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Analysis and purification of modified methoxy(polyethylene glycol) compounds of similar molecular mass by high-performance liquid chromatography

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Abstract

The chromatographic analysis of monomethoxy(polyethylene glycol) (MPEG) derivatives, with an average molecular mass of 5000, and two related PEG compounds is described. The derivatives, containing neutral, basic, and acidic groups, were separated by HPLC using a cyanopropyl-bonded analytical column with UV detection. Collected chromatographic bands were identified using ^{1}H NMR. MPEG-amine 2 was also derivatized to form the corresponding MPEG-semicarbazide 5B labeled with ^{14}C to allow for sample quantification. A two-step purification method was devised to increase the radiochemical purity of crude 5B from 50% to over 95%.

1. Introduction

There has recently been a renewed interest in poly(ethylene glycol) (PEG) compounds and their derivatives for modifying the physical properties of drug candidates. When chemically linked to a protein, PEGs can have a mediating effect on the characteristics of the protein to which it is bound, while having little biological effect themselves [1]. PEGs have been shown to slow the rate of drug release and to reduce or control the antigenicity of immunogenic proteins [1–4]. To be useful in pharmaceutical applications, the starting PEG compound must be of high purity and well characterized. Numerous analytical methods have been reported for the analysis of these compounds including reversed-phase HPLC [1,5–9], high-temperature HPLC [10], size-exclusion chromatography (SEC) [2–4,7,11–13], HPLC with evaporative light scattering detection (ELSD) [14,15], thin-layer chromatography (TLC) [12], and supercritical fluid chromatography (SFC) [10]. Previously reported methods have been successful in resolving PEG compounds from non-PEG related compounds [5], in determining molecular mass distributions of a single PEG compound [3,10,14,15], and in resolving MPEG compounds that had different molecular mass distributions [3,7,10,11,13]. However, to our knowledge, there have been no reports on the separation of PEG compounds with the same molecular mass distribution and differing only by a single functional group.

We now report a method for the evaluation of PEG related compounds that is selective based on the terminating functional group of the glycol chain. Separation was accomplished using a cyanopropyl column with a sodium perchlorate–

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acetonitrile gradient. The compounds, consisting of distributions in the 3400 and 5000 molecular mass ranges, chromatographed as broad bands which were difficult to quantify by UV detection because of the lack of a suitable chromophore. In spite of low UV sensitivity and the poor peak shapes observed with these compounds, the method is useful for determining the presence of non-PEG impurities as well as for detecting the presence of other PEG related compounds. Factors affecting the separation, including sample pH, buffer pH, salt concentration, organic concentration, and temperature, have been investigated.

2. Experimental

2.1. Equipment

Analytical chromatography was performed with a Waters Millennium 2010 HPLC system consisting of a Model 600E system controller, a Model 996 diode-array detector and a Model 717 automatic injector. Wavelengths of 192 and 195 nm with a setting of 1.0 AUFS were used for UV detection. Sample injection volumes varied from 5 to 50 μl. The 150 × 4.6 mm I.D., 5 μm Zorbax cyanopropyl column was purchased from MAC-MOD (Chadds Ford, PA, USA).

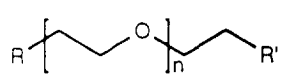
Analytical chromatography of radioactive materials was performed with a Hewlett-Packard Model 1090 M liquid chromatograph with Chemstation software. A wavelength of 192 nm was used for detection, with an injection volume of 25 μl. Radiochemical detection was performed with a Radiomatic A250 detector. ^{1}H NMR spectra were recorded on a Bruker AC-300 instrument. The specific activity of the labeled sample was measured on a Beckman LS 6000LL liquid scintillation counter.

All samples were dissolved in a mixture of acetonitrile–0.5 *M* sodium perchlorate (pH 2.5) buffer (28:72, v/v) unless otherwise specified. Preparative chromatography was completed as previously reported [16].

2.2. Materials

Chemicals and reagents used in the study were certified ACS grade while solvents were of HPLC grade (Fisher Chemical, Fair Lawn, NJ, USA). Structures of the compounds studied are shown in Table 1. The MPEG compounds (1–6) were of average molecular mass 5000 and the PEG compounds (7 and 8) were of average molecular mass 3400 (Shearwater Polymers,

Table 1
Structure of MPEG and PEG compounds studied



Compound	R	R'	Avg. molecular mass
1	CH_3O	OH	5000
2	CH_3O	NH_2	5000
3	CH_3O	OCH_2CO_2H	5000
4	CH_3O	CH_2CO_2H	5000
5A	CH_3O	$NHCONHNH_2$	5000
5B	CH_3O	$NH^*CONHNH_2$	5000
6	CH_3O	$OCO_2p\text{-}NO_2Ph$	5000
7	H_2N	OH	3400
8	H_2N	NH_2	3400

* Indicates position of [^{14}C] label.

