

## Original Research

# Dysprosium Chloride as a Nonabsorbable Gastrointestinal Marker for Studies of Stable Isotope-Labeled Triglyceride Excretion in Man

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**Key words:**  $^{13}\text{C}$ -label, cystic fibrosis, malabsorption, nonabsorbable marker, triglycerides

**Objective:** The aim of this work was to determine if dysprosium chloride ( $\text{DyCl}_3$ ) is a suitable nonabsorbable marker for studies of labeled-triglyceride excretion in cystic fibrosis patients allowing excretion to be determined accurately after analysis of one or two stools.

**Methods:** A series of 66 absorption studies were conducted in 36 young cystic fibrosis patients over a five year period. All tests consisted of ingesting a single test meal containing both  $^{13}\text{C}$ -labeled triglyceride ( $\text{TG}^*$ ) and  $\text{DyCl}_3$ ; in most studies the food colorant brilliant blue (FD&C blue #1) was administered along with the  $\text{DyCl}_3$ . Ingestion of the test meal was followed by collection of individual stools for 72 to 96 hours. Stools were analyzed for  $^{13}\text{C}$ -Excess ( $^{13}\text{C}^*$ ) and Dy.

**Results:** Excretion of Dy in cystic fibrosis patients who exhibited a wide-range of steatorrhea was quantitative. Fractional excretion of Dy and  $^{13}\text{C}^*$  in individual stools showed a high linear correlation ( $r^2 = 0.969$ ) with a slope and y-intercept close to unity and zero, respectively. As a result, estimates of  $\text{TG}^*$  excretion based on analysis of only two stools (partial pool method, PPM) were not different from those based on the analysis of all stools or stool composites. This was true both when Dy content and when stool color due to ingested brilliant blue was used to determine which stools to analyze for the PPM.

**Conclusions:** Combining the use of Dy and brilliant blue permits reasonably accurate estimates of fecal  $\text{TG}^*$  excretion after analysis of samples from two easily identified stools. This practical method can be used to address many important clinical and experimental questions regarding triglyceride digestion and absorption that may otherwise go unanswered.

## INTRODUCTION

The method of "fecal isotope balance," utilizing stable isotope-labeled substrates, is an important experimental tool for investigations of gastrointestinal digestion and absorption of selected nutrients [1–5]. With this approach, a nutrient labeled with an appropriate stable-isotope tag is administered orally and complete fecal collections are performed and then analyzed to estimate the amount of unabsorbed label. The approach

requires complete collection of stools for a minimum of three to five days [1–3]. Extended stool collection is the major drawback to this approach, which has severely limited applications of an otherwise useful method.

A potentially important application of the method of "fecal isotope balance," if a way to eliminate the need for quantitative stool collection was available, relates to studies of the digestion and absorption of triglycerides and fatty acids in health and disease. For this application,  $^{13}\text{C}$ - (or  $^2\text{H}$ )-labeled triglyceride

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Abbreviations: Dy = dysprosium;  $^{13}\text{C}^*$  =  $^{13}\text{C}$ -Excess;  $\text{TG}^*$  =  $^{13}\text{C}$ -labeled triglyceride; P\*P\*P\* = TRIPALMITIN-1,1,1- $^{13}\text{C}_3$ ; PP\*P = TRIPALMITIN-2-palmitoyl-1,2- $^{13}\text{C}_2$ ; P\*LP\* = 2-LAURYL-1,3-DIPALMITIN-dipalmitoyl-1,1,2,2- $^{13}\text{C}_4$ ; P\*MP\* = 2-MYRISTYL-1,3-DIPALMITIN-dipalmitoyl-1,1,2,2- $^{13}\text{C}_4$ ; TPM = total pool method; PPM = partial pool method.

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(or free fatty acid) would be fed and its fecal excretion assessed. The method would have many applications to studies of pancreatic insufficiency, functional bile acid deficiency, intestinal malabsorption and other disorders.

Previous investigators [6–8] have shown that radioactive chromium (<sup>51</sup>Cr<sub>2</sub>O<sub>3</sub> or <sup>51</sup>CrCl<sub>3</sub>), or barium (<sup>131</sup>BaSO<sub>4</sub>) can be used as nonabsorbable markers together with radio-labeled triglycerides to eliminate the need for quantitative fecal collections. Ditchburn *et al.* [7] showed that the time course of cumulative fecal excretion of <sup>131</sup>I-triolein was comparable to that for <sup>51</sup>Cr in 33 hospitalized patients who were fed the two radio-labeled substrates simultaneously as part of a light meal. Pederson investigated the usefulness of combining <sup>14</sup>C-triolein and <sup>51</sup>CrCl<sub>3</sub> for studies of lipid assimilation [8]. He found, in 36 patients with various gastrointestinal disorders who received the two labels as part of a breakfast meal, that the transit time for <sup>51</sup>Cr was slightly shorter than that for <sup>14</sup>C-triolein in those patients who excreted excessive amounts of the labeled fat. However, total fecal <sup>14</sup>C excretion estimated from the mean of the <sup>14</sup>C/<sup>51</sup>Cr ratio in two stools was closely correlated ( $r^2 = 0.98$ ) with measured cumulative fecal <sup>14</sup>C. Thus, radio-labeled triglyceride excretion can be accurately assessed without the need for quantitative collection of stool.

