CLINICAL INVESTIGATION

Coefficients of Fat and Nitrogen Absorption in Healthy Subjects and Individuals with Cystic Fibrosis

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BACKGROUND We sought to compare the differences of coefficient of fat absorption (CFA) and coefficient of nitrogen absorption (CNA) in healthy individuals and those with cystic fibrosis (CF) and to study the precision of CFA and CNA.

METHODS Sixteen healthy and 23 subjects with CF and pancreatic insufficiency ate a high-fat, high-protein diet for 72 hours; stool was collected between blue food dye markers to determine CFA and CNA. Subjects with CF withheld pancreatic enzymes. Tests were repeated on 5 of the CF and 10 of the healthy subjects.

RESULTS In healthy subjects, mean CFA was 93.5% ± 2.7%; mean CNA was 88.1% ± 5%. Median test-retest in 10 healthy subjects was +0.7% CFA (range, –8.1% to + 5.9%) and +0.9% CNA (range, –14.6% to +6.8%). For subjects with CF, mean CFA was 38.5% ± 14.7% and mean CNA was 52.2% ± 11.4%. Median test-retest change in 5 subjects with CF was –6.9% CFA (range, –19.7% to +42.8%) and +14.7% CNA (range, –6.4% to +42.8%).

CONCLUSIONS CFA and CNA have inconsistent precision in CF. The limitations of CFA as a measure of steatorrhea correction in CF should be recognized in studies of pancreatic enzyme supplements.

KEYWORDS cystic fibrosis; malabsorption; pancreatic enzymes; pancreatic insufficiency; pancreatic sufficiency

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INTRODUCTION

Attaining and maintaining adequate weight has long been associated with favorable outcomes in patients with cystic fibrosis (CF).[1,2] Most patients with CF are pancreatic insufficient (PI) and require life-long exogenous pancreatic enzyme replacement therapy (PERT) to prevent malnutrition.[3] PERT was available prior to the creation of the United States Food and Drug Administration (FDA) in 1931 and the subsequent Federal Food, Drug

ABBREVIATIONS CF, cystic fibrosis; CFA, coefficient of fat absorption; CNA, coefficient of nitrogen absorption; CRC, clinical research center; ELISA, enzyme linked immunosorbent assay; FDA, Food and Drug Administration; FD&C, Food, Drug and Cosmetic; PERT, pancreatic enzyme replacement therapy; PI, pancreatic insufficient

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and Cosmetic Act of 1938 that required proof of safety and efficacy before approval of drugs for marketing. Recently, the FDA announced that it will require a new drug application for all PERT with evidence to support their consistency in formulation, manufacturing and efficacy.[4] In almost all studies of PERT, the primary outcome used to measure improvement in steatorrhea is the coefficient of fat absorption (CFA), with coefficient of nitrogen absorption (CNA) used to quantify changes in azotorrhea. Much of the literature on use of CFA and CNA in patients with CF was published in the middle of the last century[5,6] and its precision in this population has not been well described. In order to improve the design of studies of PERT efficacy required by the FDA, we sought to describe and compare the differences and within-subject repeatability of CFA and CNA in healthy individuals and those with CF. We also sought to improve the reliability of these measurements by using a new dye marker to improve the accuracy of stool collections.

MATERIALS AND METHODS

As part of a study of a novel PERT, ALTU-135 (Altus Pharmaceuticals, Inc., Cambridge, MA), we tested healthy individuals on two occasions, and we prospectively planned to study some additional healthy subjects once. We also studied subjects with cystic fibrosis and PI documented by a fecal elastase < 100 μg/g stool measured by monoclonal ELISA (ScheBo Biotech Pancreatic Elastase kit, Marietta, GA) as part of a study of a new pancreatic enzyme. The study design and results have already been published.[7] A subset of these subjects were re-tested on a second occasion, and those results are presented here. Each study site's Institutional Review Board approved the protocol, and informed consent was obtained. Assent was obtained from pediatric subjects.

