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High-performance liquid chromatographic determination of PEG 600 in human urine

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ABSTRACT

Polyethylene glycols (PEGs) are non-ionic, water-soluble synthetic polymers which have been widely used for many applications. Since they are of very low toxicity and are readily excreted in urine, PEGs in the molecular weight range 400–6000 have been used extensively in the study of intestinal physiology in man. A high-performance liquid chromatographic (HPLC) method has been developed for the determination of PEG 600 in human urine, which includes a pre-column derivatisation step. The dibenzoate derivatives of PEG 600 can be quantitatively prepared, and this, coupled with ultraviolet detection at 230 nm, has greatly improved the limit of detection for the determination of PEGs by HPLC. A suitable extraction procedure has also been developed which enabled PEG levels in urine to be monitored with much greater sensitivity than any previously reported method.

INTRODUCTION

Since they were first introduced as the 'ideal' intestinal permeability probe for man [1], polyethylene glycols (PEGs) in the molecular weight range 400–6000 have been used extensively in the study of intestinal physiology. PEG has also been widely used as a food additive and in ointment bases. Only recently, however, has its toxicity been considered [2,3], and in 1982, the Food and Drug Administration [4] reported that a PEG-containing burn ointment in humans caused a syndrome of renal failure, elevated serum calcium and serum osmolality, and metabolic acidosis. The safety of using large doses of PEG 4000 in colonic lavage preparations has also been questioned [5].

For these reasons, a convenient and sensitive method for quantifying PEGs in biological fluids, and particularly in urine, is desirable. The earlier classical methods for the determination of PEGs included many colorimetric [6,7] and turbidimetric [8,9] procedures. Although these methods had low limits of detection, the analysis times were long and the methods were subject to many interferences. Titrimetric procedures [10] gave accurate and reproducible results, but lacked sensitivity. Paper [11] and thin-layer [12] chromatography have also been extensively used for the qualitative analysis of PEGs.

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Since the mid-1970s, these classical techniques have been supplemented by the faster and more reproducible column chromatographic techniques of gas chromatography (GC), gel permeation chromatography (GPC) and high-performance liquid chromatography (HPLC). With PEGs of molecular weights of 500 or less, GC can be used [1,13], but this method cannot readily be used with higher molecular weight polymers. The use of GPC for quantitating PEGs 4000 and 3350, respectively, in human urine has also been described [14,15].

For the characterisation and analysis of low-to-moderate molecular weight PEGs, HPLC is the method of choice. For PEG concentrations $>0.1\%$ (w/v), refractive index (RI) detection is satisfactory, and to date all the HPLC methods reported in the literature for the analysis of PEGs in urine have used RI detection [16,17]. PEGs have also been detected by UV absorption at wavelengths <200 nm, but even at 185 nm [18] the sensitivity is only comparable to that obtained with an RI detector.

The introduction of a UV-absorbing group can greatly improve the limits of detection for the determination of PEGs by HPLC, and three classes of PEG derivatives have been mentioned in the literature. The 3,5-dinitrobenzoate derivatives were prepared by Carey and Persinger [19] and later by Cassidy [20]. The bis(2,4-dinitrophenyl) derivatives [21] gave rise to good sensitivity but could not be prepared quantitatively. The most suitable derivatives for use in quantitative analysis were the dibenzoates reported by Murphy *et al.* [22]. The dibenzoate derivatives of PEGs 400, 1500 and 4000 were prepared by heating a sample of the PEG with benzoic anhydride and triethylamine for 60 min and the limit of detection of the method was in the range 5–10 nmol.

In this paper, a simpler and more rapid procedure, using benzoyl chloride and pyridine for the preparation of the PEG dibenzoate derivatives, is presented, and a suitable extraction procedure for the analysis of PEG 600 in urine is described.

EXPERIMENTAL

Materials

PEG 600 was obtained from Aldrich (Dorset, U.K.). Mobile phases were prepared with HPLC grade methanol and acetonitrile from Labscan Chemicals (Dublin, Ireland) and with water obtained by passing distilled water through a Milli-Q water purification system. All mobile phases were filtered through a 0.45-μm membrane filter and degassed in an ultrasonic bath.

