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High-performance liquid chromatographic method for the simultaneous determination of low-molecular-mass oligomers of polyethylene glycol in aqueous skin extracts

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Abstract

The isocratic, reversed-phase, high-performance liquid chromatographic (HPLC) method presented provides a simple and rapid analytical technique for the simultaneous determination of low-molecular-mass oligomers of polyethylene glycol (PEG) in aqueous polymer samples containing polar contaminants of biologic origin. In the present case, individual molecular mass species were quantitated in aqueous skin extracts arising from the investigation of PEG transport through mammalian skin. PEGs were isolated and purified by solid-phase extraction using large-pore kieselguhr (Extrelut QE) cartridges, which selectively retained polar contaminants from excised skin specimens. Sample recoveries were found to be dependent upon molecular mass, ranging from 18.28% to 86.10%, but were highly reproducible. The mean inter-sample error ($n \geq 5$) for the extraction of twenty-six molecular mass species of PEG was less than 3%. Satisfactory resolution of primary molecular mass components was accomplished using a base-deactivated, C_8 column (Supelcosil LC-8-DB) and a mobile phase consisting of methanol and water. Corresponding run times for the complete separation of individual oligomers ranged from 8 to 30 min, while the limit of detection for a particular molecular mass species was approximately 5 μg/ml. The method was further employed to determine the weight-average (M_w) and number-average (M_n) molecular mass distributions and polydispersity for three commercial PEG blends, as well as to characterize the methylene chloride/water distribution coefficients for twenty-two molecular mass species ranging from 282 to 1206 Da.

1. Introduction

Polyethylene glycols (PEGs) represent a family of inert, synthetic, linear polymers employed as viscosity enhancing agents, solubilizing agents and lubricants in a wide variety of commercial settings, including the pharmaceutical, cosmetic and food industries. In recent years, low-molecular-mass blends of PEGs (average molecular mass < 2000 Da) have been used to characterize the molecular size dependence of passive transport through gastrointestinal [1–4] and nasal [4,5] epithelia as well as electrically enhanced transport through hairless rat skin [6]. Accordingly, such investigations have necessitated the development of analytical methods for the quantitation of PEG oligomers in transport media containing polar contaminants of biologic origin.

Commercially available PEG blends are poly-

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disperse (multi-component) mixtures of individual oligomers formed upon controlled addition of ethylene oxide to ethylene glycol. The polymerization process typically yields a complex mixture of PEG molecules which differ in the length of the polyoxyethylene chain, resulting in a Poisson-like distribution of polymeric species. Accordingly, resulting blends are defined by the nominal average molecular mass of the individual components.

Early methods for the quantitative determination of PEGs were based upon iodine titration [7] and colorimetric determination following complexation with either ammonium cobalt thiocyanate [8] or iodobismuthate (Dragendorff's) [9] reagents. Such procedures suffered from poor sensitivity and a complete lack of specificity for individual oligomers. These methods were later replaced by gel filtration (size exclusion) chromatography [10,11]. Chadwick *et al.* [1] were the first to develop a gas–liquid chromatographic method capable of resolving individual molecular mass components from PEG 400. Since that time, several high-performance liquid chromatographic (HPLC) methods [2–4] have been developed for determination of PEG oligomers in a variety of biological fluids. Difficulties associated with these assays include cumbersome, time-consuming extraction procedures, inadequate on-column separation of oligomeric components, poor peak shape, excessive run times or insufficient assay validation.

The modified analytical method presented combines simple, isocratic, reversed-phase HPLC with solid-phase extraction (SPE) to provide rapid isolation and purification of low-molecular-mass PEGs from aqueous skin extracts, and subsequent quantitation and characterization of individual oligomeric components of polydisperse PEG blends.

