Journal of Chromatography, 578 (1992) 297–301
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6388

Short Communication

New extraction procedure and high-performance liquid chromatographic method for analyzing polyethylene glycol-400 in urine☆

Harvey A. Schwertner, Wayne R. Patterson and John H. Cissik

Clinical Investigations Directorate, Wilford Hall USAF Medical Center, Lackland AFB, TX 78236-5300 (USA)

Kathleen W. Wilson

Department of Gastroenterology, Wilford Hall USAF Medical Center, Lackland AFB, TX 78236-5300 (USA)

(First received November 12th, 1991; revised manuscript received March 16th, 1992)

ABSTRACT

We describe a new, highly efficient method for extracting polyethylene glycol-400 from urine and for its analysis by isocratic reversed-phase high-performance liquid chromatography. This method is an improvement over previously published methods in that it does not require the use of ion-exchange resins and lyophilization prior to extraction, nor does it require the separation and analysis of the individual polymers of polyethylene glycol. The procedure described in this report entails extraction with a salt–solvent combination of ammonium sulfate and dichloromethane and analysis by reversed-phase high-performance liquid chromatography. The lower limit of detection was approximately 0.25 g/l with a 2-ml urine sample. Analytical recoveries of polyethylene glycol-400 added to urine at 2.5 and 5.0 g/l averaged 97 and 96%, respectively ($n = 10$). Within- and between-day coefficients of variation were less than 5% at 2.5 and 5.0 g/l. Studies of various urine samples from patients receiving polyethylene glycol-400 revealed no interferences from other urine substances.

INTRODUCTION

Polyethylene glycol-400 (PEG-400) is widely used by gastroenterologists as a probe to assess intestinal permeability [1–3]. It has distinct advantages over other compounds such as mannitol, urea [4], xylose [5], and creatinine [6], in that it is not metabolized by intestinal bacteria. Other advantages include its high solubility in water, its rapid and quantitative excretion in urine, and its non-toxicity [1–3].

A number of gas chromatographic (GC) [1, 7–9] and high-performance liquid chromatographic (HPLC) methods [3,10] have been developed for the analysis of PEG-400 in urine. All of

Correspondence to: Dr. Harvey A. Schwertner, Clinical Investigations Directorate, Wilford Hall USAF Medical Center/SGS, Lackland AFB, TX 78236-5300, USA.

☆ The views expressed in this article are those of the authors and do not reflect the official policy of the Department of Defense or other Departments of the US Government.

0378-4347/92/$05.00

the GC and HPLC methods rely on lyophilization of the urine samples and subsequent extraction of the dried product [1,3,7–10]. In addition, the GC methods employ extensive sample pretreatment with ion-exchange resins to remove unwanted substances. Most of the GC methods also require derivatization of the ethylene glycols prior to GC analysis [7,8].

In this report we describe a new method employing a novel salt–solvent combination to enhance the extraction of PEG-400 from urine followed by HPLC with a reversed-phase column. The column is very stable and permits the analysis of the PEG-400 polymers as a single peak. The described analytical method for PEG-400 with its ease of extraction, small sample size, and high-percentage recoveries provides a suitable HPLC method for measuring PEG-400 in urine samples.

EXPERIMENTAL

Reagents and chemicals

Dichloromethane (methylene chloride, DX0831-1 glass-distilled Omni Solv) was obtained from E. M. Science (Gibbstown, NJ, USA); ammonium sulfate (A-702) was from Fisher Scientific (Fairlawn, NJ, USA) and PEG-600 and 1000 from Sigma (St. Louis, MO, USA).

Standard and control sample preparation

Calibration standards (2.5, 5.0 and 10.0 g/l) were prepared by dissolving PEG-400 (Cat. No. P3265, Sigma) in the methanol–water mobile phase (50:50, v/v). Urine-based standards (2.5 and 5.0 g/l) were prepared by dissolving PEG-400 in freshly collected urine. Both standards were stored at 4°C until used.