Benzoyl chloride, perchloric acid, sodium chloride and dichloromethane were all analytical grade and were supplied by Riedel-de Haën (Seelze, Germany). Analytical reagent grade hydrochloric acid and pyridine were purchased from BDH (Poole, U.K.). The pyridine was distilled over sodium hydroxide prior to use.

Apparatus

The chromatographic system consisted of a Waters 501 HPLC pump and a Rheodyne 7125 injection valve (fitted with a 20-μl loop). For gradient work a second Waters M6000A HPLC pump and a Waters M660 solvent programmer were used. Column eluent was monitored with either a Shimadzu SPD-6A UV spectrophotometric detector or a Waters 990 photodiode-array detector. Detector output was monitored with a Phillips linear chart recorder or with a Waters 990 plotter. Two reversed-phase columns were used: a Nucleosil 10 C_{18} column (10 μm particle size) for isocratic elution and a Spherisorb 10 ODS column (10 μm particle size) for gradient programming. Both were 25 cm × 4.6 mm columns and were purchased from HPLC Technology (Wilmslow, U.K.).

Derivatisation procedure

The quantities of reagents given in the following procedure are sufficient to derivatise up to 20 mg of PEG 600 quantitatively. However, the amount of PEG to be derivatised should normally be in the range 0–2.5 mg. The sample of PEG 600 to be derivatised is introduced into a clean dry test-tube as a solution in a volatile solvent, typically chloroform or dichloromethane. The solvent is evaporated under a stream of nitrogen at 60°C and the residue dissolved in 100 μl of dry pyridine. After addition of 20 μl of benzoyl chloride, swirl gently to mix and allow the mixture to stand at room temperature for 15 min. Add 50 μl of freshly prepared 4 *M* perchloric acid, mix thoroughly and heat at 60°C for 15 min. Allow to cool and add 400 μl of a 25% (v/v) hydrochloric acid solution followed by 2.0 ml of water. Extract with 5.0 ml of dichloromethane by vortexing for about 10 s. Allow the two layers to separate and inject a 20-μl quantity of the dichloromethane layer into the chromatograph.

Chromatographic conditions

Analysis of the total concentration of PEG 600 can be accomplished isocratically on a Nucleosil 10 C_{18} column using a methanol–water (75:25) mobile phase at a flow-rate of 1.5 ml/min. Under these conditions PEG 600 is eluted as a single sharp peak at a retention time of about 5 min. A detection wavelength of 230 nm gives optimum sensitivity.

Separation of PEG 600 into its component oligomers can be accomplished on a Spherisorb 10 ODS column using a linear solvent gradient from acetonitrile–water (40:60) to 100% acetonitrile over 17 min at a flow-rate of 2.0 ml/min. Under these conditions, up to thirteen oligomers have been resolved, using a detection wavelength of 270 nm.

Extraction of PEG 600 from urine

To a 1.0-ml volume of urine, add 0.0584 g of sodium chloride and 3.0 ml of chloroform. Carry out the extraction overnight using a blood suspension mixer and then carefully remove 2.0 ml of the chloroform layer with a pipette. Transfer

this extract to a clean, dry test-tube making sure that no traces of urine are present, and evaporate to dryness under a stream of nitrogen at 60°C. The residue obtained can then be derivatised according to the procedure outlined above.

RESULTS AND DISCUSSION

Optimisation of the derivatisation procedure

The overall reaction scheme for the reaction of PEG with benzoyl chloride in the presence of pyridine is shown in Fig. 1. The benzoylation of PEG 600 was studied in order to determine the optimum conditions for the reaction, and also to determine if any side-reactions were occurring under these conditions. Following reaction at room temperature, the reaction mixture was extracted into dichloromethane and analysed by HPLC. In addition to the PEG dibenzoate derivative, the extract was found to contain benzoic acid, benzoic anhydride and pyridine. The benzoic anhydride was readily converted to benzoic acid by treating the reaction mixture with perchloric acid, and the pyridine was removed by adjusting the pH to <2.5 before extraction. Under reversed-phase conditions, benzoic acid did not interfere with the analysis of PEG 600 because it was eluted close to the solvent front.

