Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1046 (2004) 121–126
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Separation of polyethylene glycols and their amino-substituted derivatives by high-performance gel filtration chromatography at low ionic strength with refractive index detection

Min Liu, Cao Xie, Wen Xu, Weiyue Lu*

Department of Pharmaceutics, Fudan-Pharmco Targeting Drug Research Center, School of Pharmacy, University of Fudan, Shanghai 200032, China

Received 24 February 2004; received in revised form 6 April 2004; accepted 4 June 2004

Abstract

The chromatographic analysis of polyethylene glycols (PEG) and their amino-substituted derivatives, with an average molecular mass of 2000 and 3350 is described. The PEG derivatives were perfectly separated by TSK-GEL G4000PW$_{XL}$ column, which was widely used in high-performance size-exclusion chromatography (SEC), at low ionic strength with refractive index detection. It was shown that ion-exchange interactions were mainly involved in the retention mechanism of these compounds on TSK-GEL G4000PW$_{XL}$ column, since retention volume decreased when salt concentration were added to the mobile phase and varied with the pH of the eluent. Additionally, size exclusion for PEG chains plays a role. Organic modifiers also had effect on chromatographic behaviors of PEG compounds.

Keywords: Poly(ethylene glycol); Poly(ethylene glycol)s, amino derivatives; Polymers

1. Introduction

There has been a marked interest in polyethylene glycol (PEG) compounds and their conjugates of biologically active molecules in the last two decades. Owing to PEG chains' high mobility and flexibility, PEG grafting is used to avoid biological recognition by immunogenicity and antigenicity in the case of proteins [1,2], rapid clearance from circulation by reticuleondothelial system of liposomes [3,4], and cell adherence and protein adsorption in the case of artificial biomaterials [5]. The essential step in the preparation of bioconjugates of PEG is chemical derivatization of the end groups of PEG. Among various functionalized PEG compounds, amino-substituted derivatives are most common.

Despite the common use of amino derivatization, methods for the analysis of amino-substituted derivatives of PEG with similar molecular masses by high-performance liquid chromatography (HPLC) have been rarely reported. To our knowledge, the only exception exists where separation was accomplished using a cyanopropyl column with a sodium perchlorate–acetonitrile gradient monitored by UV detection [6]. However, the compounds chromatographed with low detection sensitivity and poor shapes.

Although size-exclusion chromatography (SEC) is a widely used method for the characterization of synthetic polymer, it can only separate macromolecules, according to the hydrodynamic volume of polymer chains. However, the molecular characteristics of more complex polymers such as monofunctional or difunctional macromolecules have not indirectly correlated with the hydrodynamic volume. Since 1970's, some researchers have initiated the study of high performance liquid chromatography under critical conditions (LCCC) [7–10] and liquid exclusion–adsorption chromatography (LEAC) [11]. In critical conditions of LCCC, a part of molecule becomes "invisible" and a separation according to the units of the other type can be achieved. In LEAC, the exclusion regime cooperates with interaction of a strong adsorption type. In general, adsorption type of polymer with column packing in LCCC or LEAC is hydrophobic or polar interactions. In this paper, we found ion exchange can also take effect in LEAC or LCCC. Under near critical conditions, we can well separate PEG compounds based on ion exchange adsorption between amino groups and the packing of TSK-GEL G4000PW$_{XL}$. Taking advantage of weak ion exchange properties and lower ion

* Corresponding author. Fax: +86 21 64178790.
E-mail address: wylu@shmu.edu.cn (W. Lu).

0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.06.005

exchange capacity of the size exclusion column TSK-GEL G4000PW$_{XL}$ at low salt concentrations, we have achieved separation of PEG compounds of similar molecular weights using isocratic elution coupled with refractive index detection in LEAC. The method is highly efficient, sensitive, environmentally friendly and cost-effective. Factors affecting the separation, including salt concentrations, the pH of mobile phase and organic modifiers, have been investigated and will be discussed in detail.

