%) of radioactivity in the urine from the aqueous dose was not intact vitamin B12 and represented a non-bioavailable fraction of the oral dose. This is an unusual situation where a fragment of a decomposed vitamin is absorbed and then rapidly excreted in urine. Therefore, based on these findings, we have assumed, for the purpose of calculating bioavailability in our study, that the substantial amount of urinary ^{14}C excreted also represented a non-bioavailable fraction of the ^{14}C-vitamin B12 dose given and we have calculated % bioavailability using both urinary and fecal excretion of ^{14}C [6]:

$$\%\ Bioavailability = \frac{^{14}C\ \ intake - [^{14}C\ \ excretion\ in\ feces + urine]}{^{14}C\ \ intake} x\ 100\%$$

The % bioavailability among the five subjects, calculated using the sum of urinary and fecal ^{14}C excretion, ranged from 28.4 to 63.7% with a mean of 48.0% (Table 1).

Conclusions

This pilot study used a novel method of producing ^{14}C-B12-labelled dinner rolls to show that when the vitamin is added as a fortificant to flour it survives the fermentation and baking process, and retains its approx. 50% bioavailability when fed in small doses to healthy subjects. The Recommended Dietary Allowance for vitamin B12 established by the Institute of Medicine is 2.4 μg/d for adults [22]. This recommendation assumes that usual bioavailability of low doses of the vitamin in the crystalline form is 60%, while from foods such as meat and fish it is 50% [4,5]. Our pilot study shows that vitamin B12 added to bread

as a fortificant in the amount of 2 μg B12/100 g flour is absorbed as well as it is from meat and fish [2].

These data will be useful to organizations such as WHO and the global Flour Fortification Initiative, which at present have no equivalent data on which to base recommendations for B-12 fortification levels in flour. This pilot could lead to larger trials evaluating the efficacy of improving vitamin status in larger test populations through fortification [23].

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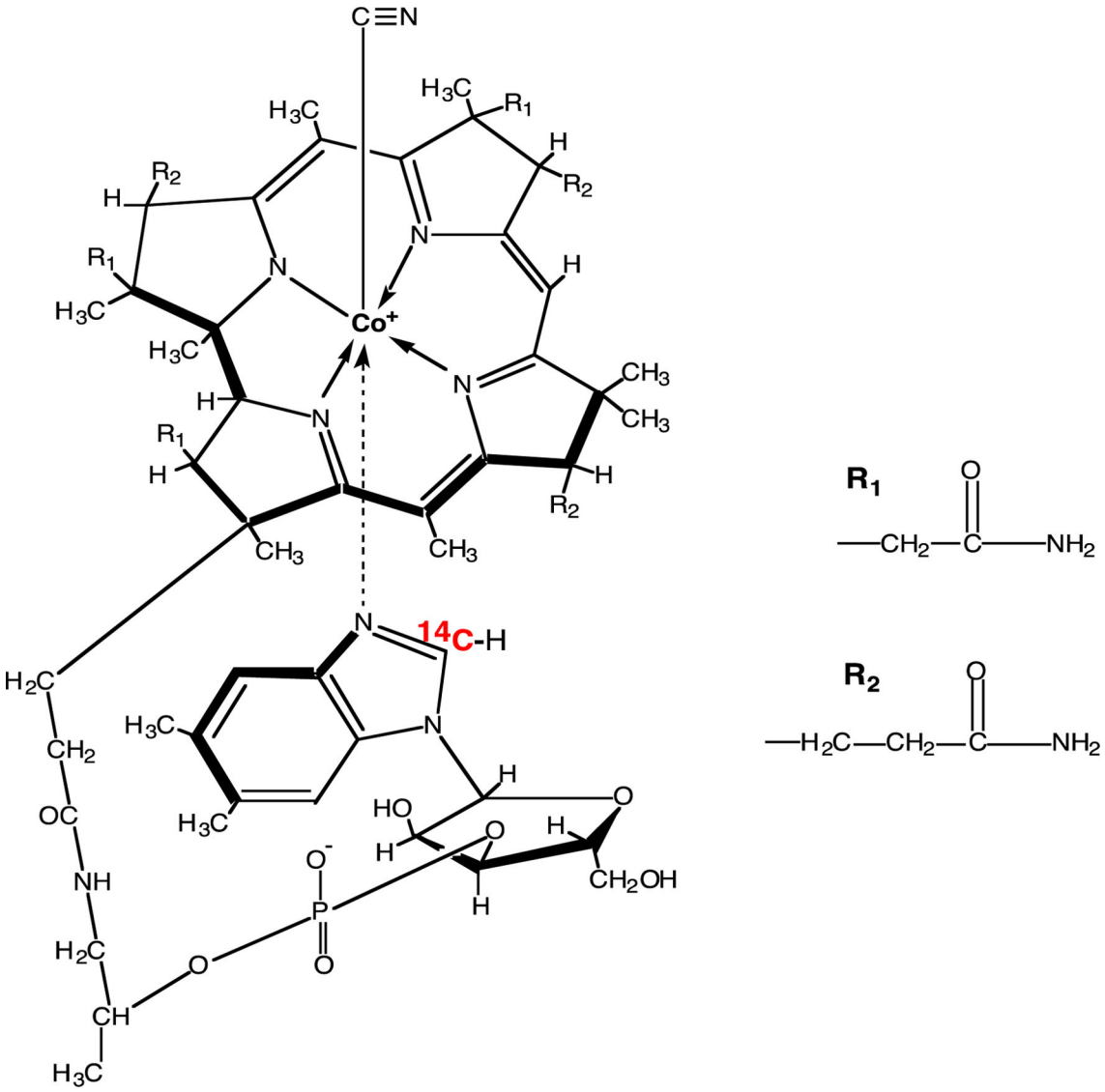