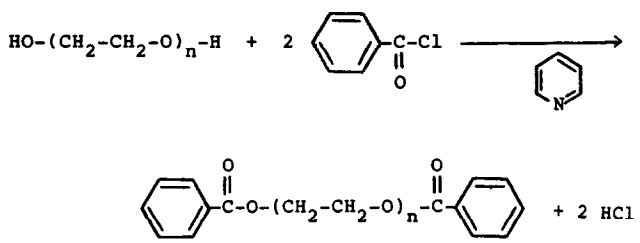

Fig. 1. Reaction scheme for the benzoylation of PEG 600.

Optimisation of the separation

The benzoyl chloride–pyridine derivatisation procedure gave a much cleaner extract to be chromatographed than the benzoic anhydride–triethylamine reaction reported by Murphy *et al.* [22]. This meant that gradient elution was not required to separate the PEG dibenzoate peak from early-eluting reaction side-products. Under isocratic conditions with methanol–water (75:25) as the mobile phase, the PEG dibenzoate oligomers were eluted as a single sharp peak, well resolved from all other components in the chromatogram (Fig. 2). These conditions also gave rise to an excellent sensitivity for the analysis of total PEG 600 in a shorter analysis time than is obtainable with gradient elution.

When the separation of the component PEG oligomers is required, gradient

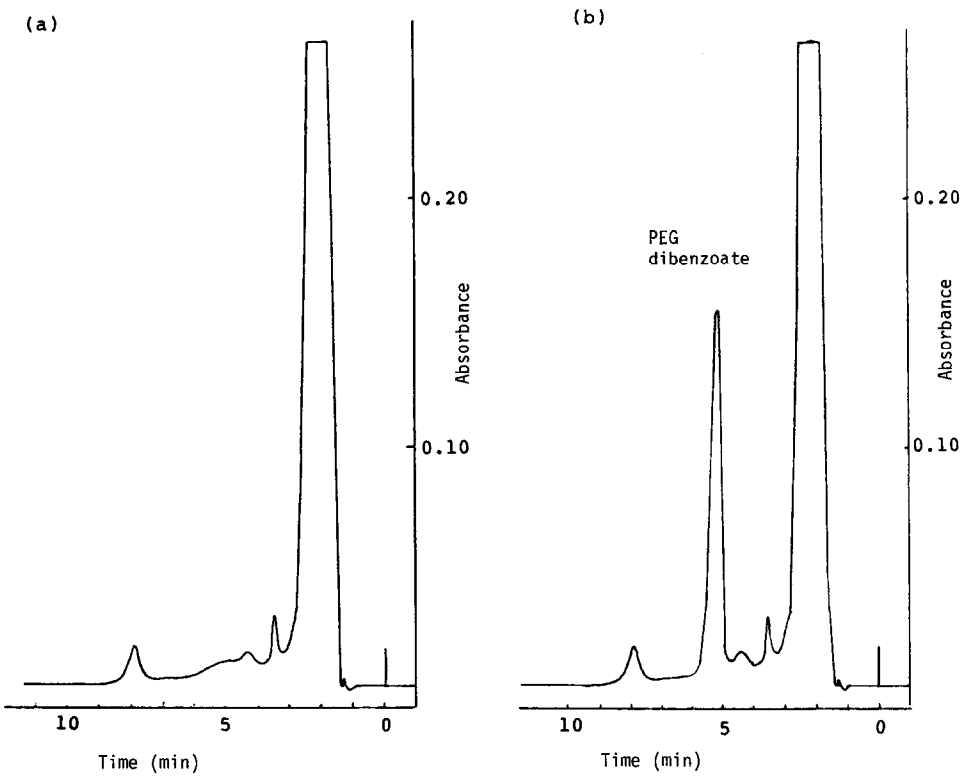

Fig. 2. HPLC under isocratic conditions of (a) blank derivatisation mixture and (b) a 50 μg/ml PEG 600 standard.

elution can be used. Fig. 3 shows the separation of thirteen PEG 600 oligomers in an analysis time of <20 min, using a linear acetonitrile–water gradient on a 10-μm Spherisorb ODS column. Better resolution of these oligomers could be achieved on a more efficient 5-μm column and/or by using a lower initial acetonitrile concentration and a longer gradient time. It was found that overall an acetonitrile–water gradient gave better separation of the oligomers than a methanol–water gradient.

