Methods

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Clinical

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Carbohydrates (CHO) are the major source of human energy requirement. Although ordinarily most of the ingested CHO are completely absorbed before reaching the colon, several disorders can result in impairment of absorption from the small intestine [1-3]. There being no quantitative fecal CHO equivalent to the quantitative estimation of fecal fat, several laboratories use semiquantitative screening tests to confirm clinical suspicion of CHO malabsorption. Established methods for the quantification of fecal CHO [2, 3] require collecting stools for 24-72 h and unpopular sample handling (e.g., extraction, distillation). Therefore, use of such tests has been mainly confined to specialized laboratories.

Fat, water, and nitrogen in stools have recently been measured by near-infrared reflectance analysis (NIRA), a method based on the measurement of the scattered radiation in the near infrared by the surface of a sample. The analysis is performed in a very short time (<1 min), without further processing of the feces and without the use of chemical reagents [4-7].

Because of the importance of an accurate and feasible measurement of the fecal CHO output in clinical gastroenterology, we have evaluated the efficiency of NIRA in comparison with "wet" chemical methods for determining fecal CHO.

Materials and Methods

NIRA is based on the matrix- and substrate-specific relation between the reflectance intensity diffused by the fecal sample surface at a specific wavelength and the composition of the sample. Each component to be measured has typical functional groups (e.g., CH, NH, OH) possessing specific absorption bands in the near-infrared area (700-2500 nm). The spectroscopic response (reflectance) from a fecal sample is related to the concentration of the compounds (functional groups) as follows [8, 9]:

\[ x = Z - f_1 \log Rf_1 - f_2 \log Rf_2 - \ldots - f_n \log Rf_n \]  

where \( x \) is the concentration of the analyte, \( Rf_n \) is the reflectance for the filter \( n \), \( f_n \) is the scaling factor for each filter, and \( Z \) is a constant of bias correction. The wavelength and corresponding spectral structure for each filter are given in Table 1. Scaling factors corresponding to each selected filter were calculated by multilinear regression [10, 11] of results obtained from a training set consisting of samples analyzed by the routine chemical method covering the whole concentration range for fecal CHO.

The method based on this principle was described by Puchant et al. [12]. In brief, the sum of squares of the residual values (differences between chemical method values and calculated values) must be as low as possible. The computer assessed every set of wavelength combinations from 1 to 12, through spectroscopic and statistical data, based on the use of a t-test where \( t = t/SD \) of \( F \).

Each combination of wavelengths was characterized by the coefficient of multiple correlation (\( r \)) between the chemical and calculated values:

\[ r = \frac{1 - S_{\text{res}} (n - k - 1)}{\text{SD}^2 (n - 1)} \]
where $S_{yn}$ is the standard error of estimate, n is the number of samples, and k is the number of filters; and by the constant of Fisher (F-ratio), which indicates the quality of the regression:

$$F\text{-ratio} = \frac{r(n-k-1)}{(1-r^2)k}$$  \hspace{1cm} (3)

As the number of wavelengths in the combination increased, r approached 1 and the F-ratio increased to >50.

For the training set, 34 stool samples from patients with various diseases and following various treatments were selected.

**APPARATUS**

Experiments were performed with an Enrif infrared analyzer (Model Fenir 8820, interference-filter-based reflectance spectrometer; Stimotron, Wendelstein, Germany) connected to an IBM-compatible computer. The optical information is provided by a constantly rotating filter wheel fitted with 12 narrow (10-nm) bandpass filters of fixed wavelength specification ranging from 1218 to 2345 nm (Table 1), which makes it possible to measure reflectance (R) as the ratio of reflected energy from the sample and the incident radiation. R was measured at 1 to 12 wavelengths, converted into log R, and entered into the computer. Because near-infrared reflectance measurements are highly dependent on the amount of scattering materials and pathlength, the 1940-nm water band was used to compensate for differences in effective pathlengths [13].

The manufacturer's software program (Perten, Rome, Italy) was used to select the best combination of filters from a group of filters that were highly correlated spectrally: Wavelengths with clearly assignable spectral responses were included and those without were excluded. Wavelengths with low constants and poor correlations were excluded on statistical grounds by use of Student's t-test. The program functioned with a combination of 1 to 12 wavelengths, giving for each combination the values of constants corresponding to each filter, the values of the correlation coefficient r, and the F-ratio.

**PATIENTS**

From January 1990 until December 1991 we studied 74 patients (39 males, 35 females) of all ages (3-71, mean 32 years) who had been admitted to our hospital (a) for the differential diagnosis of malabsorption syndromes (history of diarrhea, weight loss) or (b) with previously established causes of malabsorption or malnutrition (exocrine pancreatic insufficiency due to chronic pancreatitis or cystic fibrosis, inflammatory bowel disease, celiac sprue). A subgroup of extensively investigated patients with functional abdominal pain, but with entirely normal gastrointestinal function, served as controls. In each case, final diagnoses were established after thorough investigation according to generally accepted criteria. The study was in accordance with the ethical standards of the local committee.

**ANALYSES**

Stool was collected for 72 h in plastic containers and analyzed either on the same day or stored at -24 °C until analysis. For NIR, a stool sample of 2-3 g was homogenized (Ultraturax-Blender; Janke & Kunkel, Staufen, Germany), then placed in a disposable cup and positioned on the analyzing site of the infrared analyzer. Within a given spot sample, measurements were read at two distinct positions. While the selected filters in turn automatically rotate into the light path, reflected spectral energies from the sample are recorded by a microprocessor, and results (% substrate content) are displayed or printed in <1 min.

