The Use of Dysprosium to Measure Endogenous Zinc Excretion in Feces Eliminates the Necessity of Complete Fecal Collections1–3

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Abstract

The secretion of endogenous zinc (Zn) into the gut and subsequent excretion in the feces is understood to play a major role in maintaining Zn homeostasis in humans. Therefore, the measurement of endogenous Zn losses in the feces (EFZ) can be an important aspect of the study of Zn metabolism and homeostasis. The methods currently used to measure EFZ have the disadvantage of requiring complete fecal collections over multiple days. We have investigated the use of dysprosium (Dy), a nonabsorbable rare earth metal, in a method of determining EFZ that does not require complete fecal collections and permits the measurement of EFZ from several fecal samples. The method was evaluated using data from a study of free-living adult females in which Dy was administered 3–4 times/d over a period of 5 or 6 d to monitor completeness of fecal collections. The results did not differ from those obtained using an established isotope dilution method. We found that the method of determining the sample Dy:Zn ratio was useful for selecting samples for measurement. We conclude that the Dy method of determining EFZ is a valid and less burdensome alternative to current techniques.

Introduction

The secretion of endogenous zinc (Zn) into the small intestine and its subsequent excretion in the feces is understood to be a major Zn homeostatic mechanism in humans (1). Given its central role in the maintenance of Zn homeostasis, the measurement of endogenous Zn losses in the feces (EFZ)6 makes an important contribution to the study of human Zn homeostasis. To measure endogenous Zn in feces, it is necessary to distinguish it from unabsorbed dietary (exogenous) Zn by means of isotopically labeling one or the other. Thus, the measurement of EFZ became feasible with the development of radio and stable isotope tracer methodologies. Although relative measurements of endogenous Zn in feces had been made with radioisotopes in ruminants at least a decade earlier (2), the quantitative measurement of EFZ was first described by Weigand and Kirchgessner (3) using a radioisotope dilution technique in growing rats. Jackson et al. (4) were the first to use this method in humans, administering the stable isotope 65Zn as the tracer. About the same time, Turnlund et al. (5) reported EFZ results estimated from Zn metabolic balance data and apparent isotope tracer absorption measured by fecal monitoring of a stable isotope. Endogenous fecal Zn was subsequently measured using the same technique by Turnlund et al. (6) and others (7–9). In 1992 Hunt et al. (10–12) published the first of several reports that used the measurement of true Zn absorption by whole body counting of a radioisotope tracer and metabolic balance data to determine EFZ, thus providing a potentially more accurate measurement than possible using apparent isotope absorption. EFZ has also been determined using compartmental models (13,14) and by a method requiring complete urine and fecal collections and the measurement of tracer excretion in both (15–17). We have routinely employed the isotope dilution method of Weigand and Kirchgessner (3) using urine enrichment since our first report on the method in 1991 (18).

All the current techniques for measuring EFZ have the noteworthy disadvantage that they require, at the least, complete quantitative collection and analysis of feces. Some also require complete collection and analysis of diet or urine. This is burdensome for participants, vulnerable to collection error, and requires great sample handling and analysis effort. Also, EFZ is normally reported as a daily rate and the accuracy with which EFZ can be quantified as a daily rate is limited by the uncertainty of how reliably the excretion of endogenous Zn over a few days reflects the actual endogenous losses to the gut given the vagaries.
of bowel transit kinetics. Furthermore, complete collections are impractical, if not impossible, in some populations.

We report here on our investigation of the use of dysprosium (Dy), a nonabsorbable rare earth element, to measure EFZ in a manner that eliminates the need for complete quantitative collections of feces, urine, or diet and potentially permits the measurement of EFZ using a single fecal sample or very few samples. Because the gastrointestinal transit and excretion kinetics of Dy have been shown to be very similar to that of Zn, Dy has been used to monitor the completeness of fecal collections and to measure Zn absorption (19,20). In extending the use of Dy to the measurement of EFZ, we hypothesized that oral administration of Dy, preferably multiple times a day, in a constant and regular manner, and its measurement in the feces could be used to quantify the amount of endogenous fecal Zn as a daily rate. Furthermore, we expected that the Dy EFZ calculation would be valid only when Dy was being excreted at the same rate it was ingested and that this could be assessed by the comparison of the Dy:Zn ratio of each fecal sample with the ratio of ingested Dy:ingested Zn. As with several of the other methods, this method requires the i.v. administration of an isotope tracer. Data from a study of healthy adult females wherein Dy was used to monitor the completeness of fecal collections and measure fractional Zn absorption and EFZ was measured by the isotope dilution method, were used to evaluate the feasibility and validity of the proposed EFZ measurement method.