Fig. 1.

Structure of the cyanocobalamin form of vitamin B12 with the ^{14}C denoted in red boldface type. Figure adapted from [17].

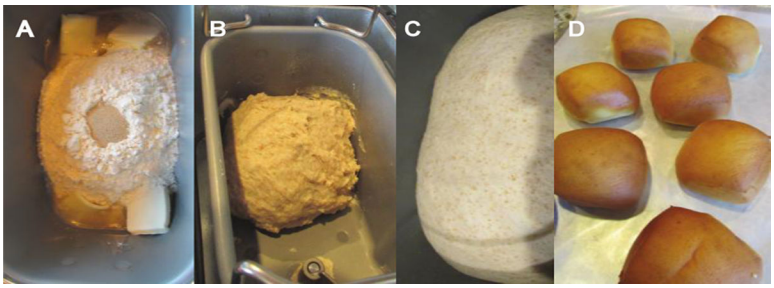

Fig. 2.

Production of ^{14}C-B12 fortified dinner rolls. (A) Starting with ^{14}C-B12 dissolved in water, (B) dough was mixed and (C) rose within the bread machine. (D) Dough was split into rolls and baked in an oven as directed in the recipe to produce uniform dinner rolls.

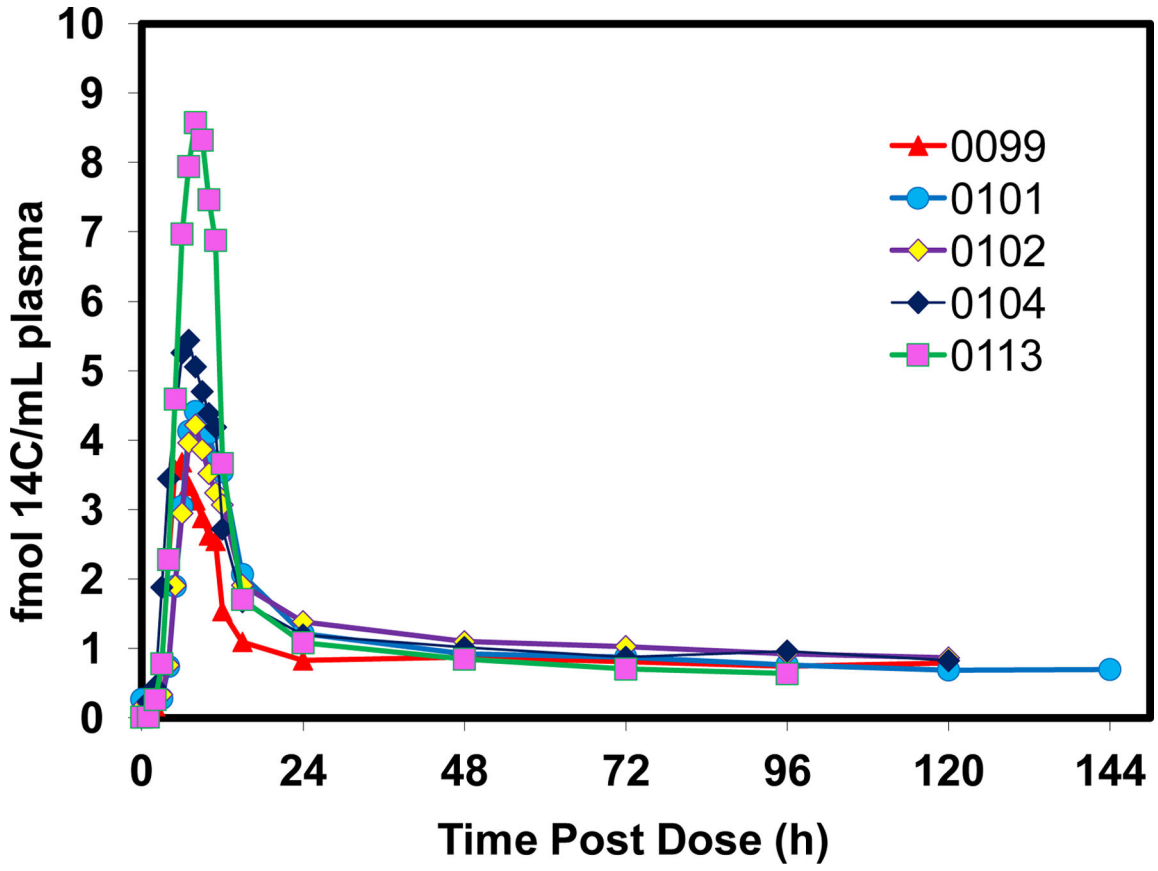

Fig. 3.

Absorption and clearance of ^{14}C in plasma following the consumption of dinner rolls fortified with ^{14}C-B12. Each subject consumed one dinner roll containing on average 0.8 μg B12 and 31.5 nCi (1.17kBq or 0.54 nmol) of ^{14}C. The individual doses are given in Table 1.