Optimisation of the detection wavelength

A photodiode-array detector was used to check the integrity of the PEG dibenzoate peak and to optimise the wavelength of detection.

The UV–visible spectrum in the range 190–350 nm was recorded for a 100-ppm PEG 600 standard derivatised according to the procedure developed. This was then compared with the UV–visible spectrum of a solution of pure PEG dibenzoate, which had already been characterised by infrared and nuclear magnetic resonance spectroscopy, and which had the same retention time under the

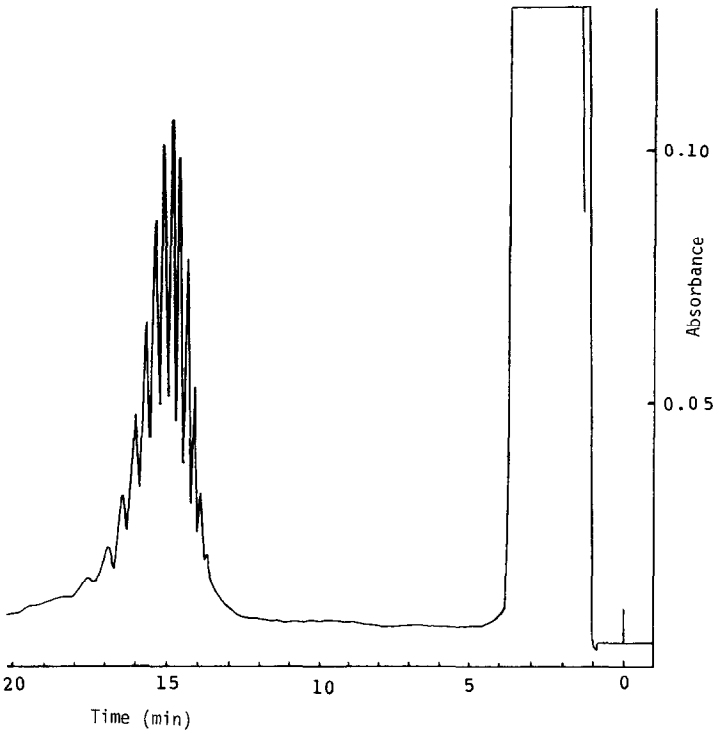

Fig. 3. Gradient elution of a 1000 μg/ml PEG 600 standard.

same chromatographic conditions. The spectra obtained were nearly identical, thus giving strong confirmation of the identity of the PEG dibenzoate peak.

The UV–visible spectrum of PEG dibenzoate exhibits two absorbance maxima at 230 and 270 nm. The absorbance at 230 nm is far stronger than that at 270 nm and therefore this is the wavelength to use for optimum sensitivity. This was confirmed using the chromatogram analysis facility on the photodiode-array detector. The chromatograms which would be obtained for a 100-ppm PEG 600 standard at different detection wavelengths can be compared in Fig. 4.

Under isocratic conditions, detection at 230 nm is possible and no interferences from solvents effects are observed in the chromatogram. However, with the acetonitrile–water gradient conditions used for the separation of the component PEG 600 oligomers, serious baseline drift was observed at 230 nm. This was caused by the increase in acetonitrile concentration of the mobile phase during gradient elution, since acetonitrile absorbs considerably more strongly at 230 nm than does water.