Subjects entered a clinical research center (CRC) and ingested a blue food dye marker (FD&C Blue #2, 500 mg, supplied as two 250-mg capsules) after an overnight fast. The site pharmacists at each study site manually filled #2 clear gelatin capsules with 250 mg of FD&C Blue #2 (also called indigo carmine), which was purchased from Spectrum Pharmacy Products (Tucson, AZ) as bulk powder. Subjects were fed a diet containing 100 grams of fat and 2 grams/kg protein divided into three meals and two snacks daily for 72 hours. No subjects were consuming medium-chain triglyceride-containing supplements. Subjects with CF withheld exogenous pancreatic enzymes with meals. Subjects ingested a second 500-mg dose of FD&C Blue #2 at the end of the 72-hour special diet period. All stools were collected starting after the first stool visibly containing the blue dye marker, up to and including the first stool containing blue dye after the second marker was ingested. Subjects re-entered the CRC one week later, and the procedure was repeated. The subjects with CF returned to the CRC for repeat testing 6-9 months later as part of a separate protocol for which Institutional Review Board approval and informed consent, and assent, when applicable, was also obtained.

All stools were immediately frozen, stored in plastic containers, and shipped to the Mayo Clinic Central Laboratory for Clinical Trials (Rochester, MN). Stool samples for fecal fat were well-homogenized, saponified with potassium hydroxide, acidified and then the lipids were extracted with petroleum ether. After the solvent was vaporized, the residue was weighed and analyzed by the modified Van de Kamer method.[8] Inter-assay precision studies were performed on two levels representing two high abnormals (50 g/24 hour and 100 g/24 hour). A total of 20 analyses at each level were conducted, and coefficients of variation were < 5%. Five serial dilutions of a sample were analyzed and demonstrated linearity with an $R^2 = 0.9997$. The lowest detectable amount of fat was 0.1 g fat/24 hours. Total nitrogen in feces was determined using the Dumas combustion method.[9] Homogenized fecal material (0.5 gram) was added to 0.1 gram of sucrose in a tin capsule, which was blanketed with oxygen prior to hermetic sealing to exclude atmospheric nitrogen interference. The capsule was then placed in the instrument, combusted, and oxidized at 960 °C. Nitrogen, in the form of nitric oxide, is released from the sample and is carried by carbon dioxide through a column containing tungsten, which reduces the nitric oxide to molecular nitrogen. Nitrogen was detected using a thermal conductivity detector and response was compared to standards of known nitrogen concentration for quantitation.

Table 1. Healthy subjects tested for CFA and CNA at two time points

	CFA			CNA		
Subjects	**Time 1**	**Time 2**	**Change**	**Time 1**	**Time 2**	**Change**
001	97.2	95.3	–1.9	93.3	89.3	–4.0
002	91.2	94.4	+3.2	90.2	91.0	+.8
003	90.1	91.5	+1.4	81.8	84.5	+2.7
004	92.8	93.3	+.5	81.9	82.8	+.9
005	86.6	92.5	+5.9	78.0	84.8	+6.8
006	94.6	94.6	0	85.7	86.0	+.3
007	90.1	92.0	+1.9	89.3	85.1	–4.2
008	98.2	90.1	–8.1	98.1	88.3	–9.8
009	92.2	93.1	+.9	87.8	88.6	+.8
010	96.7	91.6	+5.1	94.7	80.1	–14.6

CFA, coefficient of fat absorption; CNA, coefficient of nitrogen absorption

Inter-assay and intra-assay precision were performed on samples of 0.3 g/24 hour, 12.9 g/24 hour and 20.2 g/24 hour. Twenty analyses of each level were conducted and all coefficients of variation were less than 3%. Three samples of varying concentrations throughout the measurable range of the assay were serially diluted and assayed; linearity was achieved with a mean $R^2 = 0.9972$.