2. Experimental

2.1. Materials and reagents

Six commercial grades of low-molecular-mass PEGs (Polyglycol E200, E300, E400, E600, E900 and E1000) were donated by the Dow Chemical Company (Midland, MI, USA). Acetonitrile and methylene chloride (Fisher Scientific, Fair Lawn, NJ, USA) and methanol (Burdick and Jackson Laboratories, Muskegon, MI, USA) were of HPLC-grade purity. All aqueous solutions were made with purified water (Nanopure Ultrapure Water System, Barnstead/Thermolyne Corp., Dubuque, IA, USA).

2.2. Instrumentation

The basic chromatographic system consisted of either a 6000A solvent delivery system and an R401 differential refractometer (Waters Associates, Milford, MA, USA) or an SP8770 isocratic pump (Spectra-Physics Analytical, San Jose, CA, USA) and an ERC 7512 refractive index detector (Erma, Tokyo, Japan), a Chromatopac C-R6A (Shimadzu Scientific Instruments, Columbia, MD, USA) and a Model 7125 manual injector (Rheodyne, Cotati, CA, USA) fitted with a 100-μl sample loop.

2.3. Chromatographic conditions

Chromatographic separation of individual PEG oligomers was accomplished using a Supelcosil LC-8-DB (5 μm, 25 cm $\times$ 4.6 mm I.D.) base-deactivated column (Supelco, Bellefonte, PA, USA). PEG oligomers were eluted from the column isocratically at ambient room temperature, although the column was wrapped in an insulating foam layer to minimize baseline drift. The mobile phase consisted of methanol and water in the following proportions: 30:70 (Polyglycol E200 and E300), 35:65 (Polyglycol E400), 42:58 (Polyglycol E600), 44:56 (Polyglycol E900), and 46:54 (Polyglycol E1000). Prior to use, the mobile phase was filtered through a 0.45-μm, Nylon-66 membrane (Rainin Instrument, Woburn, MA, USA) and degassed by vacuum. The flow-rate was maintained at 1.0 ml/min, generating operating pressures of approximately 2500 psi. Refractive index was monitored at positive polarity, while detector temperature was maintained at 30°C.

2.4. Preparation of stock solutions and standard solutions

Individual stock solutions (2.50 mg/ml) of each polydisperse blend were prepared by introducing 250 mg of a polymer sample to a tared, 100-ml volumetric flask, followed by addition of purified water to volume. Six standard solutions, encompassing a 10- to 15-fold range of polymer concentrations, were prepared by serial dilution of the stock solution with mobile phase. Concentrations were equally distributed over 50% to 150% of the expected working range for sample analysis. When not in use, all solutions were stored in airtight vials at 4°C.

2.5. Sample preparation

Aqueous PEG samples were collected from the receptor compartments of diffusion cells following a 2-h exposure to the dermis of excised, full-thickness hairless rat skin. Samples were subsequently stored in borosilicate glass test tubes at 4°C for up to 7 days prior to analysis.

2.6. Solid-phase extraction

Isolation and purification of PEG oligomers from receptor media was accomplished by modification of a solid-phase extraction (SPE) method developed by Tagesson and co-workers [2], and later revised by Donovan *et al.* [4]. The extraction procedure served to concentrate aqueous samples as well as to remove polar contaminants likely to generate interfering peaks during subsequent chromatographic analysis. Entire sample volumes (≈ 10 ml) were added to 10-ml, large-pore kieselguhr SPE cartridges (Extrelut QE, EM Separations, Gibbstown, NJ, USA) after pre-equilibration with 25 ml of methylene chloride–acetonitrile (90:10, v/v). After 60 min, PEG oligomers were eluted from the cartridges upon addition of 3×10-ml aliquots of the organic solvent mixture, and collected in 60-ml borosilicate glass tubes. Water and polar contaminants were retained on the solid-phase matrix. Although low-molecular-mass oligomers of PEGs are highly soluble in methylene chloride, acetonitrile was incorporated into the eluting solvent in order to increase the extraction efficiency of smaller, more hydrophillic species.