Table 2
Analytical HPLC gradient elution program

Time (min)	0	2	15	16	18	19	22	27
% Acetonitrile	25	25	27	27	75	75	25	25
% Water	75	75	0	0	25	25	75	75
% 0.5 *M* Sodium perchlorate (pH 2.5)	0	0	73	73	0	0	0	0
Flow rate (ml/min)	1	1	2	2	2	2	1	1

Table 3
Initial preparative HPLC gradient elution program

Time (min)	0	1	5	15	16	17	18	21	22
% Acetonitrile	34	34	34	35	35	35	75	75	34
% Water	66	66	66	32	0	0	0	0	66
% 0.5 *M* Sodium perchlorate (pH 2.5)	0	0	0	33	65	65	25	25	0
Flow rate (ml/min)	4	15	15	15	15	15	15	15	15

Huntsville, AL, USA). Details of the synthesis of the [^{14}C]-labeled MPEG-semicarbazide (5B) have been previously described [16]. The specific activity of this material was determined to be 7.7 μCi/mg by liquid scintillation counting.

2.3. High-performance liquid chromatography

Analytical chromatography was performed using the gradient elution program described in Table 2. All gradients were linear and the column temperature was ambient.

The gradient programs employed for preparative chromatography have been previously reported [16] and are shown in Tables 3 and 4.

3. Results and discussion

3.1. The role of the buffer

The choice of the sodium perchlorate buffer was critical for the analysis of the MPEG and PEG compounds. It provided low UV absorbance in the wavelength region of interest and had good solubility in both aqueous and organic solutions. Additionally, separation of the various compounds could not be obtained on the cyanopropyl column without first complexing a minimum loading of sodium perchlorate onto the column. Equilibrating to the starting organic concentration prior to injection did not yield reproducible results until after conditioning the

Table 4
Final preparative HPLC gradient elution program

Time (min)	0	1	4	6	8	18	20	22	23
% Acetonitrile	0	0	0	32	33	45	100	100	0
% Water	0	100	100	68	67	55	0	0	0
% 5% Acetic acid	100	0	0	0	0	0	0	0	100
Flow rate (ml/min)	4	12	12	15	15	15	15	15	15

column with approximately 30 column volumes using the gradient program described in Table 2. Once conditioned, the gradient program satisfactorily maintained the sodium perchlorate concentration on the column and gave reproducible results. The selectivity of the complexed sodium perchlorate buffer was particularly effective in the case of MPEG 2, which was resolved into several components. Resolution of the first two eluting components was completely lost when water was substituted for the 0.5 *M* sodium perchlorate in the gradient program as illustrated in Fig. 1.

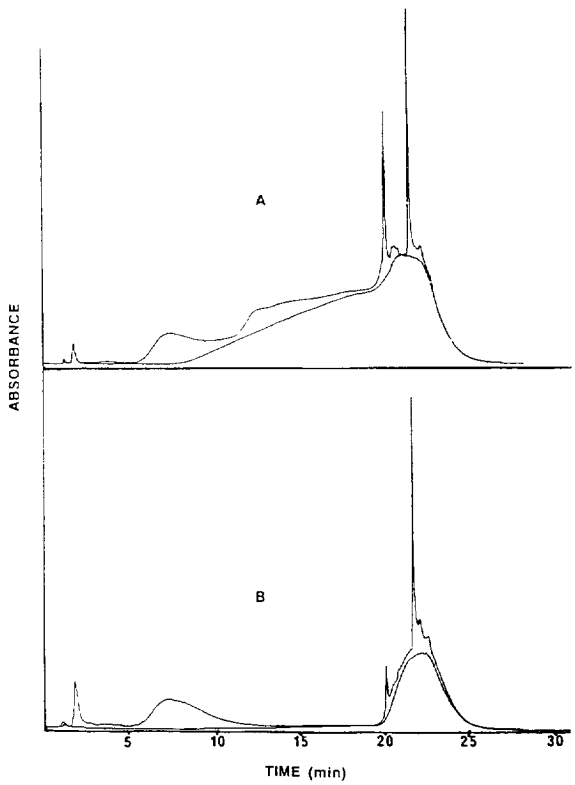


Fig. 1. Effect of buffer on the separation of compound 2, MPEG-NH_2. Chromatograms were obtained using the same column and injection vial, the only difference being that (A) used the sodium perchlorate gradient conditions outlined in Table 2 and (B) was obtained with water substituted for the 0.5 *M* sodium perchlorate. The baseline chromatogram is shown in each example.

