Measurement of Polyethylene Glycol 400 in Urine by Direct-Injection High-Performance Liquid Chromatography

G. O. Young, D. Ruttenberg, and J. P. Wright

We describe a new "high-performance" liquid chromatographic (HPLC) method for estimating polyethylene glycol 400 (PEG 400) in urine. Direct injection of diluted urine samples onto the column eliminates the necessity for lengthy preparative steps, resulting in considerable saving of both time and chemicals. High analytical recoveries (94–102%) and a rapid rate of analysis are achieved. Results vary linearly with concentrations from 0.2 to 1.6 g/L and the six major components can be individually quantified precisely at all concentrations in this range. This method shows better sample recovery, sensitivity, and reproducibility than does a previously described method. The increased sensitivity should allow the use of lower oral doses of polyethylene glycol 400 in future studies of intestinal permeability.

The amount of polyethylene glycol 400 (PEG 400) recovered in urine after ingestion of a specified dose has been widely used as an index of intestinal permeability in humans (1–3). Methods for quantifying urinary PEG 400 include "high-performance" liquid chromatography (HPLC) and gas–liquid chromatography (4–6). However, these methods require lengthy sample preparation, e.g., adsorption column chromatography and ultrafiltration or lyophilization and extraction with solvent. Such preparative procedures are expensive in terms of time and reagents and do not achieve total recovery of PEG 400. Here we describe a new technique of analysis for urinary PEG 400 by direct-injection HPLC. The results are compared with those obtained by the method of Delahunty and Holland (5), which involves lyophilization, extraction, and HPLC.

Materials and Methods

Samples

Urine was collected from 100 subjects for 6 h after the oral intake of 5.6 g of PEG 400 (lot no. 120F0189; Sigma Chemical Co., St. Louis, MO). Each specimen was thoroughly mixed, the total volume was measured, and 50-mL aliquots were stored at −20 °C without preservative.

Procedure

Direct-injection method. Urine specimens were thawed at room temperature and analyzed in duplicate. We diluted a 2-mL aliquot with 2 mL of de-ionized water (Milli-Q system; Millipore Ltd., Johannesburg, South Africa), mixed thoroughly, then aspirated the sample into a 5-mL disposable syringe and filtered it through a 0.22-μm pore size filter. We injected 250 μL of the filtrate into a chromatograph (Model SP 8800; SpectraPhysics, San Jose, CA) equipped with a 200-μL sample loop (Rheodyne, Cota CA). An isocratic mobile phase (equal volumes of methanol and water) was used to elute the PEG 400 from a 25 cm 4.6 mm (i.d.) column packed with 5-μm-diameter particulate styrene divinylbenzene (Polymer Laboratories, Amherst MA) at a flow rate of 1.0 mL/min. The effluent was analyzed with a refractive index detector (Model SP 8430) linked to a computerized integrator system (Model SP 4290, Both from SpectraPhysics). Standards consisted of PEG 400 dissolved in water to give final concentrations of 0.2, 0.8, and 1.6 g/L, and were subject to a standard curve (total area of all peaks vs PEG 400 concentration) for each analytical run. To determine the analytical recovery, we added PEG 400 to aliquots of pooled normal urine to give final concentrations ranging from 0.2 to 1.6 g/L. Reproducibility was determined by including aliquots of a single urine specimen in 10 analytical runs performed on 10 separate days.

Extraction method. Thawed urine specimens were analyzed according to the method of Delahunty and Holland (5). In this technique a 10-mL urine sample is lyophilized, extracted with 10 mL of chloroform, evaporated to remove the solvent, and then reconstituted before HPLC.

Results

Within 20 min of injection, nine peaks were readily separated and detected in standards, in PEG 400-supplemented urine, and in urine from the subjects studied (Figure 1). On the basis of retention times, these peak corresponded to PEG 400 species with Mr values ranging from 286 to 638 (5). A linear standard curve was obtained for PEG 400 concentrations of 0.2 to 1.6 g/L (Figure 2). The lower limit of detectability of PEG (i.e., the lowest concentration at which all nine peaks could be detected and integrated) was 0.05 g/L.

The six main components eluted constituted about 90

![Fig. 1. Typical HPLC chromatogram of PEG 400 (0.8 g/L)](attachment:image)

Retention times (minutes) are shown for each of the six peaks that were individually analyzed (see Table 1)

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Fig. 2. Standard curve obtained by plotting total area of all peaks (AUC) against concentration of PEG 400. Mean ± SD (10 separate analyses) is shown at each concentration.

The total and could be individually measured with an acceptable degree of analytical precision throughout the range of concentrations (Table 1). The percentage distribution of the six peaks was similar in standards, PEG 400-supplemented urine, and urine from the subjects studied (Figure 3).

