HPLC Determination of Polyethylene Glycol 400 in Urine: Oligomeric Profile in Healthy and Celiac Disease Subjects

Alexis Oliva,1 Honorio Armas,2 and José B. Fariña1,3

The absorption of orally administered polyethylene glycol (PEG) has been used to assess intestinal permeability. We describe a simple HPLC technique to determine the oligomeric profile of PEG excreted in urine. We measured the total (%) PEG excreted in 6 h and the ratio of the four smallest oligomers to the three largest oligomers (expressed as mean percentages). The proposed method differentiates distinct groups of subjects with varying degrees of intestinal permeability detected by intestinal biopsy. The percent of PEG excreted and the oligomer ratio values for healthy subjects were, respectively, 30.1 ± 3.87 and 0.35 ± 0.03; for celiac patients on a gluten-free diet, 24.5 ± 6.65 and 0.45 ± 0.18; and for celiac patients, 15.0 ± 5.93 and 1.12 ± 0.55.

Indexing Terms: intestinal permeability/ chromatography, liquid

The permeability of the intestinal mucosa to molecules varies with molecular size; hence, substances of different molecular sizes such as xylose (1), lactulose (2, 3), inulin (4), urea (5), creatinine (6), mannitol (2, 6), EDTA (7), and polyethylene glycol (PEG) (8) have been used in tests of mucosal integrity under various physiological and pathological conditions (9–11).4 Celiac disease is especially interesting because it is common (0.03% of the white population) (12), and a diagnostic laboratory test could obviate the need for biopsy. We used PEG because it has a low oral toxicity [although topical toxicity is well documented (13)], is not degraded by intestinal flora, is easily quantifiable in urine, and >90% of the dose administered is excreted in urine within 6 h after administration. Moreover, the distribution of the various homologs in urine may differentiate healthy subjects and patients. We chose a low-molecular-mass PEG, PEG 400, for the present studies because the distribution of the various oligomers and their chromatographic resolution combine to make its analysis notably easier than that of heavier PEGs.

In this study we focused on (a) the validation of HPLC determination of PEG 400 in urine, (b) the study of urinary excretion of PEG 400 in healthy subjects to determine the importance of the intersubject variability as well as the influence of gastric emptying, and (c) the estimation of variables affecting the interpretation of the oligomeric profile of PEG in urine, to establish the capacity of the PEG 400 test to differentiate subjects according to their intestinal permeability.

Materials and Methods

Sample Treatment

To validate the analytical method, we prepared six calibrators at concentrations of 0.75, 1.5, 2.25, 3.0, 3.75, and 4.5 g/L. Every concentration was replicated four times for urine samples and six times for the mobile phase. To quantify PEG 400 (Serva, Heidelberg, NY) in the urine samples, we used the method proposed by Tagesson and Sjodahl (14). Extrelut 20 columns (Merck, Darmstadt, Germany) were used as the extraction solid phase, and 5 mL of each urine sample was diluted with 10 mL of water and 5 mL of methanol. The whole volume (20 mL) was then added to the column and eluted with chloroform; the eluate was evaporated at 30°C in a nitrogen atmosphere and the dry residues of the calibrators were dissolved at 37°C in 5 mL of the mobile phase. The dry residues of the unknown samples were appropriately diluted to obtain concentrations within the calibration range. The samples to be analyzed were filtered with 0.45-μm (pore size) filters (Millipore Waters Chromatography, Milford, MA).

Analytical Method

Filtered samples (50 μL) were injected into a Waters chromatograph (Millipore Waters) equipped with an autosampler (Wisp Model 710B) and a pump (Model 510). The effluent was analyzed with a differential refractometer (Model 410) and the voltage output was integrated with a Model 730 integrator. The mobile phase was a mixture of methanol and water (30:70) at a flow rate of 1 mL/min, and an ODS column (μ-Bondapak C18, Millipore Waters) was the stationary phase. Deionized water prepared with a MilliQ apparatus (Millipore Waters) was used throughout; all other chemicals and reagents were from Merck.

Urinary Excretion of PEG 400

Three groups of subjects were used. The procedures followed were in accordance with the ethical standards of our institution’s responsible committee and with the Helsinki Declaration of 1975, as revised in 1983. No subjects were used who consumed drugs or alcohol before or during the study.

Group I: healthy individuals. This group consisted of six healthy volunteers (three men and three women, 23–28 years old, 52–75 kg) with no history of renal or...
intestinal permeability disorders. The volunteers received a medical checkup, and gave written consent to their participation in the study. They were given a single dose of 10 g of PEG 400 in 100 mL of water after a 12-h fast. A cross-assay (2 × 2 Latin square) was used, whereby the six subjects were divided into two groups of three each. The first group fasted before and during the assay, whereas the second took PEG 400 with a continental breakfast. Liquid was not restricted in either of the groups.

After 1 week the assay was repeated with the conditions interchanged. In both cases the urine samples were collected 1, 2, 4, and 6 h after administration.

**Group II: celiac patients on a gluten-free diet.** This group included five children with celiac disease (four girls and one boy, 2–14 years old, 9–62 kg) who were in the second diagnostic phase of their illness and who had been on a gluten-free diet for 2 years. Permission for their participation in the study was granted by their parents.

