

Gastric Emptying

After an overnight fast, subjects presented to the CRTU for their visit. The test administrator confirmed the subject being tested did not have a known hypersensitivity to *Spirulina*, egg, milk, or wheat allergens; was not severely lactose intolerant; and had not taken medications known to influence the rate of gastric emptying (e.g., erythromycin, metoclopramide, opiates, and anticholinergics) within three (3) days before testing. Additionally, fasting glucose levels were confirmed less than 275 mg/dL for all subjects. The subjects then provided two pre-meal breath samples to establish a baseline. After consuming the ^{13}C meal in less than 10 minutes, subjects provided six breath samples over 4 hours (45, 90, 120-, 150-, 180- and 240-minutes post-meal). Subjects were instructed not to eat, drink, or exercise during the test. Light walking was allowed. The samples were kept in secure glass tubes and mailed to a centralized laboratory for analysis by gas isotope ratio mass spectrometry (GIRMS), where the ratio of $^{13}\text{CO}_2$ to $^{12}\text{CO}_2$ over time is used to calculate gastric emptying.

Of note, it is not necessary to know the metabolic fate of all of the $^{13}\text{CO}_2$ in the GEBT meal to determine a reliable gastric emptying rate, as evidenced by the excellent agreement of GEBT gastric emptying results (breath test fraction emptied (prop_t) and breath test $t_{1/2}$ (GE $t_{1/2}$)) with scintigraphy (PMID 18406670). ^{13}C exists naturally in all foods and in all human beings at slightly different levels (around 1% of total carbon, the remainder being ^{12}C). The GEBT test meal is significantly enriched in ^{13}C by inclusion of a carefully measured dose of ^{13}C -labeled *Spirulina*: the difference in the relative amount of ^{13}C to ^{12}C in breath between samples collected prior to and then periodically after consumption of the ^{13}C -labeled GEBT test meal is the key to determining a gastric emptying rate. The consistency of the standardized ^{13}C -*Spirulina*/Egg meal and the test protocol ensure reproducibility of the results. Although only ~10% of the exogenous

¹³C administered in the test meal is excreted as ¹³CO₂ in breath, the relative rate of excretion of ¹³CO₂ in breath is proportional to the rate of gastric emptying as measured by scintigraphy.

Calculation of ¹³CO₂ excretion rates (kPCD min⁻¹) and fraction emptied/breath test t_{1/2} results.

Raw ¹³CO₂/¹²CO₂ ratio data (delta values, ‰¹³C‰) obtained from a mass spectrometer (ABCA-GIRMS) are entered in to validated spreadsheets, along with patient-specific demographic information provided on test request forms. Reports are reviewed by a second qualified individual, for accuracy and completeness of data entry.

¹³CO₂ excretion rates (kPCD min⁻¹ i.e., 1000 x percent dose excreted per minute) are calculated from delta values (¹³CO₂/¹²CO₂ ratios expressed as a delta value per mil, ‰¹³C ‰) at each breath sample collection time point t (45, 90, 120, 150, 180, 240 mins) as follows:

$$kPCD_t(\text{min}^{-1}) = \left[\frac{DOB * CO_2PR * R_S * 13}{10 * \text{dose}} \right] * 1000$$

Where:

DOB (delta over baseline) = the difference in pre-meal and post-meal ‰¹³C values (DOB = ‰¹³C_{t=i} - ‰¹³C_{t=0})

CO₂PR = estimated production rate of (total) CO₂ (mMol CO₂/min) calculated using subject height, weight, sex, and age in Schofield Equations (PMID: **4044297**).

R_S = [¹³CO₂/¹²CO₂] ratio in the reference standard Pee Dee Belemnite (PDB) which is 0.0112372

13 = atomic weight of Carbon-13

10 = A constant used to convert units

dose = weight (mg) of carbon-13 in the dose of ¹³C-Spirulina administered to the subject in the GEBT meal. Since ¹³C-Spirulina contains approximately 43% carbon-13, a 100 mg dose of ¹³C-Spirulina contains approximately 43 mg of carbon-13. This exact value is calculated on a lot per lot basis for the ¹³C-Spirulina/Egg meal.

kPCD values are calculated at each time point and plotted against time on a graph to display a gastric emptying curve.

Additionally, kPCD values at each time point are entered into established MLR equations (published in PMID: 18406670) that were derived by simultaneously measuring gastric emptying with GEBT and scintigraphy to calculate breath test fraction emptied results at 120 minutes (2 hours) and 240 minutes (4 hours), and a breath test t_½ value (derived by linear interpolation of sample times for results bracketing the 50% fraction emptied).

Hormone Assays

Total ghrelin was measured by competitive radioimmunoassay (RIA), (Millipore Research, St. Charles, MO 63304) with a reference interval between 93pg/mL-6000pg/mL. Samples above 6000 pg/mL are reported as > 12,000 pg/mL if above 6000 after dilution. Intra-assay CV's are 4.0% and 4.8% at 1209 and 2206 pg/mL. Inter-assay CV's are 10.5% and 8.6% at 446 and 1108 pg/mL.

Total glucagon-like peptide-1 was measured by a direct, double antibody radioimmunoassay (RIA) (Millipore Research, St. Charles MO 63304), with a reference range of 7.81-1,000 pmol/L. Intra-assay C.V.'s are 11.3%, 15.0% and 6.8% at 41, 279, and 320 pmol/mL

respectively. Inter-assay C.V.'s are 12.8%, 8.0% and 7.0% at 19, 46 and 211 pmol/mL respectively.

PYY was measured by the Human Peptide YY double-antibody radioimmunoassay kit (Millipore Research, Inc. St. Louis, MO 63304), with a reference range of 20-500 pg/mL.

Samples above 1000 pg/mL are reported as > 1000 pg/mL. Inter-assay C.V.'s are 4.7%, 6.5%, and 8.7% at 68, 95, and 330 pg/mL respectively