Extraction

Urine (2 ml) was pipetted into separate 125 × 16 mm glass screw-capped tubes. A sufficient amount of ammonium sulfate was added to each tube with a spatula and funnel to completely saturate the sample (approximately 2.5 g). The samples were vortex-mixed for 20 s and then extracted with 10 ml of dichloromethane for 15 min on an Eberbach shaker. The tubes were then centrifuged at 2000 *g* for 10 min at 25°C to separate the phases. The upper aqueous phase was aspirated and the organic solvent was decanted into separate 100 × 16 mm disposable glass tubes. An 8-ml volume of the dichloromethane phase was transferred with a volumetric pipette to separate disposable glass tubes or scintillation vials. Evaporation was performed under nitrogen or air at 50°C, then a 2-ml aliquot of the methanol–water mobile phase was added to reconstitute the PEG-400. The reconstituted samples were transferred with a Pasteur pipette to the 4-ml injection vials.

Instrumentation and chromatographic conditions

The HPLC system consisted of a Waters 700 Satellite WISP, a 600E system controller, 410 differential refractometer, and a Maxima 825 chromatography workstation (all from Waters Chromatography Division of Millipore, Milford, MA, USA). Chromatographic analyses were performed on a Waters μBondapak C_{18} reversed-phase column, 30 cm × 3.9 mm I.D., particle size 10 μm, at a flow-rate of 1-ml/min at ambient temperature. Of each sample 200 μl were injected and the PEG-400 concentrations were quantified by comparing the peak areas of the urine samples to those obtained with the external PEG calibration standards. The refractometer was operated at 40°C.

Application of the method

Urine samples (6 h) were collected from patients after an oral intake of 5.6 g of PEG-400. The volume of each sample was measured and aliquots were stored at −20°C until analyzed. The human studies were approved by the institutional human research review committee and informed consent was obtained.

RESULTS

Development of the extraction method and recoveries from urine

We tested the solubility of PEG-400 in dichloromethane and found it to be readily soluble (>

20 g/l). We then extracted urine samples containing 2.5 and 5.0 g/l PEG-400 and found the recoveries to be 22 and 23%, respectively. To further improve the recoveries, we added 2 ml of saturated ammonium sulfate to each 2-ml urine sample. The addition of ammonium sulfate increased the recoveries to approximately 65%. The urine samples were then saturated by adding ammonium sulfate crystals directly to the urine samples. This led to recoveries of 97 and 96% for samples containing 2.5 and 5.0 g/l (n = 10 at each concentration).

Mobile phase

We tested both methanol–water (50:50, v/v) and methanol as possible mobile phases. Methanol–water (50:50, v/v) was selected as the mobile phase since it more effectively resolved co-extractables from PEG-400. Methanol, however, produced sharper peaks especially for PEG-600 and PEG-1000. In addition, we tested a variety of mobile phases that can be used to separate the individual PEG-400 polymers if such separation is desired. Methanol–water (25:75, v/v) resolved PEG-400 into at least nine peaks.

Linearity, limit of detection, and selectivity

Standard curves were found to be linear to at least 10 g/l. The limit of detection was estimated to be approximately 0.25 g/l using 2 ml of urine and injecting 200 μl of the 2-ml sample. No efforts were made to further increase the sensitivities since they were more than adequate for our purposes.

Chromatograms showing PEG-400 extracted from spiked urine samples and from patients who had taken oral doses of PEG-400 are depicted in Fig. 1. We did notice a small peak with a retention time of 3.6 min in some of the patient samples, however, the peak did not interfere with the quantification of PEG since its retention time was different from that of PEG-400. This peak was small and more obvious when analyzing PEG-400 concentrations below 1 g/l as shown in Fig. 1. Washes of the dichloromethane phase with saturated ammonium sulfate did not eliminate the peak. Its presence appeared to be related more to its concentration in urine than to how carefully we removed the top aqueous phase. Urine samples collected from ten different individuals who had not taken PEG-400 were all free of peaks in the PEG-400 region (Fig. 1).