The use of radio-isotopes in studies involving human volunteers, however, is problematic under most conditions and is prohibited altogether in some population groups. In addition, using a radioactive fecal marker negates one of the major benefits of the stable isotope approach. Recently, however, several groups have shown that a number of lanthanide (rare earth) elements administered as simple inorganic salts exhibit negligible gastrointestinal (GI) absorption in man [3,5,9]. Because these nonabsorbable elements are present in very low amounts in human foods and the concentration of these elements can be measured with a high degree of accuracy in feces [3,5,10], only trace quantities need be administered. This reduces the potential for the marker to interfere with the absorption of the nutrient of interest. These same rare earth elements have also been shown to possess GI transit kinetics sufficiently similar to those for several minerals/trace elements that they can be used as nonabsorbable markers in order to eliminate the need for quantitative collection of stools [3,5]. Of the lanthanide elements, dysprosium (Dy) administered as DyCl<sub>3</sub> is particularly useful for this purpose [3,5]. Dysprosium in stool can be accurately quantitated using neutron activation [10], ICP-MS [3,5] or AAS [11]. Oral administration of a small dose of DyCl<sub>3</sub> concurrently with the stable-isotope-labeled mineral/trace element permits accurate assessment of fecal excretion of the latter by laboratory analysis of small samples one or two stools for the ratio (labeled mineral/trace element)/Dy.

Based on these earlier studies, we hypothesized that the simultaneous feeding of <sup>13</sup>C-labeled triglycerides and DyCl<sub>3</sub> may offer a useful approach to study triglyceride digestion and absorption in patients with a wide range of gastrointestinal and

systemic disorders without the need for complete stool collections or concerns for exposure to radioactive substrates. Such a method would be safe and convenient for use in any population group including infants and children. Herein, we report the results of several studies conducted in cystic fibrosis patients regarding whether DyCl<sub>3</sub> ingested concurrently with <sup>13</sup>C-labeled triglycerides (TG\*) in free-living patients on self-selected diets permits satisfactory assessment of fecal excretion of the latter without the need for complete collection of stools. Specifically, we explored the following methodological issues: (1) nonabsorbability of Dy ingested as DyCl<sub>3</sub>, (2) relative gastrointestinal transit kinetics of Dy and TG\* and (3) use of brilliant blue (FD&C blue #1) as a visual fecal marker to aid in *a priori* selection of stool samples for laboratory analysis of Dy and <sup>13</sup>C-Excess (<sup>13</sup>C\*; <sup>13</sup>C present in sample of interest in excess of natural abundance). These studies were conducted as part of a larger set of investigations aimed, in part, at determining the effect of changes in triglyceride structure on its subsequent digestion and absorption by patients with steatorrhea.

## MATERIALS AND METHODS

### Patients

Thirty-six children and young adult patients diagnosed as having cystic fibrosis participated in a series of studies examining the relationship between triglyceride structure and *in vivo* digestibility and absorption. All were currently receiving treatment through the Cystic Fibrosis Center at Cincinnati Children's Hospital Medical Center (Cincinnati, OH). At the time of study all were in a stable period of their disease relative to digestive function. Most studies were conducted on an outpatient basis with the entire stool collection carried out at home. A few patients began their absorption study at the end of a hospital stay initiated for pulmonary exacerbation of their disease, the timing determined by their physician in consultation with our research staff; stool collections were then completed at home. The age range of the patients studied was 12 to 24 years at the time of study. The 36 patients completed a total of 66 absorption studies over a period of five years. All absorption tests consisted of ingesting only a single test meal containing both the TG\* and DyCl<sub>3</sub> under supervision of the research coordinator, without altering the patient's routine of enzyme therapy and other dietary habits. All study protocols were approved by the Institutional Review Board of Cincinnati Children's Hospital Medical Center. All patients and/or legal guardians gave written consent. In addition to these studies carried out with cystic fibrosis patient volunteers, a series of control studies were carried out in a single normal volunteer (one of the authors) to test procedures associated with Dy administration.

### Preparation of Labeled Triglycerides

A series of <sup>13</sup>C-labeled triglycerides were purchased from Cambridge Isotope Laboratories (Andover, MA) either from their general inventory or prepared specifically for these projects. In each case, isotopic purity (>90%) and purity from contaminants were assessed both by the manufacturer and by an independent laboratory. The triglycerides utilized were TRIPALMITIN-1,1,1-<sup>13</sup>C<sub>3</sub> (P\*P\*P\*); TRIPALMITIN-2-palmitoyl-1,2-<sup>13</sup>C<sub>2</sub> (PP\*P); 2-LAURYL-1,3-DIPALMITIN-dipalmitoyl-1,1,2,2-<sup>13</sup>C<sub>4</sub> (P\*LP\*), and 2-MYRISTYL-1,3-DIPALMITIN-dipalmitoyl-1,1,2,2-<sup>13</sup>C<sub>4</sub> (P\*MP\*).