Methods

Study design. These data were from a larger study of Zn homeostasis. Thirty-two free-living, apparently healthy participants recruited from the Denver metropolitan area and 21–49 y of age were studied. All participants were female except for a single male who participated in the pilot portion of the study. All participants gave informed consent pursuant to the guidelines of the Colorado Multiple Institutional Review Board. Data from 25 of the participants studied have been included in this investigation. The criterion for inclusion was recovery in feces of at least 90% of total Dy administered. The mean Dy recovery for these participants was 99.6%. The Dy recoveries from the remaining participants ranged from 49 to 88% and were usually the result of documented or suspected incomplete fecal collections.

Although the details of the study have been published elsewhere (21), a summary of the relevant aspects of the study design follows. Participants consumed a fixed 3-d rotating diet with constant daily energy, Zn, and phytate intakes based on the individual’s 7-d diet record. The participants remained on this diet from 7 d prior to the metabolic study period until the end of the 6-d study period. An isotopic tracer (68Zn) was administered i.v. 4 or 7 d before the metabolic period and an accurately measured quantity (~50 µg, in proportion to dietary Zn in meals) of Dy was given with each of the 3 meals and, in some cases, a snack on every day of the study. Brilliant blue was given as a visible fecal marker immediately prior to the first study meal and again exactly 6 d later.

Participants collected all stools from the time of the first study meal until the second fecal marker passed completely. They also collected all urine throughout the metabolic period. We measured total Zn in stool and urine samples using atomic absorption spectrophotometry and Dy and 68Zn tracer enrichment by inductively coupled plasma MS. We measured the total dietary Zn in duplicate diet samples by atomic absorption spectrophotometry.

Dy analysis. Accurately weighed duplicate aliquots of homogenized feces were dried separately to a constant weight in an electric oven. The dried samples were then ashed in a muffle furnace at 450°C for 24 h. A few drops of concentrated nitric acid were added to the ash, which was then heated on a hot plate before redigesting at 450°C for 24 h. Ashed fecal samples were reconstituted quantitatively in 50 mL 10% HNO3. Samples, Dy standards (0–1000 µg/L), and blanks were diluted 80:1 with an internal standard solution and Dy concentrations measured by inductively coupled plasma MS. The internal standard solution was prepared by adding 5 µL 180 mg/L terbium solution and 5 µL 180 mg/L holmium solution into 250 mL 2% ultra-pure HNO3. All samples, standards, and blanks were analyzed in triplicate and external drift corrections were applied every 15 samples.

Calculation of EFZ in mg/d. The use of Dy to determine EFZ from individual fecal samples was accomplished with the following calculation:

\[
EFZ = \frac{\text{i.v. tracer in fecal sample \times Dy daily dose}}{\text{i.v. tracer enrichment in urine \times Dy in sample}}
\]

where the quantity of i.v. tracer in the sample is calculated as \(F_{\text{Zn} \times f \times \text{Dy dose}}\), the total Zn in the sample in mg, times \(f\), the i.v. tracer (\(^{68}\text{Zn}\) or \(^{67}\text{Zn}\) in these studies) enrichment of the sample, \(\text{Dy dose}\) is the total quantity of Dy given daily (µg/d), \(F_{\text{Dy}}\) is the total Dy in the sample (µg), and \(\bar{u}\) is the i.v. tracer enrichment of urine at the time the endogenous Zn in the sample was secreted into the gut. EFZ results from the individual samples were averaged to derive an EFZ value for each participant. This method, as well as the isotopic dilution technique described below, is based on the definition of tracer enrichment as the quantity of isotopically enriched tracer Zn divided by total quantity of Zn in the sample and the fact that endogenous Zn excreted in urine and feces has the same tracer enrichment, because both originate from the metabolically active, rapidly exchanging pool of body Zn.