The analytical recovery of added PEG 400 was consistently high at concentrations of 0.2–1.6 g/L (Table 2). The standard error of measurement (SEM) between duplicate determinations (calculated as \( \sqrt{\sum w^2/n} \), where \( w \) = difference between two results, and \( n \) is the number of duplicate samples) was 0.87 (\( n = 100 \)). The interassay coefficient of variation (CV) for the entire procedure was 3.3% (\( n = 10 \); mean PEG concentration, 0.96 g/L).

To compare results of the extraction and direct-injection methods, we calculated the urinary excretion of PEG 400 (g/6 h) for samples analyzed by both methods (Figure 4). Although the correlation was good (\( P < 10^{-6} \)), values obtained by direct injection (\( y \)) were generally higher than those obtained with the extraction method (\( x \)): \( y = 0.5x + 0.8 \) g/6 h.

**Discussion**

The method of direct injection described in this paper allows rapid, sensitive, and reproducible analysis for PEG 400 in urine. Twelve samples can be analyzed in duplicate in 8 h. In view of the good reproducibility (CV = 3.3%) and low SEM (0.87%), duplicate analyses may be unnecessary; thus, with the use of an automated sampler, an analysis rate well exceeding 60 samples per day is possible.

**Table 1. Precision of PEG 400 Assay**

<table>
<thead>
<tr>
<th>PEG 400 (mg/L)</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>8.8</td>
<td>8.2</td>
<td>6.4</td>
<td>3.3</td>
<td>6.4</td>
<td>6.7</td>
</tr>
<tr>
<td>0.4</td>
<td>8.5</td>
<td>3.4</td>
<td>4.5</td>
<td>5.2</td>
<td>6.1</td>
<td>6.9</td>
</tr>
<tr>
<td>0.6</td>
<td>3.8</td>
<td>2.7</td>
<td>2.6</td>
<td>6.4</td>
<td>7.2</td>
<td>7.0</td>
</tr>
<tr>
<td>0.8</td>
<td>4.0</td>
<td>3.8</td>
<td>3.0</td>
<td>5.0</td>
<td>4.7</td>
<td>5.0</td>
</tr>
<tr>
<td>1.6</td>
<td>1.3</td>
<td>1.5</td>
<td>1.5</td>
<td>4.9</td>
<td>2.2</td>
<td>1.4</td>
</tr>
</tbody>
</table>

*Peak numbers correspond to the major peaks in Fig. 1. CVs for the peak areas of each individual peak are from six separate assays, performed on six separate days.*

**Table 2. Analytical Recovery of Added PEG 400**

<table>
<thead>
<tr>
<th>PEG 400 added, g/L</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>97.2</td>
<td>3.9</td>
</tr>
<tr>
<td>0.4</td>
<td>94.1</td>
<td>2.7</td>
</tr>
<tr>
<td>0.8</td>
<td>101.8</td>
<td>5.4</td>
</tr>
<tr>
<td>1.6</td>
<td>99.1</td>
<td>5.4</td>
</tr>
</tbody>
</table>

*% recovered. \( n = 9 \) each.

**Fig. 3.** Relative abundance of each \( M_f \) fraction of PEG 400 as determined by HPLC. The three bars for each fraction correspond to PEG standards, PEG-supplemented urines, or urines from subjects (left to right, respectively), mean ± SD.

**Fig. 4.** Comparison of PEG 400 excretion (g/6 h) in 100 subjects as determined by direct-injection and extraction methods.

We found reasonably good correlation between direct-injection and extraction methods but higher values were obtained with the former. The numerous procedures entailed in the extraction method are a potential source of loss (88% recovery) (5). With the new technique, the use of fewer analytical steps results in virtually total recovery. This advantage is also reflected in the better reproducibility than that of the extraction method (CV = 7.4%). The lower limit of detection (0.05 g/L) is similar to that obtained by using gas-chromatographic analysis (4), and considerably more sensitive than the 5 g/L previously reported by those using an extraction technique (5). In addition, the lower volume of urine used in direct-injection analysis means less storage space is needed for samples. The HPLC column has now been in continual use for two years for analyzing PEG 400 in urine samples by both the extraction...
and direct-injection methods. No deterioration in peak resolution or retention time has been apparent during the last nine months, during which the minimum daily load for the direct-injection technique was 20 samples.

In the extraction method previously described, nine peaks were reported to be readily discernible, but individual analysis of the six major peaks included only those peaks that eluted before 10 min (5). In the present study, using both methods, we obtained chromatograms similar to those previously reported (5), but we found that two of the nine peaks (V and VI) had retention times >10 min and contributed a substantial proportion to the total (Figure 1). We therefore included these peaks in the analysis of the individual major peaks and used them in the comparison of the direct-injection and extraction techniques.

In conclusion, increased sensitivity and precision make the direct-injection method suitable for the analysis of urine containing PEG 400 in concentrations as low as 0.05 g/L. This could be an advantage in future permeability studies, allowing use of a substantially lower oral dose of polyethylene glycol.

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References