Following evaporation of the eluting solvent under a continuous nitrogen stream (The Meyer N-Evap Analytical Evaporator, Organomation Associates, Shrewsbury, MA, USA) at 40°C, a volume of 5 ml of methanol was added to each glass tube. The contents were vortex-mixed (Vortex-Genie, Scientific Industries, Bohemia, NY, USA) for 30 s and transferred to clean, 10-ml borosilicate glass tubes, after which the methanol was evaporated. Upon dryness, sample residues were reconstituted with 250-μl aliquots of mobile phase. Upon standing for 10 min, the contents were vortex-mixed for an additional 30 s. Prior to injection onto the column, extracted samples were clarified through an unlaminated, 0.45-μm cellulose acetate filter membrane (Alltech Associates, Deerfield, IL, USA). Resulting chromatograms were subsequently analyzed by comparing sample peak heights to those of external standards.

2.7. Determination of extraction efficiency (sample recoveries)

Standards were prepared by adding a known quantity (200–300 mg) of a polymer sample to a tared, 100-ml volumetric flask, followed by addition of phosphate-buffered saline (PBS, pH 7.4, ionic strength of phosphate buffer $\mu = 0.45$ M) to volume. A 10-ml aliquot was subsequently transferred to a second 100-ml flask, followed by further dilution with PBS to volume. Six 10-ml aliquots were carefully measured, added to SPE cartridges, and extracted as previously described. Extraction efficiency (% recovery) was determined for each oligomer by comparing resultant chromatograms to those obtained from (unextracted) standard solutions.

2.8. Sensitivity

The lower limit of detection for individual oligomers was based upon sequential dilution of standard solutions below the working range. This

limit was defined by a minimum signal-to-noise ratio of 3:1.

2.9. Identification of PEG oligomers

Positive identification of smaller PEG oligomers was accomplished by comparing absolute retention times to those of commercially available standards (Polymer Laboratories, Church Stretton, UK). Higher-molecular-mass species were subsequently identified by relative retention times.

2.10. Determination of molecular mass distributions and polydispersity

Molecular mass distributions for polydisperse PEG blends were determined according to the method of Rothman [12] and Walter and Digenis [13] for alkylphenoxy polyethylene surfactants. The frequency (F_i) of each molecular mass component (i) was defined by the area under the curve for the particular oligomer (AUC_i) and the area under the curve for the entire sample (AUC_t) according to the following relationship:

$$F_i \frac{AUC_i}{AUC_t} \cdot 100 \quad (1)$$

Weight-average (M_w) and number-average (M_n) molecular mass were then calculated by:

$$M_w = \frac{\Sigma F_i MW_i^2}{\Sigma F_i MW_i} \quad (2)$$

$$M_n \frac{\Sigma F_i MW_i}{\Sigma F_i} \quad (3)$$

where MW_i is the molecular mass of each species i. Polydispersity was then obtained by dividing M_w by M_n.

2.11. Determination of methylene chloride/water distribution coefficients

Prior to performing distribution studies, large volumes of purified water and methylene chloride were extensively mixed so that each immiscible phase was mutually saturated in the other. To each of twelve clean borosilicate glass tubes was added 2.0 ml of a dilute solution of PEGs in water (5 mg/ml), followed by 8.0 ml of methylene chloride. Each test tube was closed tightly with a Teflon-lined screw cap, after which the contents were vortex-mixed for a period of 10 min. Following additional mixing overnight (Multi-Purpose Rotator, Scientific Industries, Bohemia, NY, USA) the contents were centrifuged at 2400 rpm for 8 min (CRU-5000, Industrial Equipment Company, Needham Heights, MA, USA).

Following aspiration of the aqueous (upper) phase, a volume of exactly 1.0 ml of the organic phase from each tube was removed, placed in a clean glass tube and evaporated to dryness under a nitrogen stream at 40°C. Residues were reconstituted with 500 μl of mobile phase, vortex-mixed for 60 s, and filtered prior to chromatographic analysis. Methylene chloride/water distribution coefficients were subsequently calculated according to the convention of Leo *et al.* [14]:

$$K_i = \frac{C_1}{C_2} \quad (4)$$

where K_i is the apparent distribution coefficient of species i, and C_1 and C_2 represent the concentration of i in methylene chloride and water, respectively.