2. Experimental

2.1. Reagents

M_r 3350 PEG (PEG3350) and PEG-bis-amine (NH_2-PEG3350-NH_2) were purchased from Sigma (St. Louise, MO, USA). M_r 3400 PEG-mono-amine (PEG3400-NH_2) came from Shearwater Polymer (Huntsville, AL, USA). M_r 2000 Polyethylene glycol mono-methyl ether (MPEG2000) was purchased from Aldrich Chemical (Miwaukee, WI, USA). M_r 2000 PEG (MPEG2000) was obtained from Fluka (Milwaukee, WI, USA). M_r 2000 PEG mono-amine (PEG3400-NH_2) and bis-amine (NH_2-PEG3350-NH_2) and M_r 2000 MPEG-mono-amine (MPEG2000-NH_2) were prepared by our laboratory. Other chemicals and reagents used in the study were certified AR grade (Shanghai Chemical Reagent Company, shanghai, China).

2.2. PEG amino-substituted reaction

As shown previously [12–14], PEG2000 (70 g) was dissolved in toluene (300 ml) to which pyridine (7 ml) and thionyl chloride (20 ml) were added. After refluxing at 120 °C for 3 h, the mixture was evaporated under reduced pressure. The residue was dissolved in CH_2Cl_2, and precipitated by cold ether. The Cl-PEG2000-Cl (37 g) was dissolved in dimethylformamide (DMF, 188 ml) containing sodium azide (16.22 g), and the mixture was stirred at 120 °C for 2 h. The DMF evaporated in vacuo. The residue was taken up in CH_2Cl_2, and precipitated by cold ether. The N_3-PEG2000-N_3 (18 g) was dissolved in the solution (CH_2Cl_2:CH_3CH_2OH, 15:45, v/v), to which Pa/C (0.75 g) was added. The mixture was hydrogenated in a Parr pressure hydrogenation apparatus overnight. The catalyst was filtered, and the polymer precipitated by dry ether with a yield of 15 g.

The amino derivative of MPEG was prepared essentially as described above.

2.3. Purification of the PEG amino-substituted compounds

The PEG2000 and MPEG2000 derivatives were purified at room temperature using an SP-Sepharose cation-exchange column (26 mm × 160 mm, Amersham Biosciences, Uppsala, Sweden) on a ÄKTA explorer 100 system (Amersham Biosciences, Uppsala, Sweden) consisting of Pump P-900, Pump P-950, Monitor UV-900 and Frac-900. The column was equilibrated with 10 mM sodium acetate buffer, pH 5.7, and loaded with 10 mg of PEG sample per ml bed volume. The column was then washed with five column volumes of sodium acetate buffer (pH5.7) running a gradient of 7.5 to 60 mM with a flow rate of 5 ml/min. Fractions were collected, examined by thin-layer chromatography, desalted and lyophilized individually.

Structures of the compounds studied are shown in Table 1.

Table 1
Structure of PEG (RCH_2CH_2O–$[CH_2CH_2O]_n$–CH_2CH_2R') compounds studied

Compound	R	R′	Avg. molecular mass	Abbreviations
1	OH	OH	3350	PEG3350
2	OH	NH_2	3400	PEG3400-NH_2
3	NH_2	NH_2	3350	NH_2-PEG3350-NH_2
4	OH	OH	2000	PEG2000
5	OH	NH_2	2000	PEG2000-NH_2
6	NH_2	NH_2	2000	NH_2-PEG2000-NH_2
7	CH_3O	OH	2000	MPEG2000
8	CH_3O	NH_2	2000	MPEG2000-NH_2

2.4. HPLC analysis of PEG compounds

Analytical chromatography was performed on an Agilent 1100 series quaternary HPLC system (Palo Alto, CA, USA) equipped with a vacuum degasser, an autosampler, a thermostetted column and a refractive index detector (RID) (Fig. 1). The flow-rate was set at 0.5 ml/min. The analyses were performed using a 300 mm × 7.8 mm I.D. TSK-GEL G4000PW$_{XL}$ (10 μm particle size, 300 Å pore size, Tosoh Corporation, Minato-ku, Tokyo, Japan) with a TSK-guardcolumn at 25 °C. The mobile phase was 5 mM NaCl. Samples were prepared in 5 mM NaCl at 1 mg/ml and injected by the autosampler with an injection volume of 25 μl.

