Improved Separation of Polyethylene Glycols Widely Differing in Molecular Weight Range by Reversed-Phase High Performance Liquid Chromatography and Evaporative Light Scattering Detection

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Column liquid chromatography
Evaporative light-scattering detection
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Summary
Polyethylene glycols (PEGs) of nominal molecular weight (M) 200, 400, 600, 1000, 1500, 3000, 4000 and 6000 were chosen as model compounds and subjected to reversed-phase high performance liquid chromatography (RP-HPLC) on an octadecasilyl silica gel (C18) stationary phase using a binary gradient composed of acetonitrile and water and evaporative light scattering detection (ELSD). Satisfactory resolution of oligomers up to M of 3000 was accomplished; the higher M samples PEG-4000 and PEG-6000 could not be further resolved into the constituent oligomers and therefore, M = 4000 marks the upper limit of oligomer resolution. Despite some peak overlapping as a consequence of the more or less broad oligomer distribution, individual types of PEG samples can be distinguished from each other by their characteristic chromatographic fingerprint patterns, as shown with a mixture consisting of PEG-400, PEG-1000, PEG-3000, PEG-4000 and PEG-6000. For this reason, the method is well-suited for characterization of samples containing PEGs widely differing in M. In addition, matrix assisted laser desorption ionization time of flight mass spectroscopy (MALDI-TOF/MS) performed with PEG-600, PEG-1000 and PEG-3000 revealed that the optimum degree of oligomer resolution has been achieved by use of the present method.

Introduction
Both polyethers of the polyethylene glycol (PEG) type as well as their O-alkyl (aryllalkyl) derivatives find broad application. Their use as so-called non-ionic surfactants and emulsifiers for pharmaceuticals [1–11] has been described and, owing to their low toxicity, they also play an important role in studies of intestinal permeability and adsorption in man [12–14]. Furthermore, when covalently coupled to proteins and enzymes, extensive changes in protein pharmacology, immunogenicity and enzymatic action [15] are observed. Other novel applications of polyethylene glycol chemistry comprise the synthesis of (I) polyrotaxanes containing a polyether axis threaded with a multitude of cyclodextrin rings having implications for biomolecular recognition, molecular machines and material science [16], (II) novel crown ether-based catenanes [17, 18], (III) linearly linked crown ethers as essential structural elements of macromolecular chains [19] and (IV) polyethylene glycol oligodesoxynucleotide hybrid molecules used for DNA recognition [20].

This list highlights the fact that elucidation of the molecular composition of polyethylene glycol derivatives is of great scientific and industrial importance. For technical samples knowledge of the oligomer distribution is highly important, because either M or the ratio of hydrophobic to hydrophilic structural moieties, expressed by the so-called hydrophobic lipophilic balance (HLB) values, essentially determine the physico-chemical properties of the products.

Among the high resolution chromatographic techniques used for characterisation of PEGs, gas chromatography is only applicable for separation of oligomers with low M and, although yielding excellent resolution, supercritical fluid chromatography is not used, commonly primarily due to the more difficult handling and complexity of the SFC system compared with HPLC. Therefore, owing to the large number of available mobile and stationary phases, reversed-phase high performance liquid chromatography (RP-HPLC) is still the preponderant method for the separation of PEG oligomers [1,3,5, 6,8,10,21–28]. On the other hand normal-phase chromatography has also been reported on bare silica gel and
the so-called bonded phases, containing 2,3-propanediol (diol), aminopropyl (NH₂) and cyanopropyl (CN) substituents grafted to the bare silica gel base material [4,7,9,29–39]. Adequate separation of the 3,5-dinitrobenzoyl derivatives of monoalkylated PEG oligomers on C18 and C8 stationary phases [23] and of PEG dinitrobenzoyl derivatives on an ion-exchange matrix [39] has been also reported. Furthermore, investigations of Alexander et al. [32] revealed that chromatographic resolution \( R_s \) of PEG oligomers on C18 materials increased with decreasing carbon content, which is presumably attributable to the influence of increasing concentrations of polar sites on the stationary phase surface. When the so-called „pseudo reversed-phase” liquid chromatography, characterised by bare silica gel materials and aqueous organic eluents [40–43] is applied, a further gain in \( R_s \) is achievable. In previous investigations chromatographic separation of different types of polyether markedly differing in polarity on different reversed-phase materials [24] was reported. This study reveals good oligomer separation of the more hydrophobic polybutylene glycol (PBG) 1000 and polypropylene glycol (PPG) 1200 samples on C18 and C8 stationary phases, whereas in contrast, relatively low resolution was observed with PEG-1000. However, it should be emphasized that in this case conditions of gradient HPLC had not been optimized for PEG-1000 due to the comparative purpose of the study requiring similar chromatographic conditions for all three polyether types. For this reason, we optimized the chromatographic conditions for separation of PEG samples, markedly different in M, by means of RP-HPLC on a C18 matrix with a binary gradient of acetonitrile and water and signal monitoring by evaporative light scattering detection (ELSD).