This problem can be overcome by detecting the eluent at 270 nm. Although the sensitivity is reduced compared with that obtainable at 230 nm, the baseline drift is much less, and in practice the limit of detection was found to be better than that

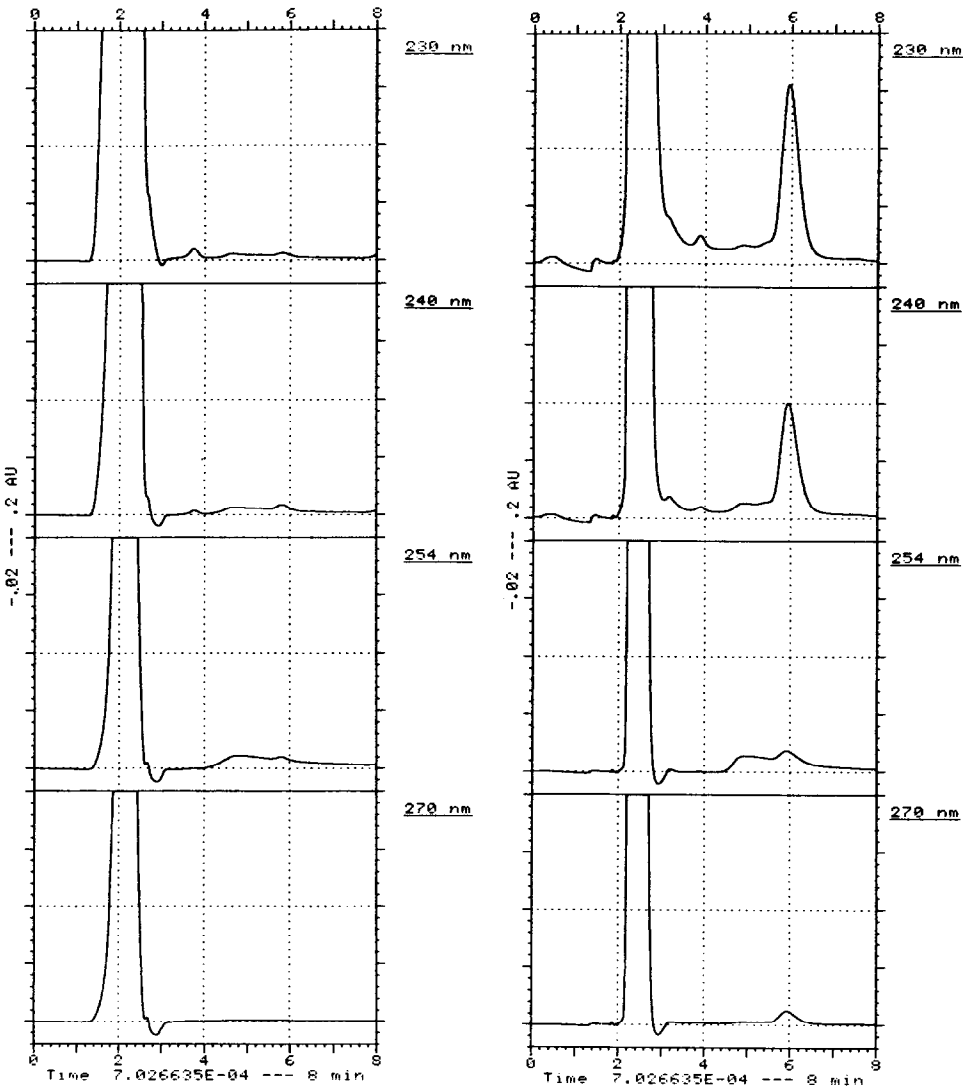

Fig. 4. Chromatographic analysis at various detection wavelengths for (a) blank derivatisation mixture and (b) a 100 μg/ml PEG 600 standard.

obtainable at 230 nm. To compensate for the reduced sensitivity, an increased injection volume can be used. Up to 100 μl of sample has been injected onto the column under gradient conditions without causing any problems of column overload.

Evaluation of peak homogeneity

The ability of the photodiode-array detector to record the full spectrum of an eluent can be of great assistance in detecting co-eluting peaks. A number of different approaches were used to evaluate peak homogeneity. One involved viewing a three-dimensional plot of the PEG dibenzoate peak under multiple viewing angles. No signs of any co-eluting compounds were observed. The peak was also viewed as a two-dimensional contour plot and the contour lines were found to be perfectly symmetrical. Skewed contour lines would have indicated co-elution.