3. Results and discussion

3.1. The role of the ionic properties in TSK-GEL G4000PWXL

As shown in Table 1, the major difference among these PEG compounds with similar molecule weights is the number of amino groups linked to the ends of PEG. It is obviously that fully resolved chromatograms of these PEG samples cannot be obtained in SEC. These PEG compounds with net positive charges at appropriate pH can adsorb to cation exchange resin. Therefore, in principle, these samples can be separated by cation exchange chromatography. Further, due to small differences in charge density, a gradient elution is generally essential for successful resolution. However, since these PEG derivatives do not contain any

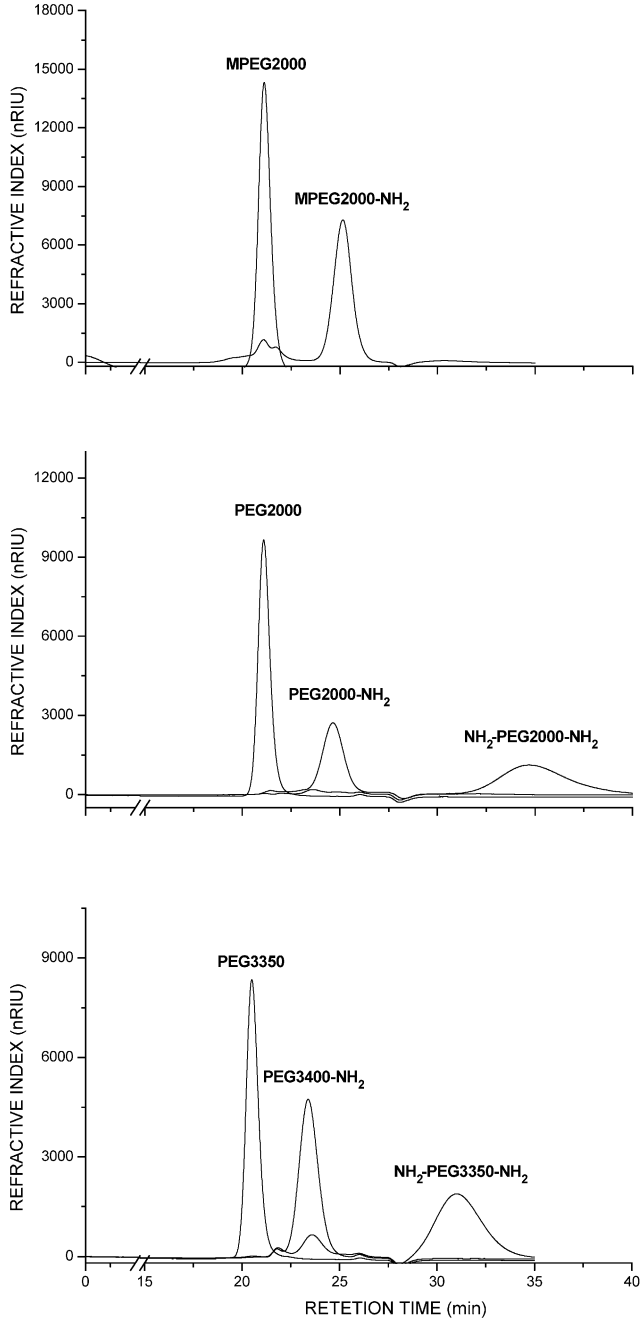

Fig. 1. The chromatograms of the PEG compounds (1∼8) obtained under same HPLC conditions (see experimental procedure for HPLC analysis).

chromophor, sensitive detection techniques, namely UV and fluorescence spectroscopy, cannot be readily applied for the analysis. On the other hand, although refractive index (RI) detection is universally applicable in detecting all chemical compounds in HPLC analysis regardless of whether or not they contain chromophor, it has limited uses only in isocratic elution, thus incompatible with the ion exchange chromatography adopting gradient elution. Even though elaborate solutions do exist such as using evaporative light-scattering detection (ELSD), electrochemical detection (ED) and mass spectrometric detection (MSD), they are not available to us [15].