### Experimental

#### Materials

The polyethylene glycol samples PEG-200, PEG-400, PEG-600, PEG-1000, PEG-1500, PEG-3000, PEG-4000 and PEG-6000\(^1\) („pract.” quality) were purchased from Fluka (Buchs, Switzerland). Acetonitrile and methanol (all HPLC grade) were from Fluka (Buchs, Switzerland). Water was purified with a Milli-Q reagent water system from Millipore-Waters (Milford, MA, USA). For RP-HPLC a Nucleosil 100 5C18 stationary phase (125 x 4.6 mm, 5 µm particle size, 100 Å pore diameter) from Macherey-Nagel (Oensingen, Switzerland) was used. For increased separation two Nucleosil 100 5C18 columns were connected together by means of a MN coupling kit obtained from Macherey-Nagel (Oensingen, Switzerland).

\(^1\) The numbers indicate the average molecular weight; M, as specified by the manufacturer.

### Analytical Equipment

The HPLC apparatus consisted of a P 4000 quaternary HPLC pump equipped with a vacuum degassing unit, an AS 3000 autosampler with a 100 µL sample loop and a PC 1000 data acquisition unit, all obtained from Thermo Separation Products (San Jose, CA, USA). For detection by means of ELSD a type Sedex 45 a apparatus from SEDERE (Vitry sur Seine, France) equipped with a 20 W iodine lamp was used.

### Chromatographic Separation and Detection

Gradient RP-HPLC was performed on the C18 column with a binary gradient of acetonitrile and water (Table I) at ambient temperature (about 22 °C) and a flow-rate of 1.5 mL min\(^{-1}\). PEG samples (20 mg mL\(^{-1}\) for PEG-200, PEG-400, PEG-600, PEG-1000, PEG-1500 and PEG-3000, 10 mg mL\(^{-1}\) for PEG-4000 and PEG-6000; w/v) were dissolved in methanol and 10 µL aliquots injected. For ELSD the nebulisation chamber was heated to 30 °C and the nitrogen flow was adjusted to 4.5 L min\(^{-1}\) corresponding to an inlet pressure of 200 kPa.

#### Table I: Gradient profile used for chromatographic separation

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>% Acetonitrile</th>
<th>% Water</th>
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<tbody>
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<td>95</td>
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<td>10</td>
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<tr>
<td>70</td>
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### MALDI-TOF/MS Investigations

The experimental conditions are described in Ref. [43]. PEG-600, PEG-1000 and PEG-3000 were used as the targets for MALDI-TOF/MS. The aim of these investigations was to elucidate, if chromatographic separation of these samples into the individual oligomers by means of RP-HPLC would match the number of repeating units obtained by means of mass spectroscopy.

### Results and Discussion

Baseline separation was only achieved with PEG-200 and PEG-400 (Figures 1a,b), but the method also permits satisfactory oligomer separation of PEG-600, PEG-