To obtain the samples of intestinal mucosa we used a pediatric Kilby capsule for intestinal biopsy, introduced via mouth or nose. From the biopsy data, celiac subjects on a gluten-free diet showed normal intestinal villi except for two subjects who exhibited slight partial atrophy.

All the children received PEG 400 (0.175 g/kg body weight) diluted in 100 mL of water after a 12-h fast. Urine was collected during the 6 h after administration.

**Group III: celiac patients.** This group consisted of 16 children (eight girls and eight boys, 1–17 years of age, 9–81 kg) who were in the first stage of celiac disease and hence were on a diet in which gluten was still present. The permeability test was performed exactly as above.

The celiac patients showed subtotal intestinal atrophy, except for two who showed moderate partial atrophy.

### Results and Discussion

As a preliminary step to the study of urinary PEG excretion, the chosen analytical method was validated both in the mobile phase, with no impurities that might affect determination, and in urine. The separation of PEG 400 by HPLC resolved several peaks corresponding to the various oligomers of $M_w$, 282–634. To quantify PEG 400 we used the total area of all peaks. The percentage of the total area for individual peaks allowed estimation of the distribution of the oligomers.

Up to eight PEG oligomers could be resolved (Fig. 1), although we selected the seven best quantifiable in urine. The minimum quantifiable concentration of PEG 400 in the mobile phase was 0.75 g/L. The mean extraction recovery for urine was 83% (n = 24), with CV 6.74%, a value close to that obtained by Delahunty and Hollander (15) with chloroform extraction. As with the mobile phase, the analytical technique detected 0.75 g/L PEG 400 concentrations in urine, the proposed linear model being acceptable with a CV of 5.9%. The regression lines and their respective equations are shown in Fig. 2.

To clarify and complement the information available (16, 17), we studied PEG 400 urinary excretion in six healthy adults; PEG 400 urinary excretion is virtually the same in adults as in children (17), and the use of healthy children as controls would pose obvious practical difficulties.

The experimental design used to carry out the assay was a $2 \times 2$ Latin square. The statistical model took the following form:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + e_{ijk}$$

where $Y_{ijk}$ was the observation made of the $i$th individual during the $j$th period and in the $k$th test; $\mu$, overall mean; $\alpha_i$, effect due to the $i$th individual; $\beta_j$, effect due to the $j$th period; $\gamma_k$, effect due to the $k$th assay; and $e_{ijk}$, random error normally distributed with zero mean and $\sigma^2$ variance.

We measured the total PEG excreted ($X_{ij}$) in 6 h and the ratio $C$ of the four smallest oligomers to the three largest oligomers (expressed as mean percentages).

The experimental design enabled us to carry out a three-way analysis of variance (ANOVA) for the selected variables. Table 1 shows the findings for each subject.

Independent of the response used, the ANOVA results, taking as response $X_{ij}$ and the ratio $C$, showed that the intersubject variability was not significant ($\alpha = 0.05$, $P = 0.257$ and 0.168, respectively), although the influence of the ingestion of food (assay factor) differed according to which response was used in the ANOVA.

Although not significant when $X_{ij}$ is used ($P = 0.799$),
gastric emptying primarily influences the oligomeric profile of PEG 400 in urine when C is used ($P = 0.0175$), so we confined ourselves to using the data of the fasting control subjects for later contrast with the other groups.

Figure 3 shows the oligomeric profile for the selected control subjects (fasting) at each interval of urine collection. The profile is completely unaltered throughout the collection period, so the test may be simplified as far as collection times are concerned; in principle, a urine collection period of 1 h would enable us to obtain the same results when interpreting the oligomeric profile.

The results of the test carried out on the other groups are given in Table 2. In agreement with the results obtained by Lifschitz et al. (18), the celiac patients show an important reduction in $X_u$.

The differences among the three groups are evident from the mean oligomeric profiles in urine for each group (Fig. 4). There is a clear inversion between the percentages of smaller and larger molecules in the celiac patients in group III.

To determine the statistical significance of these differences between the three groups, a one-way ANOVA
was carried out, with the following equation being used as the statistical model:

\[ Y_{ij} = \mu + \alpha_i + e_{ij} \]  

(2)

where \( Y_{ij} \) is the \( j \)th observation of the \( i \)th subject, \( \mu \) is the overall mean, \( \alpha_i \) is the effect of the \( i \)th treatment, and \( e_{ij} \) is the random error normally distributed with zero mean and \( \sigma^2 \) variance.

The ANOVA results for \( X_u \) and quotient \( C \) showed that there are significant differences (\( \alpha = 0.05, P = 0.0022 \) and 0.0002, respectively) among the three groups. This enabled us to apply Scheffe’s test to contrast pairs of means (Table 3) and consequently to determine which groups of subjects differed from each other.

From this comparison, the use of \( X_u \) and quotient \( C \) equally allows differentiation between normal subjects (group I) and celiac patients (group III), and between celiac patients and those with the gluten eliminated from their diet for 2 years (group II).

The results of the test also allow us to infer that the difference between groups I and II is not significant; it is our opinion that the control group used for contrast with celiac patients could be composed exclusively of subjects consuming no gluten, with these subjects serving as their own controls in future comparisons, when gluten might be reintroduced into their diet. This would require that the number of subjects in this group be increased, which means that the minimum significant difference would be reduced, allowing a clearer distinction to be made between means.

References