An apparent solution to the problem can be obtained if PEG compounds differing in charge density can be separated by ion exchange chromatography with isocratic elution. This will pave the way for the use of RI detection. However, suitable ion-exchange resins that allow for isocratic resolution of PEG derivatives are difficult to come by because most commercial products are of high exchange capacity (100∼300 μmol/ml gel) and binding affinity and designed for gradient elution. We have found that TSK-GEL PW Type columns widely used in high-performance size-exclusion chromatography (SEC) are ideally suited for the separation of various amino derivatives of PEG using isocratic elution under near critical conditions.

In an ideal SEC separation, the mechanism is purely sieving with no adsorption interactions between the column matrix and sample molecules. In practice, a small number of weakly anionic groups on the surface of TSK-GEL PW matrices can cause sample elution profile to deviate from that under ideal situations (the matrices are slightly negatively charged with an ionic capacity of 6 to 8 μmol/ml gel, whereas the ionic capacity of an ordinary ion-exchanger is significantly higher) [16]. This deviation can be particularly problematic at low ionic strength of an eluent where cationic samples strongly retarded by ionic adsorption can elute later than theoretically expected. In order to eliminate such undesirable ionic interactions in SEC, it is common to use an eluent with ionic strength greater than 0.1 mM. However, the weakly anionic property of TSK-GEL at low ionic strength enables efficient separation of polyethylene glycols and their amino-substituted compounds of similar molecule mass by isocratic elution. As shown in Fig. 2: the elution volumes of PEG amino derivatives with the same molecular mass increase with increment of the number of amine. At the same time, PEG samples with same amino number still elute in a SEC order. A possible explanation

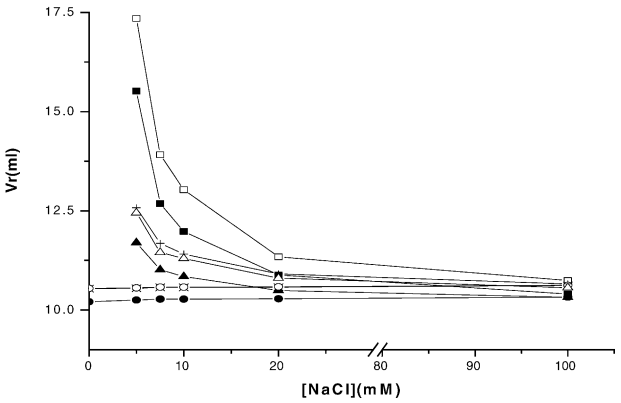

Fig. 2. Injection of PEG compounds on TSK-GEL G4000PW$_{XL}$ column: retention volume as function of NaCl concentration. (●) PEG3350; (○) PEG2000; (×) MPEG2000; (▲) PEG3400-NH_2; (△) PEG2000-NH_2; (+) MPEG2000-NH_2; (■) NH_2-PEG3350-NH_2; (□) NH_2-PEG2000-NH_2; (see experimental procedure for HPLC).

could be as follows: the separation mechanism in TSK-GEL column combines with adsorption interaction for the amino groups and size-exclusion for the PEG chain under near critical conditions, namely liquid exclusion–adsorption chromatography. However, the adsorption type is ion-exchange instead of traditionally hydrophobic or polar interaction. Moreover, ion-exchange interaction dominates the separation of PEG amino derivatives using 5 mM NaCl as a mobile phase.

3.2. Effects of salt on the retention of PEG derivatives

Different NaCl concentrations were used to study the effects of salt on separation between PEG derivatives and ion groups on the TSK-GEL G4000PW$_{XL}$. As shown in Fig. 2, chromatography of non-amino-substituted compounds 1, 4 and 7 tested showed no difference in retention using mobile phases containing NaCl ranging from 0 to 100 mM, while the retention of amino-substituted compounds 2, 3, 5, 6 and 8 gradually decreased with increased salt concentrations. It is worth noting that the more the number of amino groups on the PEG molecule, the more rapidly the retention time decreased. This result is consistent with ion-exchange interactions as the retention mechanism for the PEG compounds for the following reasons. First, the compounds without ionizing groups do not interact with the column matrix, and their retention is expected to be independent of different salt concentrations. Second, ionic interactions between groups of opposite charges are known to be weakened progressively in the presence of increasing amounts of salts that compete with the PEG derivatives and the column matrix. Consistently, when water was used as eluent, nonionic polymers such as compounds 1, 4 and 7 eluted as expected, while ionic compounds 2, 3, 5, 6 and 8 retained on the column. With increasing salt concentration of the mobile phase, the interaction for amino groups with the packing of TSK-GEL column becomes weak, the size-exclusion for PEG chains grows predominant, and the eluent order changes again from ion exchange interaction to exclusion. Up to 100 mM NaCl as eluent, the PEG compounds were separated according to their molecular mass.