3.2. Method of detection

PEG compounds are frequently detected using an evaporative light scattering detector (ELSD) [14,15] or a refractive index detector (RI) [1,3,5,11,13] because of the low UV absorbance with these compounds. An ELSD was not available to us, and the use of an RI detector is severely limited for gradient applications. Since the compounds separated in this study differ only by one functional group, the use of a gradient elution program was essential for successful resolution. While the studies in this paper were completed using a UV detector, the use of an ELSD should enhance the performance of the method.

UV detection allows for gradient elution, however the problem of low sensitivity of the PEG compounds remains. UV detection has been used with some success with these compounds at low wavelengths [8,10] and by derivatization with a chromophore [6,7]. In this study, the lack of UV sensitivity was improved by using large concentrated samples (4.5 mg/injection). The high sample loading caused retention times to decrease with increased loading. To obtain reproducible results, all comparative chromatograms were prepared with the same sample loading and concentrations. The lack of pure reference materials prevented quantification of the chromatograms. However, interpretation of the results was aided by examining peak shape and absorbance spectra obtained using diode-array detection and, ultimately, by incorporation of a radiolabel and radioactive flow detection. All of the PEG and MPEG distributions chromatographed in broad bands with extensive peak tailing. Obtaining baseline resolution in most cases was not possible due to the large peak widths. Detection sensitivity improved as the monitoring wavelength was decreased, with the UV maxima observed at the lowest wavelength recorded (192 nm). At this wavelength, many of the non-MPEG/PEG impurities had very narrow peak widths and high UV absorbance. The UV absorbance of these impurities decreased at the lowest recorded wavelengths, with UV maxima between 210 and 220 nm. These differences allowed for qualitative

identification of PEG and non-PEG compounds, but quantification of results was not possible.

Detection at low wavelengths also made it difficult to achieve a reproducible, flat baseline with the gradient system employed. A blank injection showed a small peak at the void volume and a slow rise in the baseline was observed over the entire chromatogram as the concentration of the sodium perchlorate increased. Blank baselines are shown with all spectra for reference.

3.3. Band broadening

To effectively chromatograph the MPEG and PEG distributions it was necessary to increase the selectivity of the terminal functional groups, which comprise less than 1% of the total molecular weight, while minimizing the size of the broad bands observed with high molecular weight distributions. To limit band broadening, the effects of temperature and salt concentration were examined.

The MPEG and PEG compounds were analyzed at various temperatures up to 80°C using the sodium perchlorate gradient system. Increasing temperature has been previously reported to improve separation with MPEG compounds [10]. The acetonitrile concentration was adjusted as the temperature was increased to give acceptable retention times. The chromatograms obtained showed no corresponding increase in selectivity with increasing temperature and all of the compounds tested eluted with increased band widths and lower resolution values. Therefore, all subsequent analyses were performed at ambient temperature.

The best selectivity for the compounds was obtained with a water–acetonitrile gradient in the absence of buffer. However, under these conditions very wide peak widths were obtained. A gradient using sodium perchlorate with no change in organic concentration produced narrower peak widths but with reduced selectivity. Therefore, in order to obtain both tight bands and high selectivity, it was necessary to optimize the gradient for both buffer and organic composition. The best chromatographic results were achieved with a gradient using a large increase in salt concentration (72%) accompanied by a small increase in organic concentration (2%).

3.4. The effect of pH

The effect of pH on both the mobile phase and the sample solution was examined. Chromatography of the MPEG and PEG compounds tested showed no differences in retention using mobile phase compositions in the pH range of 2.5 to 5.9. A pH of 2.5 was chosen based on the high buffering capacity and good pH stability observed over several weeks of use.