3. Results and discussion

3.1. Chromatographic conditions

Fig. 1 illustrates the on-column chromatographic separation of the primary molecular mass components of Polyglycol E400, E600 and E1000. A number of reversed-phase columns and mobile phase compositions were initially evaluated in an effort to optimize the resolution of individual oligomers, while minimizing run time. Traditional C_{18} and C_8 columns were found to yield unsatisfactory results. The base-deactivated C_8 column, however, permitted satisfactory (baseline or near-baseline) resolution of all major oligomers contained within the six

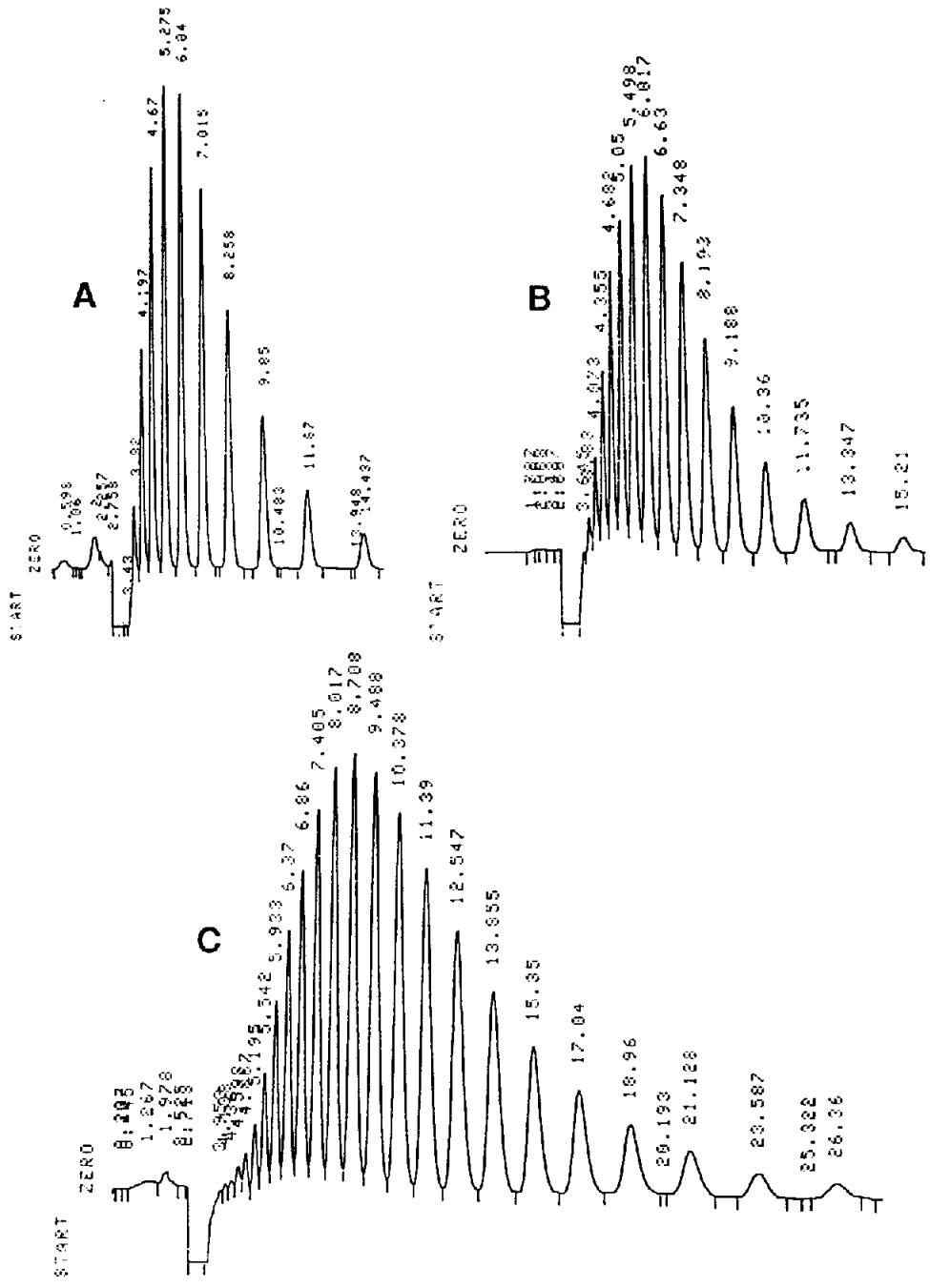

Fig. 1. Chromatographic separation of low-molecular-mass oligomers of (A) Polyglycol E400, (B) Polyglycol E600, and (C) Polyglycol E1000 following solid-phase extraction from aqueous skin extracts.

commercial blends, and resulted in well-defined, symmetric peaks with reasonable run times (< 30 min). Resolution of 27 oligomers, ranging in mass from 238 to 1382 Da, was accomplished through modification of the mobile phase composition as previously described.