Precision

Within- and between-day assay coefficients of variation (C.V.s) were determined by analyzing PEG-400-based urine standards. The within-day C.V. was 2.1% with the 2.5 g/l samples and 1.3% with the 5.0 g/l samples. Between-day assay C.V.s were 4.6 and 4.4% for the 2.5 and 5.0 g/l samples (n = 10). Analyses were performed using single injections of each sample and with calibration curves that were prepared with each analysis.

Analysis of patient samples

Urine samples from seventeen patients receiving 5.6 g of PEG-400 were analyzed on two separate days and the results were within 2.5% of

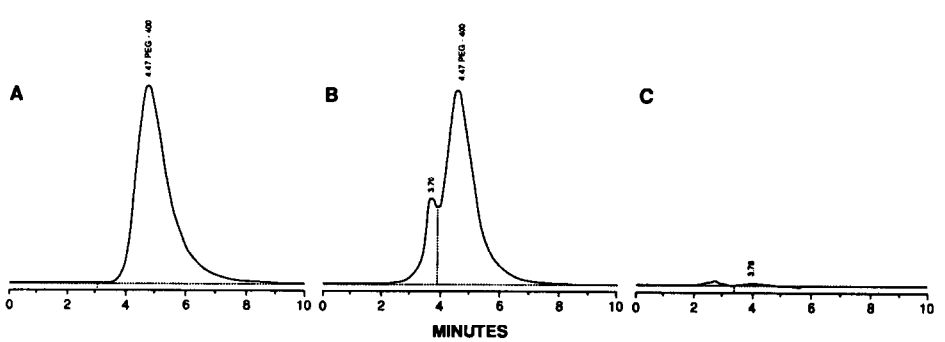

Fig. 1. Chromatograms obtained from extracts of spiked urine (A), urine of patients receiving PEG-400 (B) and urine from patients not receiving PEG-400 (C). Chromatograms A and B were normalized to full scale.

each other (r = 0.999). The 6-h urine samples contained approximately 1405 mg or 25.1% of the original dose of 5.6 g. We compared the PEG-400 concentrations obtained with this procedure to those obtained with the procedure using lyophilization and chloroform extraction [10]. The mean concentration using our method was 2.06 g/l for twelve patient samples, whereas the PEG concentration obtained with the lyophilization and chloroform extraction procedure [10] was 0.32 g/l. The PEG concentrations obtained with the two methods were not found to be related (r = 0.192). In studies involving a larger population (n = 27), the 6-h recovery of PEG-400 was 1416 mg or 25.2% of the administered dose. The urinary PEG-400 concentration was 3.39 g/l.

The chromatograms of the PEG standards were found to superimpose those obtained from patients receiving PEG-400, thereby indicating that the molecular distribution of the various polymers remained unchanged after oral intake. The retention times of PEG in the patient samples were also identical to the urine standards containing PEG-400. Both findings support the view that individual polymers do not need to be analyzed. We also analyzed PEG-600 and PEG-1000. Their retention times were 5.16 and 7.75 min, respectively. They cannot be used as an internal standard since they were not sufficiently resolved from PEG-400 under our chromatographic conditions. They, however, can be analyzed by this method or by methods employing a modification of the method.

DISCUSSION

In this report, we describe a new simple method for extracting PEG-400 from urine and for its analysis by HPLC. This method uses a salt–solvent combination of ammonium sulfate and dichloromethane to enhance the extraction of the highly water-soluble PEG-400 polymers. All of the previously published gas and liquid chromatographic methods employed time-consuming lyophilization steps followed by extraction of the lyophilized product [1,7–10]. Such lyophilization procedures typically take 20–24 h to complete. The procedures also require lyophilization equipment and such equipment is not available in most laboratories. Most importantly, our study contradicts the widely held view that "the lyophilization step is essential because a biphasic extraction will not recover the water-soluble PEG in the organic phase" [10].