Validation of the method

The main criteria used for validating an HPLC method include selectivity, linearity, sensitivity, limit of detection, limit of determination and precision.

The selectivity of the method was assessed by injecting a blank sample. As shown in Fig. 2a, there were only minor interfering peaks present in the blank derivatisation mixture injected under the same chromatographic conditions as a 50-ppm derivatised PEG standard.

The linearity of the relationship between response (y) and concentration injected (x) was tested over the likely range of study. The linearity of the calibration equation was checked visually and the least-squares method was used to determine the regression equation. The confidence limits on the intercept were also calculated and the calibration graph was found to go close to or through the origin. A typical calibration graph in the range 0–50 ppm PEG 600 gave rise to a linear relation expressed by the equation $A = 3.0 \cdot 10^{-3}$ ppm PEG 600 with a correlation coefficient (r) of 0.9998.

The limit of detection is generally defined by the mass of analyte which produces a signal-to-noise ratio of 2 or 3. It was determined experimentally for PEG 600 to be 1 μg when an injection volume of 20 μl was used.

The sensitivity is the ratio of the signal to mass of the analyte. It is given by the slope of the calibration graph and it was found to be $3.0 \cdot 10^{-6}$ A.U. per ng of PEG 600.

The repeatability of the procedure was assessed by replicate successive injection of a standard solution at different levels of concentration. The coefficients of variation were found to be 0.93 and 1.27% at the 20 and 100 ppm concentration levels, respectively.

The stability of the PEG dibenzoate derivatives in solution was tested to determine for how long the solutions could be kept before analysis. It was found that solutions prepared in dichloromethane were stable for a number of days if stored at 40°C. If the solutions were stored at ambient temperatures for more than a few hours, an increase in concentration was observed due to evaporation of the solvent.

HPLC determination of PEG 600 in urine

In order to use the derivatisation and HPLC procedure developed to analyse for PEG 600 in urine, a suitable extraction procedure was required. The benzoylation reaction required an extraction step which would yield a dry residue of PEG 600 for derivatisation. A liquid–liquid extraction with chloroform was tried. This was initially based on the method reported by Clark *et al.* [15] in which 1.0 ml of urine was extracted with 2.0 ml chloroform. A range of spiked aqueous and urine samples were extracted for 1 h. A recovery of 77 ± 5% was obtained for the aqueous samples and a plot of peak height *versus* concentration was linear. However, the results obtained for the urine samples showed much lower and non-reproducible recoveries.

The reason for the poor extraction of PEG 600 from urine was thought to be the presence of trace amounts of urine in the chloroform extracts. To eliminate this source of error, an extraction was carried out using 3.0 ml of chloroform to extract 1.0 ml of spiked urine, but instead of removing the supernatant and evaporating all of the chloroform extract, a 2.0-ml volume of the chloroform layer was carefully removed with a pipette and transferred to a clean, dry test-tube. It was then evaporated to dryness and the residue obtained was derivatised as per usual. This gave a much more reproducible recovery of PEG 600 from urine.

The effect of salt concentration on the efficiency of extraction for a 250-ppm solution of PEG 600 was studied, and a "salting out" effect was observed. The optimum salt concentration for the extraction of PEG 600 from urine was determined and it can be seen in Fig. 5 that the best recovery of PEG 600 was attained at a sodium chloride concentration of 1.0 *M*.