3.2. Calibration curves and linearity

Fig. 2 illustrates a typical set of calibration curves for the 7 major oligomers of Polyglycol E400. Calibration curves were generated by measuring the peak heights of each primary oligomer over a 10- to 15-fold range of polymer concentrations. Resulting correlations were highly linear, generally yielding r^2 values between 0.998 and 1.000, and never less than 0.991.

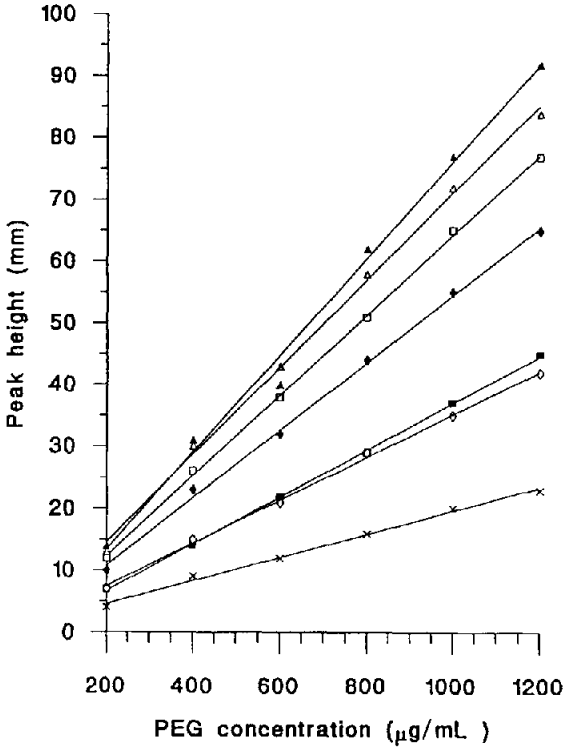

Fig. 2. Representative standard curve for low-molecular-mass oligomers of Polyglycol E400: (□) PEG 282; (■), PEG 326; (△) PEG 370; (▲) PEG 414; (◇) PEG 458; (◆) PEG 502; (X) PEG 546. Lines denote linear least-squares fits of the data. Each point represents a single determination. The mean $r^2 = 0.998$ ($n = 7$).

Calibration curves based upon peak area were also found to be highly linear, although correlation coefficients were slightly lower in most cases. It is interesting to note that most y-intercepts were found to be non-zero, undoubtedly reflecting a lack of linearity at PEG concentrations below the range studied in the present application.