Analysis of Data

The coefficient of fat absorption was calculated as follows:

$$\frac{(\text{grams of fat consumed} - \text{grams of fat excreted}) \times 100}{\text{grams of fat consumed}}$$

The same equation using the number of grams of nitrogen was used to calculate the coefficient of nitrogen absorption (CNA). Data are presented using descriptive measures such as mean and standard deviation. Individual data points are presented to enable the reader to appreciate inter- and intra-subject variability.

RESULTS

Sixteen healthy subjects (13 male) and 23 subjects with CF (14 male) completed the study with a mean age of 31.2 ± 12.0 years (range, 16.0 – 47.8 years) and 23.5 ± 7.8 years (range, 15.2 – 44.5 years), respectively. No subjects withdrew prior to completing stool collection. Subjects with CF reported a variety of gastrointestinal adverse events during the period of time off PERT, including abdominal pain, upper abdominal pain and flatulence. Following ingestion of each dose of the marker, blue dye was seen clearly in stools of all subjects with a mean of 80 ± 22.3 hours (range, 40-147 hours) between appearance of the first blue dye marker and appearance of the second blue dye marker. There was no significant difference in the time to visibility of the second marker in those with CF (78 ± 17.4 hours) and without CF (82 ± 26 hours). None of the subjects noted that urine was blue-green tinged following ingestion of the dye marker. The weight of all stools analyzed in CF subjects was three-fold heavier than that of healthy subjects (1534 ± 349 g vs. 463 ± 161.4 g) with 1.5 times the normal number of stools (8.6 ± 2.6 vs. 5.4 ± 2.9). The number of stools in the healthy controls may be skewed higher as a result of loose stools in one subject, who had 13.5 stools during the collection period.

In all healthy subjects, mean CFA was 93.5% ± 2.7% and mean CNA was 88.1% ± 5.0%. Of note, the mean value for CFA in healthy subjects in this study is consistent with the usual reported normal value of 93%, however, the lowest value was 86.6%. The median test-retest change in the 10 healthy subjects studied on two occasions was +0.7% CFA (range, –8.1% to +5.9%). Median test-retest change in CNA was +0.9% (range, –14.6% to +6.8%). Individual values are listed in Table 1. For all subjects with CF, mean CFA was 38.5% ± 14.7% and mean CNA was 52.2% ± 11.4% (Table 2). The median test-retest change in 5 subjects with CF studied on two occasions was –6.9% CFA

Table 2. Subjects with cystic fibrosis and pancreatic insufficiency, tested off enzymes for CFA and CNA at two points in time

	CFA			CNA		
Subjects	**Time 1**	**Time 2**	**Change**	**Time 1**	**Time 2**	**Change**
011	49.7	30.0	–19.7	53.9	47.5	–6.4
012	14.4	33.7	+19.3	39.7	55.4	+15.7
013	41.3	34.4	–6.9	49.0	63.7	+14.7
014	45.7	31.7	–14	44.0	40.8	–3.2
015	17.0	59.8	+42.8	33.7	76.5	+42.8

CFA, coefficient of fat absorption; CNA, coefficient of nitrogen absorption

(range, –19.7% to +42.8%). The median test-retest change in CNA was +14.7% (range, –6.4% to +42.8%). Individual values are listed in Table 2.