3.3. Effects of pH on the retention of PEG derivatives

The effect of pH of the mobile phase was also examined. As shown in Fig. 3, while pH had no effect on the retention of compounds 1, 4 and 7 containing no amino groups, it affected elution of mono and bis-amino PEG compounds. In the pH range of 4–9, PEG samples with similar molecular mass had been well separated. However, beyond the range, the resolution decreased.

According to the ion-exchange theory, the working pH range of an ion exchanger, over which the ion exchange groups remain charged and maintain consistently high capacity, is represented by the vertical part of the titration curve for the matrix. Based on the titration data of TSK-GEL

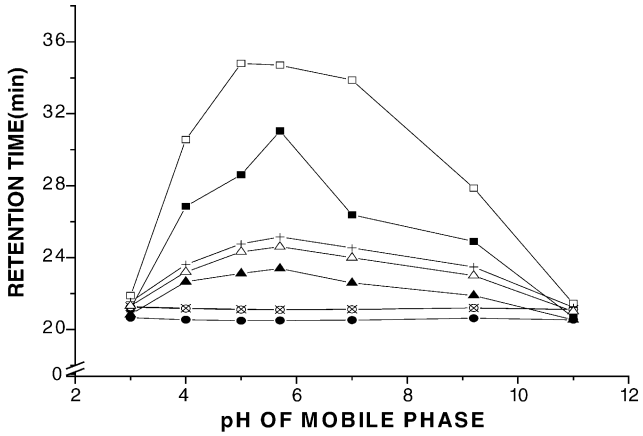

Fig. 3. The relative changes in retention time of PEG compounds as a function of mobile phase pH. (●) PEG3350; (○) PEG2000; (×) MPEG2000; (▲) PEG3400-NH_2; (△) PEG2000-NH_2; (+) MPEG2000 -NH_2; (■) NH_2-PEG3350-NH_2; (□) NH_2-PEG2000-NH_2 (elution: 5 mMNaCl pH adjusted with HCl and NaOH solution and see experimental procedure for HPLC analysis).

G4000PW$_{XL}$ packing, the range of the vertical part is indeed from 4 to 9, which is the same as the suitable pH range determined for the PEG samples. We therefore conclude that high-resolution separation of PEG amino-substituted compounds on the TSK-GEL PWXL type column is attributed to the interaction between PEG samples and anionic groups on the resin surfaces. Since the surface of TSK-GEL PW matrices contain weakly ionic groups, its working pH range is sharper than strong ion exchanger that is completely ionized over a wide pH range. Therefore, pH of the eluent plays an important role on separation performed on the TSK-GEL PWXL type column. This also explained why PEG compounds had poor resolution at pH3.0 and 11.0. The ion exchange groups of the column resin could not remain charged at pH 3.0, while at pH 11.0, PEG amino-substituted compounds carried no net charge. This resulted in not binding PEG samples to the ion exchanger and poor resolution.

Although the ionic exchange interaction for amino group results in good separation of PEG compounds, we can not neglect SEC on TSK-GEL PW type column. The elution curves, shown in Figs. 2 and 3, illustrate how the TSK-GEL PW packing perform differently for same amino-substituted PEG compounds with different molecular mass, such as compounds 1, 4 and 7, compounds 2, 5 and 8, compounds 3 and 6. The dates demonstrate that elution order is, for similar PEG compound types, a function of molecular weight, the largest materials eluting first, and the smallest last. This result can be used for exclusion–adsorption (ion exchange) chromatography (LSAC). An obvious adsorption–exclusion transition was observed during we adjust the pH of mobile phase: as can be seen from Fig. 4, when pH was gradually decreased up to 3.0 or increased up to pH 11.0, the interaction for the amino groups was substituted by size exclusion for PEG chains.