We calculated urine enrichment in 2 ways. The mean enrichment for the whole study period (similar to the value calculated for the isotope dilution method described below) was used, but, because the Dy method calculates EFZ for individual fecal samples, we assumed that more accurate results could be obtained by estimating the actual urine enrichment at the time of secretion of the endogenous Zn into the gut. The time interval between endogenous Zn secretion into the gut and its subsequent excretion was estimated as the mean total transit time (from ingestion to excretion) of the visible markers minus 5 h [mean gastric one-half emptying time plus one-half of the mean small intestine transit time in healthy adults (22)]. This interval was then subtracted from the sample excretion time to find the appropriate time to determine urine enrichment. The urine enrichment corresponding to this time was estimated from a nonlinear regression analysis (using an exponential decay function) of all the urine enrichment data.

We also determined EFZ using the isotopic dilution technique of Weigand and Kirchgesner (3), which employs the following equation:

\[
EFZ = \frac{\text{total i.v. tracer in feces \times study duration}}{\sum \text{i.v. tracer enrichment in urine \times study duration}}
\]

where the summation includes all fecal samples between the appearances in feces of the visible markers (\(m1\) and \(m2\)) administered at the beginning and end of the study period, \(F\) is total Zn in the sample (in mg), \(f\) is the i.v. tracer enrichment of the sample, \(\bar{u}\) is mean i.v. tracer enrichment of urine over the study period, and \(t\) is time (d) between administration of the visible markers.

Use of sample Dy:Zn ratio to identify appropriate samples for EFZ determination. The selection of fecal samples that were suitable for determining EFZ by the Dy method was accomplished by calculating the ratio of Dy:Zn in each sample and comparing this value to the ratio of the daily dose of Dy to total daily dietary Zn (TDZ). The criterion of sample Dy:Zn > 50% of Dy dose:TDZ for inclusion in the calculations was applied and evaluated.
Results

We found the comparison of sample Dy:Zn ratios to daily Dy dose:TDZ to be a reliable technique for evaluating individual sample results and, because samples with Dy:Zn ratios >50% of Dy dose:TDZ usually exhibited more consistent and realistic EFZ values (Fig. 1A, B), the acceptance of EFZ values based on that criterion appeared to be appropriate. It was evident from the data that the threshold had to be at least 45–50% to exclude obviously meaningless EFZ values. But, although this was the minimum acceptable threshold, experimentation with higher thresholds did not improve the overall agreement of results with the isotope dilution method. The number of samples averaged to determine a participant’s EFZ ranged from 3 to 8 with a median of 6. In 76% of cases, the first sample useful for calculating EFZ was the second or 3rd fecal sample after the start of the study; this was usually the second sample containing some of the first visible marker.

All the EFZ data were found to have normal distributions (P-values ranged from 0.12 to 0.61). The EFZ results (mean ± SD) from the Dy method using the mean urine enrichment (mean u), the Dy method using urine enrichment from regression analysis (regression u), and the isotope dilution method were 2.84 ± 0.53, 2.90 ± 0.56, and 2.77 ± 0.69, respectively. The 2 Dy methods did not differ from one another (P = 0.06) or from the isotope dilution method (P = 0.41 for mean u and P = 0.16 for regression u) (paired t tests). Isotope dilution was correlated with Dy (mean u) (r = 0.77; P < 0.00001) and Dy (regression u) (r = 0.78; P < 0.00001). Bland-Altman analysis of agreement of the Dy (mean u) compared with isotope dilution results showed the mean difference (Dy – isotope dilution) of 0.07 mg/d and 95% limits of agreement (mean difference ± 1.96 × SD of the differences) of −0.79 and 0.94 mg/d (Fig. 2). Bland-Altman analysis of the Dy (regression u) compared with isotope dilution results showed the mean difference of 0.13 mg/d and 95% limits of agreement of −0.73 and 0.98 mg/d.

Discussion

The primary goal of this investigation was to assess the agreement between a new method of measuring EFZ using Dy and the isotope dilution method of Weigand and Kirchgessner (3). Because this was the first attempt to measure EFZ in individual fecal samples after commencement of the regular daily ingestion of Dy, it was important to have a means of judging the validity of sample EFZ results and criteria for accepting valid results. The use of the sample Dy:Zn ratio for this purpose and its comparison to the daily Dy dose:TDZ ratio proved to be successful. Although experimentation with the threshold of >50% of Dy dose:TDZ for sample acceptance showed it to be appropriate, it may still be possible that this criterion can be refined or a more complex criterion developed to improve sample selection reliability.