### Preparation of DyCl<sub>3</sub>

High-purity (>99.9%) Dy metal (Aldrich 26,302-8) was dissolved in 2 N HCl with gentle heating. The pH of the resulting solution was adjusted to ~2.0 using 2 N NaOH. This solution was then filtered into a 100 mL volumetric flask and the volume adjusted. The final solution contained 1.00 mg Dy/100 μL, and all doses were prepared from this stock standard solution. This stock standard solution was also diluted appropriately and used to prepare analytical standards for analysis of Dy in all fecal samples.

### Clinical Protocols

An initial study was performed in nine cystic fibrosis patients to investigate the relative gastrointestinal transit kinetics of TG\* and DyCl<sub>3</sub>. The labeled TG\* used in this initial study was P\*P\*P\*. <sup>13</sup>C-labeled tripalmitin was considered an ideal TG\* to use in this feasibility study because it is highly lipophilic and was expected to be poorly absorbed [12]. As such, it represented a “worst case scenario” for excretion of a TG\* (fat-soluble) to demonstrate similar excretion kinetics with the nonabsorbable marker Dy (water-soluble). Any disparity in excretion kinetics between P\*P\*P\* and Dy was expected to be especially apparent in cystic fibrosis patients with steatorrhea. The fact that absorption was expected to be poor also meant that sufficient <sup>13</sup>C-label would be excreted in the stool to allow accurate measurement of <sup>13</sup>C-excess. In addition, P\*P\*P\* was commercially available and did not require custom synthesis.

The clinical protocol was as follows: after an overnight fast or four hours after the previous meal, each patient consumed 0.70 g of P\*P\*P\* mixed into ~20 g peanut butter and fed as part of a test meal along with an oral dose of DyCl<sub>3</sub> solution. The DyCl<sub>3</sub> solution was added to 50 mL milk and consumed intermittently during the meal along with two water rinses. The composition of the test meal was varied according to patient's preferences, but provided ~30% of the patient's daily caloric needs and ~40% of calories as fat. During the meal each patient also consumed his/her normal complement of therapeutic enzymes. Quantitative collection of stool was initiated immediately after consumption of the test meal and continued for 120 hours. Control studies in the normal volunteer were carried

out in a similar manner, except that the P\*P\*P\* dose (1.00 g) was not incorporated into peanut butter, but stirred into skim milk and consumed with a light breakfast.

The remainder of the studies, carried out to examine the effect of changes in TG\* structure on TG\* absorption/excretion in patients with steatorrhea, were carried out in a manner similar to that described above with the following changes: (1) TG\* doses were mixed manually with 10–20 g of reduced calorie butter substitute (I Can't Believe It's Not Butter! Light, Lipton, Englewood Cliffs, NJ). (2) Dose of TG\* used was increased to 2.00 g to insure sufficient <sup>13</sup>C-enrichment of stool samples for good analytical precision. Increasing the TG\* dose was necessary in these later studies either because the TG\* used contained fewer <sup>13</sup>C-atoms/molecule of TG\* (i.e., PP\*P) or because the digestibility of the TG\* was expected to be greater due to a reduction in the chain length of the fatty acid in the sn-2 position compared to P\*P\*P\* (i.e., P\*MP\* and P\*LP\*) [12]. (3) DyCl<sub>3</sub> dose was incorporated into a gelatin capsule which also contained 100–300 mg D-glucose and 50–60 mg brilliant blue (FD&C blue #1); the Dy capsule was consumed midway through the test meal. (4) Seventy-two hours after consumption of the test meal, a second gelatin capsule was consumed containing only D-glucose and brilliant blue. For all of these protocols, complete stool collections began immediately after consumption of the test meal and continued until fecal excretion of the second dose of brilliant blue was noted by the patient. Excretion of the brilliant blue visual marker was readily observed as it imparted a green color to at least a portion of one or more stools.

For all studies, stools were collected individually and frozen until prepared for analysis. In our initial study designed to examine the relative gastrointestinal transit kinetics of P\*P\*P\* and Dy, each stool was thawed, weighed and physically mixed before an accurately weighed portion was saved for analysis. The saved portion was dried at 100° C for 48 hours and then re-weighed. Each dried stool aliquot was then pulverized to form a fine powder; stools containing a large amount of unabsorbed dietary fat retained a pasty consistency after drying. An accurately weighed portion of each sample was then analyzed for Dy and <sup>13</sup>C\*. For the remainder of the studies, the focus of which was to evaluate the relationship between fecal excretion of each TG\* and 72-hour stool fat, stool composites were made using the excretion of the two doses of brilliant blue to mark the beginning and end of the composite period. In some cases, we also saved a portion of one or more individual stools for determination of total <sup>13</sup>C-label excretion by the partial pool method (PPM). It was not feasible to do so for all studies because of the time and expense involved. Composite and single stool samples were dried and prepared for analysis as described above.

### Analytical Methods and Calculations

Dy content of subsamples taken from each sample of dry powdered stool was determined by neutron activation analysis

according to the method of Gray and Vogt [10]. Briefly, samples and a series of Dy-spiked fecal standards were irradiated in the fast pneumatic irradiation port of the University of Missouri research reactor (MURR) for five seconds. After a delay of 15 seconds, the intensity of the 108-keV  $\gamma$ -ray for the nuclear transformation <sup>164</sup>Dy(n, $\gamma$ )<sup>165m</sup>Dy (t<sub>1/2</sub> = 1.26 minutes) was recorded on a high-resolution  $\gamma$ -spectrometry system for 120 seconds. The Dy content ( $\mu\text{g/g}$  dry wt) of each sample was estimated from the linear regression equation for the corresponding set of calibration standards prepared by spiking dry baseline fecal samples with known increments of Dy from the stock solution used for oral administration.