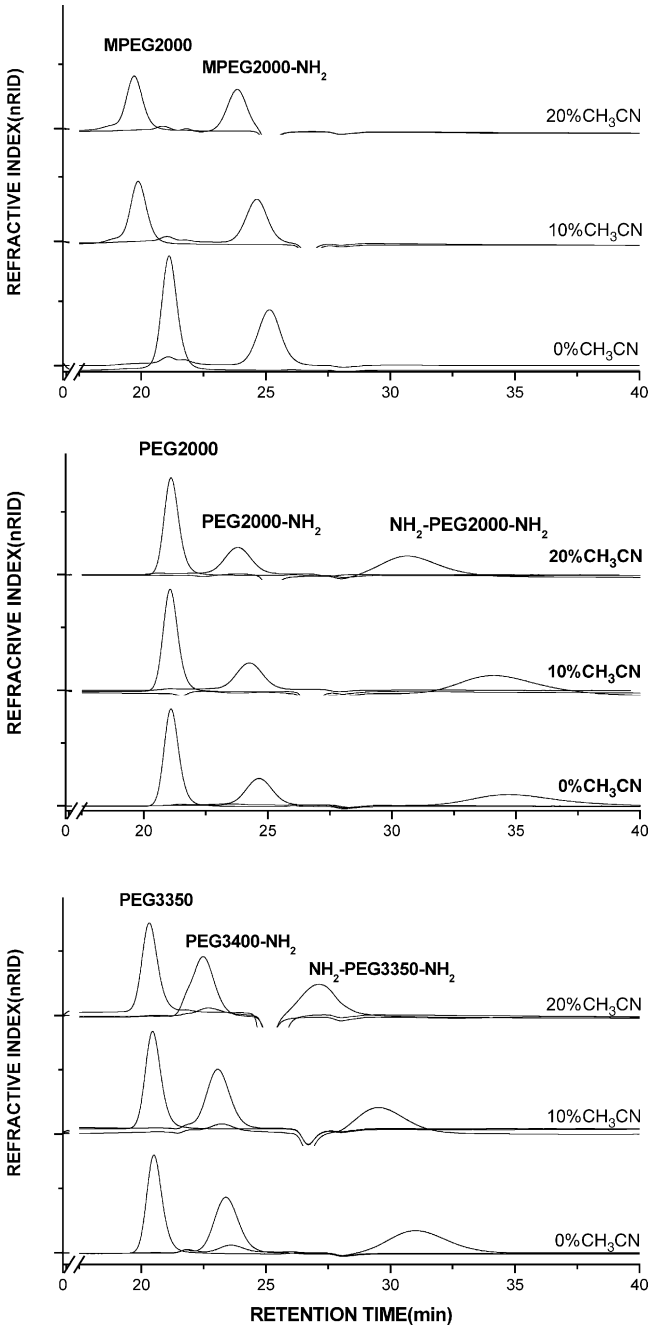

Fig. 4. Retention time of PEG amino-substituted compounds on TSK-GEL G4000PW$_{XL}$ as function of percent of acetonitrile in the mobile phase (elution: 5 mMNaCl containing with CH_3CN of different concentration). From left to right, the peaks represent in the graph PEG–monoamino-PEG–diamino-PEG).

3.4. Effects of organic solvent on the retention of PEG derivatives

The effect of organic solvents such as acetonitrile on interactions between PEG derivatives and TSK-GEL PW type resin was investigated. It showed in Fig. 4, that retention time of PEG amino-substituted compounds decreased when acetonitrile was added to the mobile phase and PEG samples without amine groups maintained invariable. The only exception existed when MPEG2000 was determined. We found that acetonitrile could also shorten the retention of MPEG2000 on TSK-GEL PW column, although MPEG2000 had no amine group. The possible explanation was due to methoxy at the end of MPEG2000. Methoxy increased the hydrophobic characteristics of MPEG2000 so that MPEG2000 eluted more rapidly than PEG compounds in the acetonitrile solution. We also tested various eluent systems of either acetone or methanol as organic modifiers to effect on the elution time of PEG samples. Table 2 showed, the organic modifiers with different dielectric constant(ε) made different affect on the elution of PEG amino-substituted compounds. Similarly, the chromatographic behaviors of bis-amine substituted compounds had more significant change, compared with mono or none amine compounds. This phenomenon may be attributed to that organic modifiers would bring about the change of pH in the eluent, which effect the dissociation of ion group in the insoluble matrix of TSK-GEL column and the charge of PEG amine derivatives.