3.3. Sample recoveries and precision

The efficiency of the SPE procedure for low-molecular-mass oligomers of PEG was evaluated by comparing the peak heights of PEGs in the extracted standards to those obtained from (unextracted) standard solutions. Recoveries were then calculated according to the calibration curves previously established. Results of the extraction procedure are summarized in Table 1. The efficiency of the extraction procedure was found to be highly dependent upon the molecular mass of the PEG oligomer, yielding sample recoveries ranging from 18.28% to 86.10% for PEG 282 and PEG 854, respectively. Recoveries were found to increase with increasing molecular mass, undoubtedly reflecting the increased affinity of larger molecules for the eluting solvent. Aqueous solubility of PEG oligomers is known to decrease with increasing molecular mass.

The addition of acetonitrile to the eluting solvent appeared to improve recoveries of the smallest PEGs (< 600 Da), but was limited to 10% of the organic phase by volume. On account of their water-miscible properties, the presence of polar organic modifiers at higher concentrations frequently results in elution of water (and associated water-soluble contaminants) from the solid-phase matrix along with the compounds of interest. However, incorporation of acetonitrile up to 10% of the eluting solvent volume resulted in clean chromatograms with no interfering peaks. In addition to isolating PEG oligomers from polar contaminants, the SPE procedure served to effectively concentrate PEG samples for accurate quantitation. Final sample concentrations (post-extraction) ranged from 6.88 to 34.44 times that of original aqueous sample concentrations.

Table 1
Inter-sample reproducibility for the chromatographic determination of low-molecular-mass oligomers of PEG following solid-phase extraction from a dilute solution of (A) Polyglycol E400, (B) Polyglycol E600, and (C) Polyglycol E1000 in phosphate-buffered saline ($n = 6$).

	Molecular mass (Da)	Retention time (min)	Solid-phase extraction			
			Mean recovery (%)	Standard deviation	Coefficient of variation (%)	Extraction[a] factor
(A)	282	3.7	17.01	0.51	2.97	6.81
	326	4.1	17.23	0.62	3.60	6.89
	370	4.7	20.25	0.53	2.62	8.10
	414	5.4	23.90	0.81	3.37	9.56
	458	6.4	27.12	0.80	2.93	10.85
	502	7.6	31.78	1.07	3.36	12.71
	546	9.2	36.20	1.37	3.79	14.48
(B)	370	3.6	18.28	0.00	0 00	7.31
	414	3.9	22.13	0.55	2.49	8.85
	458	4.2	25.99	0.63	2.42	10.40
	502	4.6	31.11	0.84	2.71	12.44
	546	5.1	35.59	0.82	2.32	14.23
	590	5.6	40.58	0.89	2.20	16.23
	634	6.2	44.77	0.91	2.04	17.91
	678	7.0	48.88	1.11	2.26	19.55
	722	7.9	52.46	1.22	2.32	20.99
	766	9.0	56.59	1.40	2.48	22.64
	810	10.3	58.97	1.61	2.78	23.19
(C)	722	6.1	79.15	2.57	3.24	31.66
	766	6.6	79.83	4.69	5.88	31.93
	810	7.1	85.03	3.72	4.38	34.01
	854	7.7	86.10	3.36	3.91	34.44
	898	8.4	83.68	3.23	3.86	33.47
	944	9.2	82.58	1.87	3.47	33.03
	986	10.0	81.29	2.49	3.06	32.52
	1030	11.0	79.55	2.25	2.83	31.82
	1074	12.1	79.65	1.93	2.42	31.86
	1118	13.4	80.73	2.66	3.30	32.29
	1162	14.8	82.48	2.91	3.53	32.99
	1206	16.4	83.07	2.31	2.79	33.23
	1250	18.2	81.09	1.55	1.92	32.44
	1294	20.3	79.12	4.12	5.21	31.65
	1338	22.5	75.36	3.19	4.23	30.15
	1382	25.6	69.74	0.00	0.00	27.89

[a] Extraction factor = concentration (post-extraction)/concentration (pre-extraction).

It should be noted that a number of solvents, both halogenated and non-halogenated, were initially screened for their ability to elute all PEG oligomers from the solid-phase matrix. In general, those solvents which yielded higher recoveries were also associated with the appearance of multiple, interfering peaks in subsequent chromatograms. On account of large sample volumes, however, other types of solid-phase extraction cartridges were not considered.