Stool samples were also analyzed for total carbon and the ratio <sup>13</sup>C/<sup>12</sup>C (atom% <sup>13</sup>C) in triplicate using an Europa Scientific 20/20 isotope ratio mass spectrometer equipped with Automated Nitrogen Carbon Analyzer. Total carbon and carbon isotopic composition were measured on fat extracts of dried stool composites and/or individual stools. Aliquots of dried stool (~250 mg) were re-hydrated with 2 mL deionized water and four drops of concentrated HCl and then the fat was extracted according to the method of Jeejeebhoy *et al.* [13]. This method was adopted so that total fat and <sup>13</sup>C\* could be determined from the same aliquot of dried stool. A set of fat extract standards was prepared for each TG\* studied. These standards, whose isotopic contents covered the expected range, were prepared from unlabeled stool extract and spiked with known increments of TG\*. An appropriate set of standards was analyzed with each batch of sample extracts. The content of TG\* equivalents (mg) in each sample was estimated using the regression equation for <sup>13</sup>C\* vs. TG\*. Excretion of <sup>13</sup>C-label from each ingested TG\* is expressed in terms of mg equivalents of TG\* to facilitate comparisons between the dose of TG\* ingested and the excretion of <sup>13</sup>C-label in all forms (i.e., undigested TG\*, labeled free fatty acid, sn-1,2-diglyceride, etc.) as well as between TG\*s containing differing amounts of <sup>13</sup>C-label.

<sup>13</sup>C-label excretion data from our initial study designed to examine the relative transit kinetics of <sup>13</sup>C\* and Dy was measured in a slightly different manner. For this study only, in which the absolute value of <sup>13</sup>C-label excretion was not important, total carbon and isotopic analyses were carried out on dry stool samples directly without fat extraction. The mg equivalents of TG\* excretion were then calculated using a theoretical, instead of measured, value for the <sup>13</sup>C/<sup>12</sup>C content of the TG\* used (P\*P\*P\*).

Utilizing Dy ( $\mu\text{g/g}$  dry stool) and TG\* (mg/g dry stool) data, derived as indicated above, from either composite fecal pools or individual stool samples and the Dy dose ingested ( $\mu\text{g}$ ), total TG\* excretion (mg) was calculated as follows: total TG\* excretion = [(sample TG\* content)/(sample Dy content)] X Dy dose ingested. Excretion of TG\* determined in this manner for a composite pool represents the total pool method (TPM) for calculating TG\* excretion, whereas data calculated

for a single or small number of individual stools represents the partial pool method (PPM) for calculating TG\* excretion.

### Statistical Methods

SPSS Version 10 was utilized for statistical analysis (SPSS, Inc., Chicago, IL). The Kolmogorov-Smirnov non-parametric test was used to examine the normality of Dy excretion data. Linear regression analysis was used to compare the kinetics of Dy and <sup>13</sup>C excretion. Where appropriate, sample means were compared using Student's *t* distribution (comparison to theoretical value) or paired *t* test; *p*-values of less than 0.05 were considered to indicate statistically significant differences.

## RESULTS

### Analytical Performance

The accuracy of Dy measurements was evaluated in comparison with the lowest expected amount present in a collected stool. For the administered level of Dy (1000  $\mu\text{g}$ /subject), a stool containing only 1% of the dose would contain 10  $\mu\text{g}$ . Since the analytical method utilizes ~1% of the stool, such a sample would contain 0.1  $\mu\text{g}$  Dy. Data showing the results of triplicate Dy analyses for stool samples spiked with known amounts of Dy (0.1 to 10.1  $\mu\text{g}$ ) are summarized in Table 1. Precision of Dy analyses was always better than 5%, even for stools containing as little as 0.1  $\mu\text{g}$  Dy. Therefore, measurement of Dy in stools is not a limiting factor.

Precision and accuracy of Dy analyses in stool were also very consistent over the time course of the studies. Data from eight sets of Dy standards analyzed over the five-year study period are shown in Table 2. The %RSD for each set of standards was always better than 5% and ranged from 1.79% to 4.4%. These findings are consistent with previously published reports [5,10].

Measurement performance data for total carbon and carbon isotope analyses from a single absorption study carried out in a

**Table 1.** Typical Data Regarding the Accuracy of Dy Measurements in Stool Samples

Sample #	$\mu\text{g}$ Dy Added	Counts/ $\mu\text{g}$	RSD (%)
1-1	0.1013	32428	2.34
1-2	0.1013	31106	
1-3	0.1013	31194	
2-1	0.5065	32600	4.16
2-2	0.5065	31662	
2-3	0.5065	34353	
3-1	1.013	32977	3.79
3-2	1.013	33170	
3-3	1.013	30958	
4-1	5.065	31369	0.98
4-2	5.065	31762	
4-3	5.065	31156	