It is possible with these methods to quantify endogenous Zn in individual fecal samples with little error. Assuming good laboratory/analytical technique, the primary uncertainty in the calculation is in the estimation of urine enrichment. Additional uncertainty is introduced to measure EFZ as a daily rate, the...
usual form of EFZ quantification. With the isotope dilution method, this involves collecting all stools over multiple days and using a visible marker to demarcate the beginning and end of collections for a defined study interval. Even with the use of fecal markers, there can be notable uncertainty regarding which marker-containing stools or what portion of individual stools should be included in the measurement. This is potentially the principal source of error with the isotope dilution method. The Dy method uses regular ingestion of Dy and its measurement in individual fecal samples to quantify EFZ as a daily rate. The uncertainty here is how closely the fraction of daily Dy dose contained in a stool reflects the quantity of endogenous Zn in the stool as a fraction of true EFZ. It would be expected that the administration of Dy with all meals and snacks, as was done here, would minimize this uncertainty.

The Dy method EFZ results from using 2 ways of estimating urine enrichment bordered on being significantly different, but the mean difference was only 2% of the mean EFZ and possibly reflected better accuracy of the regression technique. The small difference between techniques supported the use of either technique when all fecal samples from the study period were being used. If, however, fewer samples were to be used to measure EFZ, it would be more important to use the regression technique to ensure the accuracy of urine enrichment estimations for individual samples. Generally, the estimation of urine enrichment by regression analysis permits more flexibility in fecal sampling. It should also be noted that the timing of i.v. tracer administration affects the sensitivity of EFZ determinations to urine enrichment estimation, because the rate of decay of enrichment decreases over time, making the timing of enrichment measurements less critical as more time elapses after isotope administration.

Although the principal finding was that the difference in mean results from the 2 methods was not significant, further useful information on the agreement between methods was provided by a Bland-Altman plot of differences for the individual participant data (Fig. 2). The plot shows that the small difference in the means (0.07 mg/d) is due to most Dy values being higher than those of the isotope dilution method but that the variation between methods otherwise appears random. No clear explanation for the tendency of Dy values to be higher was found, although a slight difference in how mean urine enrichment was calculated was considered a possible cause of bias between methods. Other than urine enrichment estimation, the potential causes of error in both methods discussed above were understood to be random in nature and the primary sources of the random variability in differences. Although the great majority of data exhibited differences <0.5 mg/d, the Bland-Altman limits of agreement quantified the larger range within which 95% of differences are expected to lie. The one obvious point exhibiting a large difference was due to an unusually high EFZ value (4.7 mg/d) from the isotope dilution method; an examination of the participant data and analysis indicated no basis for discarding this datum, however. Another important characteristic of the data that the plot made evident was that the bias and variability in the differences were independent of EFZ magnitude. In summary, the Bland-Altman analysis generally confirmed the adequacy of the agreement between the methods while displaying its limitations, particularly with variability in differences between individual data. Finally, it should be pointed out that an evaluation of the relative accuracy of the methods was not undertaken, because the isotope dilution method, although an established method, is not considered the gold standard.

The development of the Dy method created the possibility that EFZ could be measured using a single fecal sample. Whereas in the best cases (Fig. 2A) the use of a single sample measurement would have provided reliable results for an individual participant, most of the data were not this predictable. The mean relative SD of participant EFZ results was 0.25 and the absolute values of individual sample measurements deviated from the participant mean EFZ by ~0.5 mg/d on average. Regardless of whether this variability is attributable to the method or natural processes, it appears that single sample measurements are not reliably accurate with the method as described here and that it would be prudent to use a minimum of 3 fecal samples to ensure reasonable accuracy.

We conclude that the new method for measuring EFZ using Dy is a valid alternative to other methods and, in fact, may be considered a preferable method, because it eliminates the difficulties and limitations of conducting complete fecal (or urine) collections and analysis, thereby permitting the measurement of EFZ in situations and populations where other methods are impractical.

Acknowledgments
L.V.M., X.Y.S., K.M.H., J.E.W., and N.F.K. were responsible for the research design including project conception, development of overall research plan, and study oversight. L.V.M., X.Y.S., J.E.W., and L.S. conducted the study and performed data collection. L.V.M., X.Y.S., L.S., and J.E.W. were responsible for data analyses and LVM for statistical analyses. L.V.M. and K. M.H. wrote the paper and they along with N.F.K. were responsible for the final content. All authors read and approved the final version of the paper.

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