In spite of the variability in recoveries among oligomers of different molecular mass, results were highly reproducible for each individual oligomer. Inter-sample variability ranged from 0.00% to 5.88% ($n \geq 5$), while the mean inter-sample error for 26 oligomers was approximately 3%, a value which is only slightly greater than the mean inter-sample error obtained for repeated injections ($n = 6$) of corresponding aqueous standard solutions. Inter-day recoveries were found to be more variable than inter-sample recoveries, however, and were attributed to minor differences between batches of the solid-phase matrix (*i.e.* mean particle size, total sorbent surface area, *etc.*). As a result, care was taken to ensure that all samples from a particular experiment were extracted using the same lot of SPE cartridges.

3.4. Sensitivity

The lower limit of sensitivity for satisfactory, simultaneous quantitation of all major peaks within a polymer sample ranged from 100 μg/ml (for Polyglycol E200) to 400 μg/ml (for Polyglycol E1000). The limit of sensitivity for each individual molecular mass species was found to be approximately 5 μg/ml.

3.5. Molecular mass distributions and polydispersity

Fig. 3 illustrates the distribution of low-molecular-mass oligomers of Polyglycol E600, E900 and E1000. The plots demonstrate a typical Poisson distribution of molecular mass components resulting from the random polymerization of ethylene oxide monomer. The most prevalent oligomers in these three commercial blends were found to be PEG 590, 898 and 986, consistent with the nominal weight-average molecular mass by which they are named. Similar distributions for Polyglycol E200, E300 and E400 blends could not be obtained, however, due to interference of the solvent front with the peaks of PEG oligomers less than 238 Da.

Relevant molecular mass distributions for Polyglycol E600, E900 and E1000 were calculated according to eqs. 2 and 3. For each of the three blends, the calculated M_w values were within 5% of the nominal M_w of the polymer, consistent with the molecular mass range for commercially available PEG blends. Based upon calculated values of number-average (M_n) and weight-average (M_w) molecular mass for each multi-component mixture, resulting polydispersities were found to range from 1.03 to 1.04, as expected.

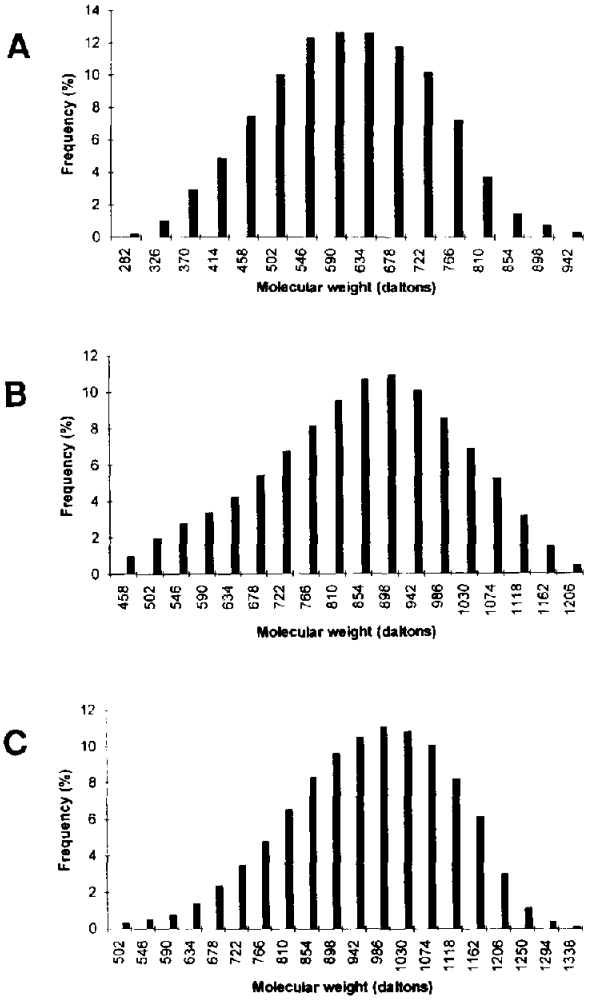

Fig. 3. Distribution of low-molecular-mass oligomers of (A) Polyglycol E600, (B) Polyglycol E900, and (C) Polyglycol E1000 as determined by HPLC, according to the method of Rothman [12] and Walter and Digenis [13].