**Table 2.** Consistency in Precision of Dy Analyses over Time

Date	μg Dy Added (Range)	%RSD for Set of Standards
10/97	1 to 5.0010	3.96
4/98	1 to 5.0010	2.20
9/98	1 to 4.0008	1.79
3/99	1 to 4.0008	2.08
4/99	1 to 4.0008	2.09
7/00	1 to 4.0008	3.13
11/00	1 to 4.0008	4.33
5/01	1 to 4.0008	4.44

normal volunteer (1.00 g dose P\*P\*P\*) are shown in Table 3; data are shown as mean and %RSD for each individual stool. As shown, atom% <sup>13</sup>C measurements were highly reproducible in all samples with RSD values of 0.6% or better; total carbon measurements were less reproducible. Using total carbon and isotope ratio data from stool #2 excreted 24 hours after dosing, the estimated value for <sup>13</sup>C-label excretion, as mg TG\* equivalent, in this stool is 496.7 mg with an overall uncertainty of ±34.0 mg (6.8% RSD). This level of overall uncertainty is based on quadruplicate measurements and reflects realistic values to be expected in routine analyses.

### Nonabsorbability of Dy Marker

A total of 66 tests were performed in 36 CF patients. Of these, 11 collections were considered incomplete based on self-report of the patient, Dy and <sup>13</sup>C\* recovery data along with stool pattern and visual marker excretion data. Total Dy recovery values for the remaining 55 studies (28 subjects) ranged from 83% to 118% of dose; the mean of the recoveries, 102 ± 8 (SD), was not statistically different from 100%. Considering the data set as a whole, there was a slight bias toward over recovery in the data from our initial study in which total Dy recovery was based on the sum of individual stool data. Mean Dy recovery for our initial study alone was 108 ± 11 (SD; n = 8), which is significantly different (*p* < 0.05) from the mean for

**Table 3.** Analytical Measurement Parameters for Total Carbon and Isotopic Analyses<sup>1</sup>

Stool # Post Dosing	Carbon Content		<sup>13</sup> C-Content	
	(% dry wt)	(%RSD)	(Atom%)	(%RSD)
Baseline <sup>2</sup>	53.7	3.7	1.0973	0.02
1	54.5	5.4	1.1697	0.32
2	53.2	6.2	1.2344	0.57
3	57.0	1.9	1.1150	0.09
4	55.2	2.5	1.1035	0.08
5	57.1	9.0	1.0982	0.02
6	57.2	3.9	1.0978	0.01
7	56.4	4.4	1.0972	0.01

<sup>1</sup> Data for individual stools collected by a single normal volunteer after ingestion of a 1.00 g dose of P\*P\*P\*.

<sup>2</sup> Baseline stool collected just prior to ingestion of TG\* dose.

the remainder of studies in which stool composites were analyzed (100 ± 7; n = 47). Exclusive of data from our initial study, Dy excretion data were normally distributed.

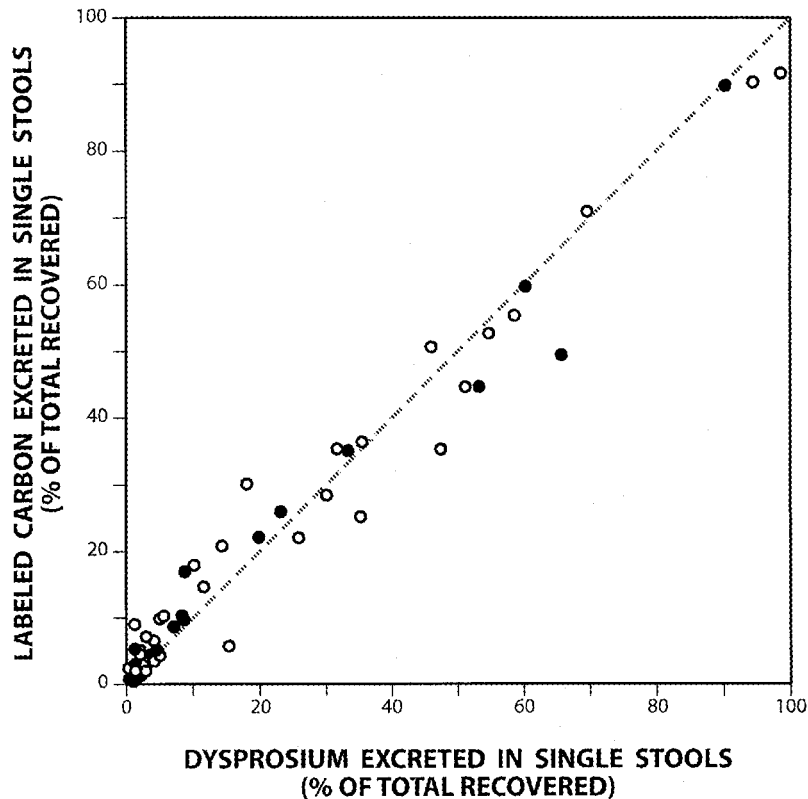
Dy excretion data from a series of control studies carried out in a normal subject are shown in Table 4. These individual absorption studies were carried out over a five-year period and represent different conditions for Dy marker ingestion. From these results, it is clear that Dy ingested as DyCl<sub>3</sub> along with a meal or physically mixed with a standard formula (hot or cold) is excreted quantitatively; the mean excretion value for all control studies, excluding #94-3, was 100 ± 7%. For control study #94-3, in which the DyCl<sub>3</sub> dose was physically mixed into an egg yolk during the frying process, Dy excretion was only 78%.