Table 2
Effect of organic solvent with different dielectric constant(ε) on the retention time of PEG amino-substituted compounds on TSK-GEL 4000PW$_{XL}$ column (Elution: add differently organic solvent to 5 mM NaCl)

5% (v/v) Solvent	Acetone ($\varepsilon = 20.7$)	Methanol ($\varepsilon = 32.6$)	Acetonitrile ($\varepsilon = 36$)
PEG3350	20.44	20.50	20.51
PEG3400-NH_2	22.54	22.91	23.13
NH_2-PEG3350-NH_2	25.00	28.50	29.30
PEG2000	21.06	21.10	21.10
PEG2000-NH_2	23.31	23.89	24.32
NH_2-PEG2000-NH_2	29.79	32.11	33.20
MPEG2000	21.07	21.12	21.13
MPEG2000-NH_2	23.71	24.25	24.57

4. Conclusions

Depending on weak ionic characteristics on TSK-GEL G4000PW$_{XL}$ column, the novel high-performance liquid chromatography appeared to be a satisfactory method to analysis amino-substituted PEG derivatives with similar molecular mass under near critical conditions.

It demonstrated that lower ionic capacity on the TSK-GEL PW type column resulted in highly efficient separation and available detection of the amino derivatives of PEG2000, PEG3350 and MPEG2000 with only a lowly ionic strength and isocratic eluent program. This interactions between the column matrix and PEG compounds are mainly ion-exchange interaction since the retention volume decreased by addition of salts and varied with pH of the eluent. Additionally, size exclusion for PEG chains can also play a role in separation of PEG samples. The orange modifiers also changed the elution behaviors.

Acknowledgements

Project supported by the National Science Foundation of China (No. 30271550) and by the Research Fund for the Doctoral Program of Higher Education (No. 20030246050).

References

[1] T.D. Brumeanu, H. Zaghouani, C. Bona, J. Chromatogr. A 696 (1995) 219.
[2] J.E. Seely, C.W. Richey, J. Chromatogr. A 908 (2001) 235.
[3] H. Li, J.H. Song, J.S. Park, K. Han, Int. J. Pharm. 258 (2003) 11.
[4] T.S. Levchenko, R. Rammohan, A.N. Luckyanov, K.R. Whiteman, V.P. Torchilin, Int. J. Pharm. 240 (2002) 95.
[5] C. David, M.C. Millot, B. Sébille, J. Chromatogr. A 753 (2001) 93.
[6] W.H. Leister, L.E. Weaner, D.G. Walker, J. Chromatogr. A 704 (1995) 369.
[7] A. Gorbunov, B. Trathnigg, J. Chromatogr. A 955 (2002) 9.
[8] T. Macko, D. Hunkeler, D. Berek, Macromolecules 35 (2002) 1979.
[9] A.M. Skvortsov, G.J. Fleer, Macromolecules 35 (2002) 8609.
[10] I. Park, S. Park, D. Cho, T. Chang, Macromolecules 36 (2003) 8539.
[11] C. Rappel, B. Trathnigg, A. Gorbunov, J. Chromatogr. A 984 (2003) 29.
[12] S. Zalipsky, J.L. Chang, F. Albericio, G. Barany, React. Poly. 22 (1994) 243.
[13] S. Zalipsky, C. Gilon, A. Zilkha, Eur. Polym. J. 19 (1983) 1177.
[14] S. Furukawa, N. Katayama, T. Iizuka, I. Uraba, H. Okada, FEBS Lett. 121 (1980) 239.
[15] K. Rissler, J. Chromatogr. A 742 (1996) 1.
[16] Scientific Report of Tosoh Corporation, TSH-GEL PWxl columns: a new series of polymer-based columns for high performance gel filtration, Chromatography 29 (1985) 1.