A range of spiked urine samples (1.0 ml) containing 50–250 ppm PEG 600

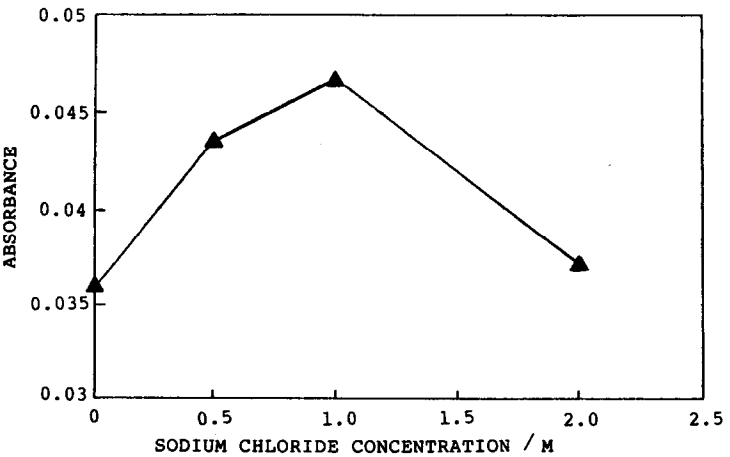

Fig. 5. Effect of sodium chloride concentration on the extraction of 250 μg/ml PEG 600 from urine.

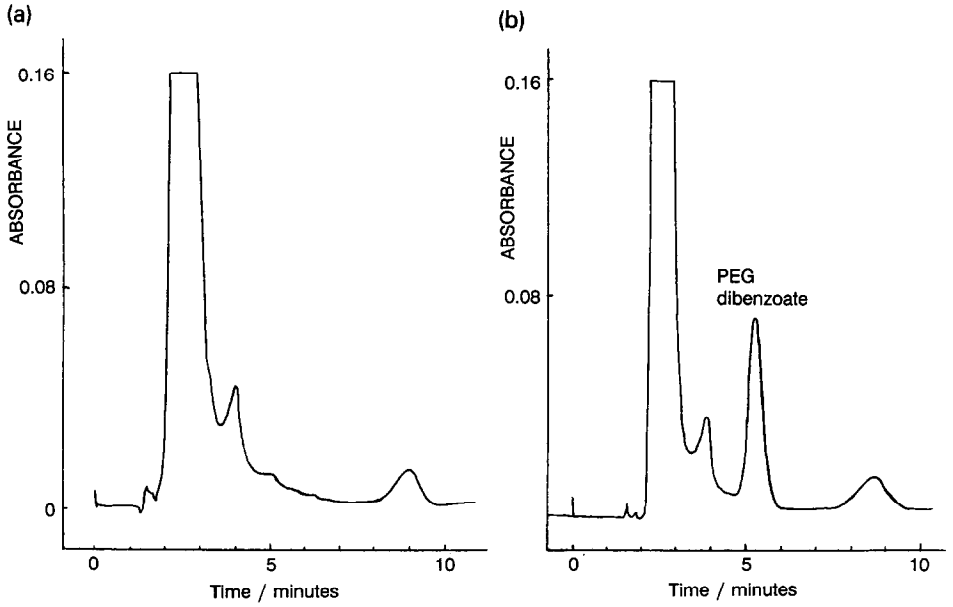

Fig. 6. HPLC of (a) blank urine sample and (b) urine sample spiked with 200 μg/ml PEG 600.

were then extracted into 3.0 ml of chloroform in the presence of 1.0 *M* sodium chloride. Each extraction was carried out in duplicate and the recovery of PEG 600 was 82 ± 2%. Only minor interfering peaks were observed in the chromatograms obtained for the extracted urine samples (Fig. 6), and the calibration graph in the range 50–250 ppm PEG 600 was linear with a correlation coefficient of 0.9997. For the determination of PEG 600 in urine, the limit of detection and the limit of determination were found to be 7 and 20 μg, respectively, per 1 ml of urine.

CONCLUSION

A convenient and sensitive HPLC method for quantifying PEG 600 in urine has been developed. The method included a pre-column derivatisation step in which the dibenzoate derivatives of PEG 600 were quantitatively prepared in a considerably shorter reaction time than previously reported. The procedure also gave a cleaner extract to be chromatographed resulting in a shorter analysis time for the determination of total PEG 600. In addition, the method could be used to measure the relative concentrations of individual oligomers if gradient elution was employed. Overall, PEG urine levels could be followed using this analytical technique with much greater sensitivity than previously reported methods. This would be particularly useful in intestinal permeability studies where, because of the increased sensitivity of the method, lower doses could be administered. It

could also be applied to plasma samples with perhaps some modification of the extraction procedure, and this would be useful in studies assessing the toxicity of PEGs in humans.

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