### Relative Transit Kinetics of Dy and <sup>13</sup>C-Label

Analysis of individual stool samples for Dy and <sup>13</sup>C\* were performed for all nine patients who participated in our initial study and for control studies 1–3; the TG\* administered in each case was P\*P\*P\*. Data comparing the fractional recovery of Dy and <sup>13</sup>C\* in individual stools are shown in Fig. 1 for all stools containing measurable amounts of both. If, as proposed, total <sup>13</sup>C-label excretion is to be determined accurately from a sample of one or a small number of stools, the fractional excretion of Dy and <sup>13</sup>C\* must show a highly linear correlation for all stools regardless of their sequence, within the expected error limits. The observed correlation coefficient (*r*<sup>2</sup> = 0.969) was highly significant (*p* < 0.001) reflecting the quantitative correspondence in the fecal excretion of <sup>13</sup>C\* and Dy. In addition, the slope of the regression line (*B* = 0.910) and the *y*-intercept (2.08% of recovered dose) were close to unity and

**Table 4.** Dy Recovery Data for Control Tests in a Single Normal Subject

Study #	Dy Recovery (% of Dose)	Mode of Dy Ingestion
94-1	97.3	DyCl <sub>3</sub> in H <sub>2</sub> O with light breakfast
94-2	102.9	DyCl <sub>3</sub> in H <sub>2</sub> O with light breakfast
94-3	78.2	DyCl <sub>3</sub> solution (100 μL) carefully stirred into egg yolk during frying. Egg consumed as part of a breakfast meal.
97-1	113.3	DyCl <sub>3</sub> (100 μL) added to capsule containing D-glucose. Dy capsule ingested with a lunch meal of turkey casserole, roll and coffee.
97-2	91.0	Same as above.
97-3	95.7	Same as above.
99-1	96.2	Dy capsule dissolved in cold infant formula and then consumed completely.
99-2	104.8	Dy capsule dissolved in boiling hot infant formula and consumed once cool.



**Fig. 1.** Observed correlation between excretion of <sup>13</sup>C\* and Dy ( $r^2 = 0.969$ ); dashed line represents the line of identity. The data include 39 stools from nine cystic fibrosis patients (○) and 16 stools from three tests in a single healthy adult (●).

zero, respectively. In the context of the scope of these observations, the data also show no marked differences in the relative transit of Dy and <sup>13</sup>C\* between the CF patients and the normal volunteer.

### Estimation of Total <sup>13</sup>C-Label Excretion

Based on all of the above data it appears that Dy meets the criteria of nonabsorbability and identical transit behavior relative to <sup>13</sup>C\* from simultaneously ingested TG\*. Thus, it should be possible to accurately estimate total <sup>13</sup>C-label excretion by determining the <sup>13</sup>C\* and Dy content of one or a small number of stools collected after isotope administration. Using the data from our initial study in which individual stools were analyzed for <sup>13</sup>C\* and Dy, we compared the value of total <sup>13</sup>C-label excretion, expressed as mg equivalents of P\*P\*P\*, estimated from the complete five-day fecal collection (total pool method, TPM) with estimates based on data from a sample of one or two stools collected after isotope administration (partial pool method, PPM); the data are shown in Table 5. For this study, the TPM-values represent the sum of <sup>13</sup>C-label excretion determined for each individual stool for eight of nine CF patients; one patient from this study (#1) did not provide a complete stool collection, and the patient's data is not included. Total <sup>13</sup>C-label excretion estimates made using the PPM utilized

<sup>13</sup>C\* and Dy data from either the first stool to contain significant Dy [PPM(1)] or an average of the value for the first stool with significant Dy plus the next stool [PPM(2)]. Compared with the five-day collection value, total <sup>13</sup>C-label excretion values based on the first stool to contain significant Dy slightly under-estimated <sup>13</sup>C-label excretion in seven of eight patients (paired  $t = 3.55$ ,  $p < 0.01$ ). However, the two-stool estimate yielded values that were not different from the five-day collection value (paired  $t = 0.144$ , NS) and only varied from the total pool value by more than 10% in one of eight patients. The same calculations were carried out for control studies 94-1 and -2 with similar results. The corresponding five-day collection and two-stool values for the control studies were 927 vs. 915 and 886 vs. 808 mg, respectively.

The initial comparison made between the TPM and PPM utilized data from our first study in which quantitative stool collections were carried out and from which Dy data for each stool were available. While these data clearly illustrate the potential usefulness of the PPM, utilizing stool Dy content to select stool(s) for <sup>13</sup>C\* analysis has important practical limitations. Therefore, we investigated the practical utility of administering a small amount of the food colorant brilliant blue (FD&C blue #1) to visually mark the stool(s) to be analyzed both for Dy and <sup>13</sup>C\*. Individual stool Dy content and color data were available for 18 excretion studies. Recovery of Dy

**Table 5.** Estimation of <sup>13</sup>C-label Excretion after P\*P\*P\* Ingestion Based on Five-Day Stool Collection (TPM) vs. 1 or 2 Individual Stools (PPM)

Subject #	TPM	PPM	
		PPM (1) <sup>1</sup>	PPM (2) <sup>2</sup>
		(mg equivalents of P*P*P*)	
2	512	416	550
3	656	645	645 <sup>3</sup>
4	487	508	527
5	477	337	424
6	753	636	736
7	675	608	608 <sup>3</sup>
8	559	515	515 <sup>3</sup>
9	566	453	658
Mean ± SEM	586 ± 36	517 ± 39	583 ± 34

<sup>1</sup> Total <sup>13</sup>C-label excretion was estimated using <sup>13</sup>C\* and Dy data for the first stool to contain a significant amount of Dy: PPM(1).