DISCUSSION

Pancreatic insufficiency leads to malabsorption of fat and protein in individuals with CF, and providing exogenous pancreatic enzyme replacement therapy can control maldigestion of food. However, other gastrointestinal factors can contribute to malabsorption in patients with CF. These include intraluminal factors (such as acidification of the intestinal tract due to lack of pancreatic, biliary and mucosal buffering ability, a decreased bile salt pool potentially leading to poor micelle formation, or bacterial overgrowth of the small bowel) and mucosal factors (such as thick intestinal mucus or other poorly defined features of an intestinal epithelial surface with dysfunctional cystic fibrosis transmembrane regulator protein), among others.[10] CFA is a composite methodology that integrates factors at all of these levels. CFA can be abnormal in the presence of pancreatic sufficiency if there is significant hepatobiliary or mucosal disease.[4] In this study, the range of CFA was much larger in subjects with CF than in healthy individuals. This likely reflects malabsorption due to PI as well as confounding effects of non-pancreatic gastrointestinal co-morbidities in subjects with CF. Furthermore, although the number of subjects studied was small, the intrasubject variability was much greater in those with CF than healthy subjects. It is possible that the shorter interval between stool collections in healthy controls (1 week) than in the CF subjects (6-9 months) contributed to the lower intrasubject variability in the healthy controls. However, lack of precision of repeated measures of CFA in subjects with CF has been documented in another uncontrolled study.[11] The calculated standard deviations presented here show that change in CFA is highly variable in the CF population. These data suggest that in the next generation of studies, statistical methods based on ranks should be considered, as these do not require standard deviation inputs to calculate sample size. A larger sample size than we have studied here would need to be tested for precision of CFA to estimate specifically how many subjects would be needed to detect a 10%, 20%, or 30% median difference in a crossover or in a parallel randomized trial.

In this study, we used a novel marker, the food dye FD&C Blue #2, to improve the reliability of our stool collections. CRC nurses had reported to us that carmine red, a marker that has been traditionally used in timed stool collections, is difficult to identify in stool. Similar comments have also been made regarding the use of charcoal as a stool marker. Therefore, we conducted a small pilot study using 1 gram of charcoal taken orally. Only 1 of 7 subjects could easily detect it in stool. In a pilot study using 250 mg, 500 mg and 1 gram of FD&C Blue #2, 100% of healthy subjects (8 of 8) could easily detect blue dye in stool at the 500 mg and 1 gram doses. Two of these eight subjects noted blue tinged urine in the 500 mg and 1 gram dose cohorts, suggesting a small amount of absorption. FD&C Blue #2, also known as indigo carmine, is a commercially available food dye that is also a commercially available drug product labeled for intravenous or intramuscular use (Indigo Carmine Injection, USP). There are case reports in the literature of a small number of individuals who have had adverse events to oral administration of FD&C Blue #2 including rash, itching, gastric upset,

and angioedema in patients with a known food additive allergy.[12-14] FD&C Blue #2 does not interfere with the fecal fat analysis (Schmidt S, Mayo Clinical Research Laboratory, Rochester, MN, personal communication, Sept 2002). It contains a small amount of nitrogen, but this is a minor component compared to the amount of nitrogen normally excreted in stool.

The CFA and CNA in subjects with CF is lower in this study than in previous reports.[6,11,15,16] Among other explanations, CFA and CNA will be falsely elevated if a timed stool collection is shortened because incomplete collection of stool will contribute fewer grams of excreted fat or protein to the CFA or CNA calculation. It is possible that other studies in the literature that report higher CFA and CNA may have had less complete stool collections because of inability to visualize difficult-to-see stool dye markers and the natural, human tendency to minimize the odious task of stool collection. FD&C Blue #2 is easy to see in feces, thus improving the accuracy of timed stool collections.

An ideal outcome measure has both precision and accuracy.[17] Although CFA is a reliable integrated measure of fat malabsorption in healthy individuals, it is less useful in patients with CF. This study demonstrates the severity and wide range of steatorrhea among pancreatic insufficient individuals with CF, which likely reflects both pancreatic and non-pancreatic etiologies of malabsorption. In a smaller group of subjects, we also found inconsistent precision of CFA and CNA. In the absence of another measurement, CFA will likely be the primary outcome measure in studies of pancreatic enzyme supplements; however, its limitations as a measure of steatorrhea correction in CF should be recognized. The new methodology we describe and the data presented here should help to improve the design of these studies until a better outcome is available.

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