Samples that had been frozen were thawed, homogenized as above, and centrifuged at 5000g for 10 min. The supernatant was filtered through 10-μm (pore size) filters (Millipore, Bedford, MA) before assay.

For comparison we measured fecal total CHO by using anthrone in a modification of the spectrophotometric method [14, 15]. This method, first described by Dreywood [16], is based on the appearance of a characteristic blue-green color ($\lambda_{max} = 680$ nm) due to formation of a hexose-anthrone complex in the presence of sulfuric acid. The method is highly specific for all CHO (>95%), but is not sensitive to organic acids that do not include CHO [5]. The mean CV of this assay after 10 repeated measurements was 4.8%.

**STATISTICS**

Unless otherwise stated, the results were expressed as mean ± SE. For statistical analysis, Student's unpaired t-test was used.

**Results**

Table 1 shows the optimum set of wavelength combinations for fecal carbohydrate analysis.

**Calibration and validation.** Comparison of the concentrations measured by the anthrone and the infrared method training set in 34 stool samples (calibration set) showed $r = 0.962$ and $S_{yr} = 1.65$ g/kg wet weight (Fig. 2A). For the validation data, shown in Fig. 2B, $r = 0.869$ and $S_{yr} = 3.1$ g/kg wet weight. The residual
Fig. 1. CHO measured by NIRA is as many as five spot samples from a single stool, shown as the difference between the mean value from the spot samples and the value from the whole homogenized stool.

Values (differences between results obtained by the two methods) are shown in Fig. 3.

The mean CHO concentration was 6.5 ± 3.4 g/kg wet weight (range 2.7–24.5 g/day) by the NIRA and 5.3 ± 2.4 g/kg wet weight (range 2.5–19.3 g/day) by the anthrone method, not significantly different (P > 0.05).

Recovery. An aliquot of stool homogenate was analyzed for CHO content. Three different amounts of lactulose solution were added to the homogenate, in amounts yielding CHO-to-stool ratios of 3, 2, and 1 g of CHO per 1.0 g of stool. Samples were then rehomogenized and several aliquots of each homogenate analyzed. The recovery of CHO was 98.7–102.5% (n = 6) regardless of the amount of CHO added, suggesting that the method can be used to determine both high and low quantities of CHO excretion.

Stability of stool CHO. To determine the effect of bacterial degradation on stool CHO content in the time between passage of stool and analysis, we added a known amount of lactulose solution to fresh stool homogenate and then rehomogenized as above. Freezing at −24 °C for 72 h was chosen to evaluate the effect of storage during the collection period. Three aliquots of stool, stored at this temperature and analyzed every 72 h over a 2-month period, yielded stable results (Fig. 4).

Discussion

The importance of quantitative fecal CHO output measurement in the diagnosis of malabsorption syndromes has been recognized in several previous studies [1–3]. However, traditional methods for quantifying fecal CHO are complex and time consuming and require unpleasant sample handling.

Our data indicate that NIRA is a valuable practical alternative to conventional chemical methods for measuring fecal CHO. The absence of any potentially hazardous reagent (therefore

Fig. 4. Stability of three different stool samples analyzed for carbohydrate content during storage at −24 °C over 2 months.
excluding the need for associated precautions), homogenization, extraction, distillation, and other onerous procedures allows this method to be applied in any hospital laboratory, not just in specialized centers. Moreover, the short time required for the analysis by NIRA (<1 min) provides immediately available results. Finally, comparison of NIRA in homogenized stools and the traditional anthrone method gives a precise correlation, implying that NIRA is as accurate as the conventional methods.

Repeated analysis on different portions of the same stool collection, performed in a few minutes, can avoid homogenization, usually the most unpleasant part of the classical methods. Five readings reduce the difference from the posthomogenized value to <7%, but for clinical application three readings may suffice.

We emphasize that the use of NIRA is based on calibration with a chemical method, which is the first and indispensable step when starting the measurements. This means that the reflectometric results depend on the availability and accuracy of the chemical methods used in the individual laboratory as well as on the choice of the biological matrix. Therefore, NIRA cannot be “more accurate” with respect to the true value than the reference chemical methods are. Benini et al. [17] previously demonstrated that changes of stool matrix (e.g., in liquid stool) may influence the results of nitrogen analysis. In such cases, one should adjust the calibration with an adapted stool matrix or change the chemical method. In this study, we did not focus on these possibilities, but also saw no indication from our patient groups that this might be clinically relevant.

Further, as Benini and colleagues found for fat and nitrogen [17], NIRA can underestimate fecal CHO. This is of little consequence for a clinical screening method, but applying the NIRA method to nutrient balance studies could lead to systemic errors. The difference between reflectometric and chemical values can also be partly explained by the imprecision of the chemical methods. So Grimble et al. [18] found that the Kjeldahl method underestimated fecal nitrogen because of acid digestion of refractive nitrogen. Similarly, the anthrone method can fail to detect all CHO if the appropriate extraction solvent is not used. Thus, by using different chemical methods, one can expect better correspondence between NIRA values and chemical values.

For routine purposes in a clinical setting, we consider NIRA a valuable and precise alternative to the anthrone method. Taking into account the simultaneous measurement of fat, nitrogen, and water justifies the current instrumentation price of ~$30,000 (US), which could be reasonable according to the number of analyses performed; also, only one trained analyst is required.

We conclude that NIRA represents a useful replacement for the conventional laboratory methods in the investigation of malabsorption. In the future, NIRA may offer a new and powerful diagnostic approach to malabsorption syndromes, by simultaneous determination of several variables, such as dry weight, fecal fat, and fecal nitrogen [6, 7], in addition to CHO reducing sugars, for evaluating the digestive and (or) absorptive function of the gut.

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References