<sup>2</sup> Total <sup>13</sup>C-label excretion was estimated using <sup>13</sup>C\* and Dy data for the first stool to contain significant Dy plus the next stool; the two estimates were then averaged: PPM(2).

<sup>3</sup> For these subjects Dy was excreted almost quantitatively in a single stool. The next stool contained too little Dy to provide a reliable estimate of total <sup>13</sup>C-label excretion, thus, PPM(1) = PPM(2).

(% of dose) in the first stool to contain significant green color (>10% of stool appeared green) averaged 42.8 ± 23.3% (SD) with a wide range of values from 8.8% to 84.7%. On the other hand, Dy recovery for this stool plus the next averaged 77.7 ± 17.4% with the lowest value being 45.4%; only two of the 18 patients studied excreted less than 60% of the Dy dose in these two stools. The patient with the lowest two-stool Dy value excreted four stools containing almost equal amounts of Dy and all were similarly green. Excretion of Dy in stools prior to the presence of green color was not observed. Thus, while the visual marker data are only qualitative (presence or absence of color), the presence of green color in the stool clearly marks the beginning of Dy excretion. For the cystic fibrosis patients

**Table 6.** Comparison of the TPM vs. PPM for Estimating <sup>13</sup>C-Label Excretion Using Stool Color to *a priori* Select Individual Stools for Analysis

TG* Ingested <sup>1</sup>	Subject #	<sup>13</sup> C-Label Excretion (mg Equivalents of TG*)	
		TPM	PPM
P*LP*	2	235	224
	4	248	241
P*MP*	1	304	340
	2	477	406
	3	458	484
	4	889	931
PP*P	1	655	582
	2	1184	1188
	4	1427	1142
	6	962	1029

<sup>1</sup> The TG\* dose administered in all studies was 2.00 g.

studied who often had multiple stools per day, 2–3 stools contained significant green color (average of 2.9). Stool sequence alone could not have been used to predict the timing of Dy excretion since the stool number of the first stool to contain Dy after dosing ranged from 1 to 7. Stool fat values for these patients who received their normal complement of therapeutic enzymes during the test ranged from 4.3 to 55 g/day.

Based on these observations, we examined <sup>13</sup>C\* and Dy excretion data from our later studies for all individuals for whom both composite and individual stool data were available to determine if stool color could be used *a priori* to select the correct stools for analyses. Because the purpose of these later studies had been to compare fecal excretion of various TG\*s with dietary fat (72-hour stool fat), samples of individual stools were not available for all patients. Individual data comparing the TPM and PPM of estimating total <sup>13</sup>C-label excretion from the ten cystic fibrosis patients for whom such data were available are shown in Table 6; excretion data for three labeled TG\*s are included P\*LP\*, P\*MP\* and PP\*P. The PPM, based on stool color to *a priori* select stools for analysis, accurately predicted total <sup>13</sup>C-label excretion. Mean excretion was 684 and 657 mg for the TPM and PPM, respectively, and a paired comparison of individual excretion estimates indicates the results are not different for the two methods ( $t = 0.842$ , NS  $p > 0.20$ ). In six out of ten patients, the PPM and TPM agreed to within 5% or better. In only one case (patient #4, PP\*P) did the PPM estimate vary from the TPM estimate by more than 15%. These data indicate that the PPM utilizing the food colorant brilliant blue to visually mark stools for analysis yields reasonably accurate estimates of total <sup>13</sup>C-label excretion from ingested TG\* after analysis of samples of only two stools.

## DISCUSSION

The goal of the work presented herein was to determine if simultaneous oral administration of Dy (as DyCl<sub>3</sub>), TG\* and the food colorant brilliant blue as part of a normal meal would permit accurate assessment of total <sup>13</sup>C-label excretion after analysis of a sample of one or two visually marked stool(s). In order for this approach to be feasible several specific criteria must be met: (1) Dy excretion must be quantitative, (2) Dy and <sup>13</sup>C-label from TG\* must exhibit quantitatively similar excretion kinetics and (3) brilliant blue excretion in stool must mark the correct stools to utilize in the PPM. We tested all aspects of the approach directly in cystic fibrosis patients who were pancreatic insufficient and who exhibited a range of steatorrhea.

Dy excretion by the cystic fibrosis patients was quantitative [102 ± 8% (SD)] as has been shown to be the case for healthy adults [3,5], thus, criterion 1 was met. It is important to note, that in all of the patient studies, Dy was administered as DyCl<sub>3</sub>. Incorporation of Dy into a food product may increase its absorption significantly as we observed in one control study when the Dy dose was mixed into an egg yolk while frying;

organic complexes of Dy have been shown to be absorbable [14]. Thus, quantitative excretion of Dy under ingestion protocols other than those used in the studies reported herein would need verification.