The pH of the sample solution, however, was found to effect retention times even though the mobile phase was sufficiently buffered and maintained at pH 2.5 throughout the study. The relative retention and retention order of the MPEG compounds could be manipulated by adjusting the pH of the sample solution, while maintaining a constant concentration of the organic component. The retention time of basic compound 2 increased as sample pH was increased while retention of acidic compounds 3 and 4 decreased with increasing sample pH. Neutral compound 1 was unaffected by pH adjustment (Fig. 2). Therefore, the optimal pH of the sample solution was dependent upon the particular compound being chromatographed.

3.5. Analysis of starting materials and MPEG-semicarbazide

The method worked well in assaying the homogeneity of all of the compounds used in the study. Representative chromatograms of several compounds are shown in Fig. 3. The chromatography of compound 1 gave a single broad peak, which was subsequently identified by isolation and ^{1}H NMR analysis. However, chromatography of the other compounds showed the presence of additional components. The MPEG acids, compounds 3 and 4, each showed a peak shoulder trailing the main peak. Chromatography of compounds 5A and 5B revealed the presence of multiple peaks, in addition to a late eluting compound. Chromatography of com-

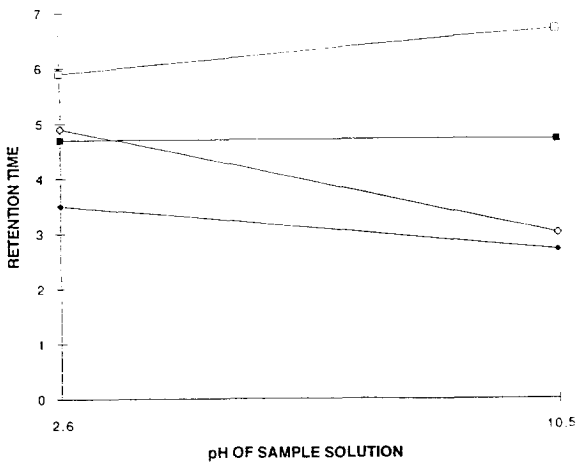


Fig. 2. The relative changes in retention times of MPEG compounds as a function of sample solution pH: (■) 1, MPEG-OH; (□) 2, MPEG-NH_2; (◆) 3, MPEG-OCH_2CO_2H; (◇) 4, MPEG-CH_2CO_2H (see Experimental for HPLC conditions).

pound 6, the only MPEG compound analyzed containing an aromatic group, gave a single off-scale peak with a retention time of approximately 22 min. Compounds 7 and 8 were separated into several well resolved peaks.

Chromatography of MPEG 2 resulted in the separation of two broad, PEG-like bands as well as two more retained peaks. ^1H NMR spectra confirmed the identity of the first major peak to be compound 2. Impure compound 2 was further reacted to provide unlabeled 5A and [^{14}C]-labeled MPEG-semicarbazide, 5B. Due to the initial low purity of compound 2, several products were obtained from the reactions and HPLC purification was required. The major peak in the MPEG-semicarbazide chromatograms was isolated using the sodium perchlorate gradient program outlined in Table 3. A second chromatographic purification was required to remove unseparated reaction impurities as well as the sodium perchlorate salts carried through from the initial chromatography. Several different mixtures were evaluated to determine the optimal conditions for washing the perchlorate salts off the column. These solutions included aqueous mixtures of 5% $NaHCO_3$, 5% CH_3COOH, and 0.1% TFA, and were examined using the