The method described in this paper has certain advantages over the GC methods used for analyzing PEG-400 [1,7–9]. The GC methods, for example, require extensive sample clean-up and the use of ion-exchange resins to remove interfering substances [1,7–9]. Many also require complex derivatization steps and extraction of the derivatives [7,8]. Derivative stability is frequently a problem [1,7,8], as is column stability [7,8]. In addition, the GC analyses take 50–60 min to complete [7,8]. With our HPLC method, the extraction procedure is simple, derivatization is not required, the reversed-phase column is very stable, and the chromatographic analysis can be completed in less than 10 min.

In this study, we found that the average retention time of the polymers from patients receiving PEG-400 were identical to those of the standards. This result is similar to those obtained by others [3,10] and suggests that the individual polymers do not have to be resolved by molecular exclusion chromatography or by GC. If analysis of the individual polymers is desired, they can be readily analyzed by this chromatographic procedure by using methanol–water (25:75, v/v) as a mobile phase.

We compared the results obtained with our HPLC method to those obtained with a previously published HPLC procedure [10]. With our method, the average 6-h output of PEG-400 after an oral administration of 5.6 g was 1405 mg or 25.1% of the administered dose. When we lyophilized the urine samples and extracted them with chloroform [10], the 6-h output was 368 mg or about 6.6% of the administered dose. The 6.6% recovery of administered dose that we obtained with lyophilization and chloroform extraction is somewhat similar to the 2.9% recovery claimed by the authors [10]; however, both

results are very low compared to the 25% recovery achieved with our extraction method or the 23% recoveries reported by several others using GC methods [1,9]. We do not know why the recoveries are so different in the two methods, however, it could be the manner in which the lyophilized product is extracted. In the GC procedures, acetone [1,7] or water [9] was used to extract PEG from the lyophilized product, whereas, in the HPLC method, chloroform was used [10]. We do want to reiterate that the recoveries mentioned in this paragraph pertain to recoveries of administered dose and not to recoveries of PEG-400 from urine. With our procedure the recoveries from urine were 96–97% when ammonium sulfate was added prior to extraction. Without ammonium sulfate, recoveries of PEG-400 from urine were 22–23%.

Based on the above findings, we feel that our extraction and analytical method has distinct advantages over those used in previously published procedures. We also feel that the extraction method described here can be successfully used in the GC methods for PEG-400 analysis.

ACKNOWLEDGEMENTS

The assistance of Velma Grantham and Lou Ann Caywood in the preparation and review of this manuscript is appreciated.

REFERENCES

1 V. S. Chadwick, S. F. Phillips and A. F. Hofmann, *Gastroenterology*, 73 (1977) 241.
2 V. S. Chadwick, S. F. Phillips and A. F. Hofmann, *Gastroenterology*, 73 (1977) 247.
3 D. Holland, C. M. Vadheim, E. Brettholz, G. M. Petersen, T. Delahunty and J. I. Rotter, *Ann. Intern. Med.*, 105 (1986) 833.
4 J. S. Fordtran, F. C. Rector, M. F. Ewton, N. Soter and J. Kinney, *J. Clin. Invest.*, 44 (1965) 1935.
5 I. S. Menzies, *Gut*, 13 (1972) 847.
6 C. A. Loehry, J. Kingham and J. Baker, *Gut*, 14 (1973) 683.
7 J. B. Bouske and S. F. Phillips, *J. Chromatogr.*, 183 (1980) 72.
8 T. Sivakumaran, R. T. Jenkins, W. H. C. Walker and R. L. Goodacre, *Clin. Chem.*, 28 (1982) 2452.
9 H. J. McClung, P. A. Powers, H. R. Sloan and B. Kerzner, *Clin. Chim. Acta*, 134 (1983) 245.
10 T. Delahunty and T. Hollander, *Clin. Chem.*, 32 (1986) 351.