Our data also indicate that the relative transit kinetics of Dy and <sup>13</sup>C\* are nearly identical (see Fig. 1). Thus, criterion 2 has also been met. The high degree of correlation observed in the current study between <sup>13</sup>C\* and Dy excretion in individual stools compares favorably with similar data for <sup>54</sup>Fe, <sup>70</sup>Zn and <sup>26</sup>Mg [3,5]. The correlation coefficient observed in the current study ( $r^2 = 0.969$ ) is only slightly less than observed for <sup>54</sup>Fe ( $r^2 = 0.988$ ) and <sup>70</sup>Zn ( $r^2 = 0.990$ ), and greater than observed for <sup>26</sup>Mg ( $r^2 = 0.937$ ). The <sup>54</sup>Fe and <sup>70</sup>Zn studies were carried out in normal adult volunteers. On the other hand, the <sup>26</sup>Mg studies were performed in Crohn's disease patients many of whom had multiple stools per day and some degree of malabsorption, similar to the cystic fibrosis patients who participated in the current study. These two factors may account for the slight reduction in the observed correlation coefficients in the patient groups compared to normal subjects.

Based on these observations, we tested the hypothesis that the PPM could be used to accurately determine total <sup>13</sup>C\*-excretion. In order to evaluate the best sampling approach to use for estimates made using the PPM, total <sup>13</sup>C-label excretion was estimated using (1) the first stool to contain significant Dy and (2) an average of the value for the first stool with significant Dy plus the next stool. The latter approach has been found to yield the best PPM estimates for <sup>70</sup>Zn and <sup>26</sup>Mg [5] as well as for radiolabeled <sup>14</sup>C-triolein in which case <sup>51</sup>CrCl<sub>3</sub> was used as the nonabsorbable marker [8]. Using Dy content as our selection criteria, the two-stool PPM was found to yield total <sup>13</sup>C\*-excretion values comparable to the TPM for both the cystic fibrosis patients and the studies performed in the control subject (see Table 5).

Practically speaking, however, it is not possible to *a priori* select stools for Dy and <sup>13</sup>C\* analyses based on stool number or sequence. If patients or volunteers are required to collect a large number of stool samples in order to assure that the two containing significant Dy and <sup>13</sup>C\* are saved, the PPM offers less of a practical advantage. With this in mind, we examined Dy and <sup>13</sup>C\*-excretion data from our studies in which the food colorant brilliant blue was simultaneously ingested to determine if excretion of this readily observed fecal marker could be used to identify the correct stools to utilize for the two-stool PPM. Using stool color to select stools for analysis, the PPM accurately predicted total <sup>13</sup>C-label excretion (see Table 6). Individual estimates made using the two methods were highly correlated ( $r^2 = 0.944$ ). Both methods also yielded the same relative rankings for <sup>13</sup>C-label excretion by individuals within a TG\* group as well as the relative excretion of the TG\*s studied (i.e., <sup>13</sup>C-label excretion was significantly lower after P\*LP\* ingestion than after P\*MP\* or PP\*P ingestion). Thus, criterion 3 has also been met.

Total stool fat during the test period for the above mentioned patients ranged from 7 to 64 g/day. The magnitude of the difference between the TPM and PPM values for individual patients did not correlate with the degree of steatorrhea observed nor with Dy recovery in the two stools utilized for the PPM. There may, however, be a limit to the proposed use of the PPM in extreme cases of steatorrhea. In one patient who declined enzyme therapy (data not shown in Table 6), studied following P\*LP\* dosing, there was significant disparity between Dy and <sup>13</sup>C\* excretion. Fecal fat in this patient was found to be 126 g/day with large amounts of solid fat visible in the collected stools. In this patient, the first green stool plus the next contained 75% of the Dy dose but only 29% of the TG\* dose.

While the degree of accuracy provided by the PPM may not be sufficient for all purposes, the approach utilized herein provided reasonably accurate estimates of total <sup>13</sup>C-label excretion. In addition, the PPM predicted the relative digestibility of the TG\*s studied and the results of the two methods were highly correlated. Within the limits of the data available, the accuracy of the approach appears to be independent of the TG\* studied as well as the degree of steatorrhea observed—except in the extreme.

## CONCLUSIONS

As a quantitative fecal marker, Dy has the characteristics of being nontoxic and nonradioactive and can be quantitated with a high degree of accuracy. Similar to what has been observed in normal adults [3,5], we have shown that Dy is excreted quantitatively by cystic fibrosis patients with a wide range of steatorrhea. The data presented herein have also shown that by combining the use of Dy as a nonabsorbable marker with the visual marker brilliant blue, a practical tool for quantitating fecal excretion of ingested TG\* is available based on the analysis of small samples of only two stools. While the proposed PPM may not replace the TPM in situations requiring the greatest degree of accuracy, many experimental and clinical applications exist where quantitative collection of stool is not possible or desirable. Use of the PPM, as described herein, would permit important research and clinical questions to be addressed that would otherwise go unanswered.

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