3.6. Methylene chloride/water distribution coefficients

Although low-molecular-mass oligomers of PEG are insoluble in nonpolar organic solvents, these polymers demonstrate appreciable solubility in polar, halogenated hydrocarbons. Accordingly, the distribution of PEGs between methylene chloride and water was examined in order to demonstrate the relative hydrophillic–hydrophobic nature of the oligomers as a function of molecular mass. Fig. 4 illustrates the distribution of PEG molecules between these two immiscible phases. Resultant values were found to increase with increasing molecular mass, ranging from 0.085 to 7.65 for PEG 282 and PEG 1118, respectively. Above PEG 1118, distribution coefficients were observed to decrease sharply. This behavior is consistent with the observed recovery of PEG molecules following elution from the polar solid-phase matrix, and likely reflects a change in the relative contributions of hydrogen bonding between repeating ethylene oxide groups and the molecular solvation shell, as well as nonspecific hydrophobic interactions between carbon atoms of adjacent subunits at molecular mass above 1100 Da. As expected, the distribution of individual PEG oligomers between methylene chloride and water were found to be independent of the multi-component PEG source.

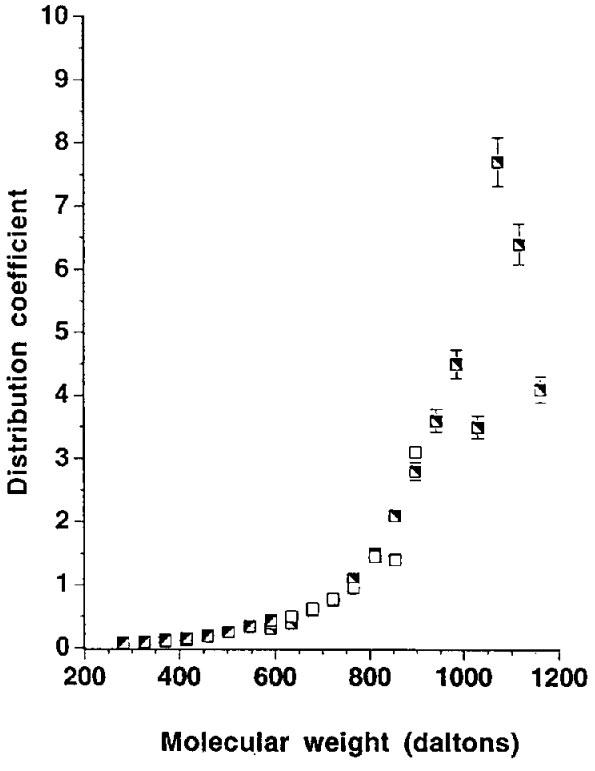

Fig. 4. Distribution of low-molecular-mass oligomers of PEG between methylene chloride and water. Source: (◩) Polyglycol E400; (□) Polyglycol E600; (◪) Polyglycol E100. Each data point represents the mean of 12 determinations. Error bars denote the standard deviation.

4. Conclusions

The HPLC method presented provides a simple, rapid, selective and highly reproducible technique for the simultaneous determination of low-molecular-mass oligomers of PEG in aqueous skin extracts. The present method should be useful to investigators seeking to resolve the molecular size dependence of biological membranes or to characterize the transport properties of synthetic membranes. In addition, this technique permits the rapid characterization of polydisperse PEG blends without the need for costly size exclusion chromatography columns and associated computer software.

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