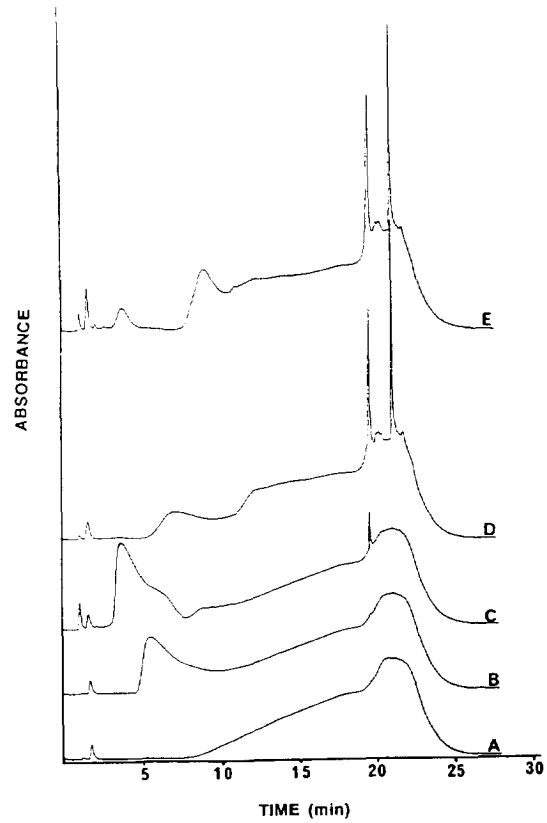


Fig. 3. The chromatograms of several representative compounds obtained under identical HPLC conditions (see text for conditions). MPEG-OH (1) is relatively pure while the other compounds show the presence of additional peaks: (A) blank, (B) 1, MPEG-OH, (C) 3, MPEG-OCH_2CO_2H, (D) 2, MPEG-NH_2, (E) 8, H_2N-PEG-NH_2.

gradient method described in Table 4. Each was found to have a great effect on both sample solubility and column stability.

A sodium bicarbonate (pH 8.3) wash resulted in increased retention and a loss of resolution. Following its use, an additional 5% acetonitrile was needed to elute the compounds off the column and reduced selectivity was observed. This was most likely a result of a partial stripping away of the bonded phase from the base silica. The presence of a cyanopropyl by-product in an isolated sample of compound 2 was confirmed by ^1H NMR. To avoid this problem, either acetic acid or trifluoroacetic acid could be substituted for the sodium bicarbonate solution in the gra-

dient program. No decrease in column stability was observed with either of these acids following their use. The final purification procedure was completed using 5% acetic acid in the gradient program (Table 4).

The preparation of MPEG-semicarbazide with a radioactive label (5B) allowed for the direct quantification of results which was not possible with UV detection. The initial radiochemical purity of 5B isolated from the reaction mixture was approximately 50% as measured using a radioactive flow detector (Fig. 4). The product was purified by preparative chromatography with the sodium perchlorate gradient system (Table 3). Further purification and removal of the sodium perchlorate salts was accomplished using the system described in Table 4. The radiochemical purity of the isolated product was determined to be 95% based on radioanalysis with a radioactive flow detector using a scintillation cocktail. The increase in chromatographic purity was even more significant as shown in Fig. 4. The identity of the purified [^{14}C]MPEG-semicarbazide was confirmed by ^{1}H NMR.

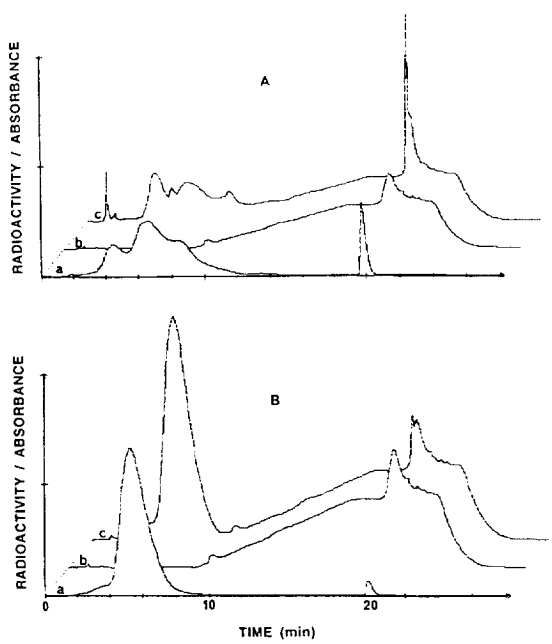


Fig. 4. Chromatograms of (A) crude 5B and (B) purified 5B obtained using UV (192 nm) detection and a radioactive flow detector. The HPLC purification increased the radiochemical purity from approximately 50% to over 95%: (a) [^{14}C] radiochromatogram, (b) baseline with UV detection, (c) UV detection (see text for HPLC conditions).

4. Conclusions

A combined organic and sodium perchlorate gradient on a cyanopropyl column has been shown to separate a number of PEG and MPEG compounds with similar molecular mass distributions. The use of a sodium perchlorate buffer allowed for detection at very low wavelengths while displaying unique chromatographic properties, obtained by interaction between the bonded phase and the perchlorate salt. The distributions chromatographed in broad bands but could be separated using a slight organic gradient coupled with a salt gradient to maintain good peak resolution. Additional selectivity was achieved by manipulating the pH of the sample solution. The method was used to determine the relative purity of PEG and MPEG compounds and to purify a sample of [^{14}C]-labeled MPEG semicarbazide (5B) from 50% to over 95% radiochemical purity.

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