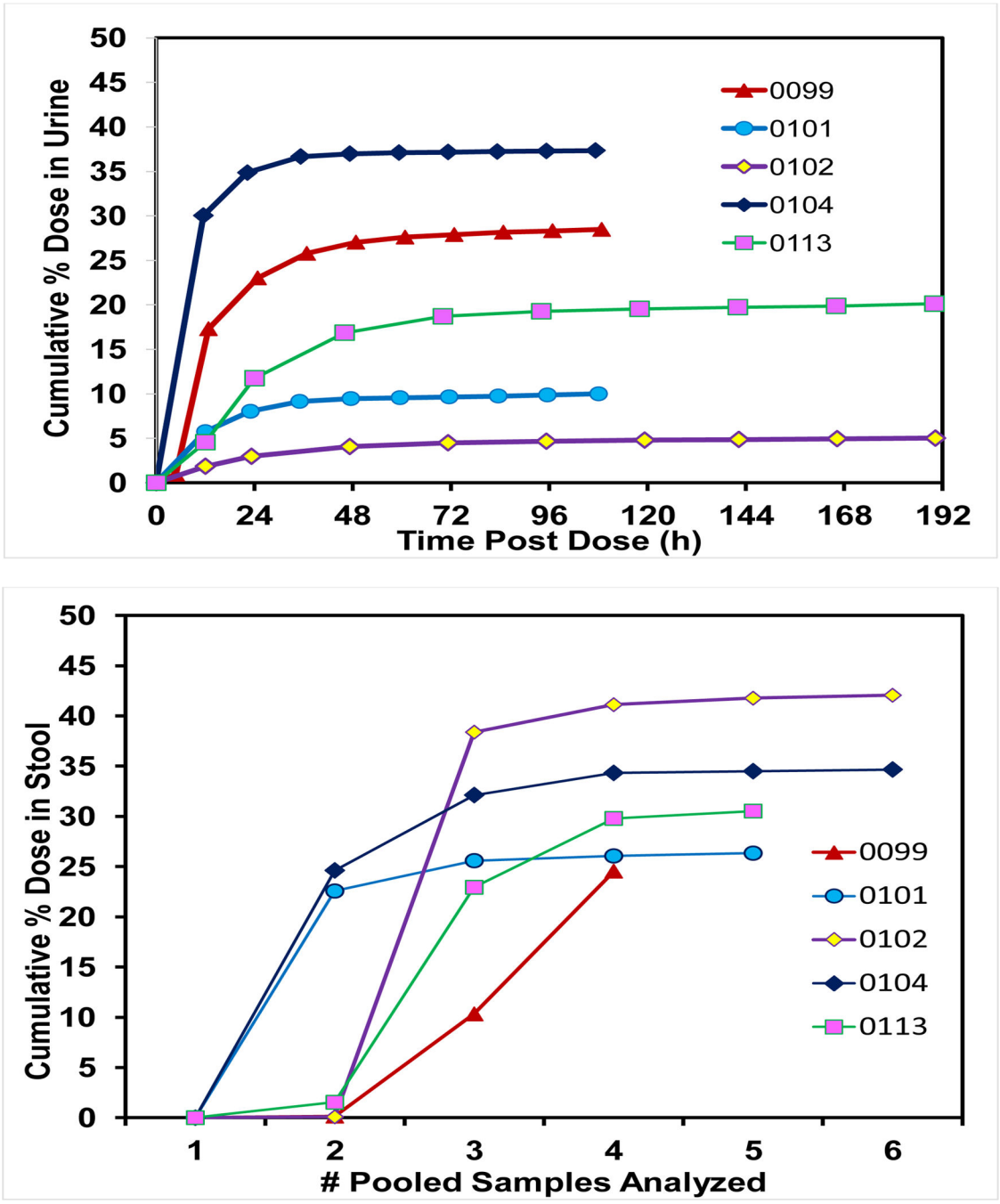

Fig. 4.

Percent of dose ^{14}C activity excreted in urine (A) and stool (B). Urine was pooled into 12-h fractions on the first day and then in 24-h fractions the remainder of the study. Each stool during the study was homogenized separately and analyzed.

Table 1

Study subject characteristics and doses

Age (y)	Gender	Serum B-12 (pmol/L)	Pepsinogen I (ng/mL) †	nCi dose	B-12 Dose (μg)	Total nCi Excreted (urine/stool)	% Bioavailability
62	F	302	76	31.03	0.79	16.47	46.9
61	F	251	112	31.48	0.80	14.92	52.6
69	M	263	149	31.70	0.81	11.49	63.7
66	F	592	109	31.59	0.81	22.61	28.4
78	F	472	90	31.64	0.81	16.02	49.4

† Normal serum pepsinogen I concentrations are >25 ng/mL.