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Liquid chromatographic–electrospray ionization mass spectrometric analysis of neutral and charged polyethylene glycols

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Abstract

Poly(ethylene glycols) (PEGs) are widely used water soluble and biocompatible polymers. PEGs are suitable as paracellular probes in biomembrane permeability studies because they are hydrophilic and various oligomers have defined molecular sizes. In previous studies corneal and conjunctival permeability for neutral PEGs has been measured, and the results were used to calculate the number and size of the cellular pores. In this study we have developed a high-performance liquid chromatographic–electrospray ionization-mass spectrometric method for analysis of neutral PEGs and positively charged aminoPEGs simultaneously. The new method is fast, accurate, sensitive and specific for high throughput analysis.The method was used to evaluate the paracellular permeability of PEGs through a corneal epithelial cell culture. Paracellular pores are negatively charged and it was in our interest to characterize the interactions of positive charge and size of the molecules with the paracellular pores.

Keywords: Poly(ethylene glycol)

1. Introduction

Poly(ethylene glycols) (PEGs) are widely used water soluble and biocompatible polymers [1,2] that consist of repeating ethylene oxide units. The water solubility of PEGs increases as the molecular mass decreases, 44 mass units per each ethylene oxide unit. PEGs are suitable for paracellular penetration studies because they are hydrophilic and various oligomers have defined molecular sizes. Previously we determined the corneal and conjunctival permeability for neutral PEGs. The results were used to calculate the number and size of the paracellular pores. In general, it is accepted that the paracellular route is negatively charged at physiological pH and due to the charged carboxylic groups arising from amino acid residues of tight junction proteins [3,4]. This route of absorption is important for small-sized hydrophilic compounds. Protonated small-sized hydrophilic compounds such as amino acids permeated the pores faster than their negatively forms [4].

Analytical methods such as high-performance liquid chromatography (HPLC) with a UV detection system and radiolabeled compounds with scintillation counter have been widely used in studying the permeability of cellular monolayers. UV detectors are not selective and they have limited sensitivity for many compounds and the radiolabeled compounds are not always available [5,6]. Mass spectrometry detection is very promising analytical alternative for permeability studies. HPLC sample inlet techniques

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and soft ionization methods as electrospray ionization (ESI) coupled with mass spectrometry make it the ideal technique for analysis [7,8]. A difficulty with PEG determination is that the mass spectral signal is spread out over an oligomer distribution rather than concentrated into a few ions. Thus, a mixture of various PEG oligomers will show relatively low ion abundances compared to a single pure compound [9].

To evaluate the combined effects of charge and size on the permeation of PEGs via the paracellular route, we developed a new HPLC–ESI-MS method for neutral PEGs and positively charged aminoPEGs. The new HPLC–ESI-MS method is fast, sensitive and specific at low concentrations and it performs quantitative analysis of charged and neutral PEGs simultaneously. In this study the chromatographic separation of PEGs was achieved in 5 min. A recent study ([10]) reported an approach that measures the caco-2 paracellular permeability of neutral PEGs separated by HPLC–MS [10]. However, the total chromatographic run time for each sample was 40 min, which is rather long for routine analysis. To our knowledge no direct quantitative HPLC–MS method for aminoPEGs has been published.

Corneal epithelial cell culture were tested using a mixture of PEGs (mean molecular masses of 200, 400, 600 and 1000) and mixture of charged aminopoly(ethylene glycol)s (mean masses 350 and 750). The samples and standards were analyzed with the combination of HPLC and ESI-MS. The method was used to evaluate the paracellular permeability of charged and neutral PEGs through a corneal epithelial cell culture.

2. Experimental

2.1. Chemicals

PEGs (mean molecular masses of 200, 400, 600 and 1000) were purchased from Pressure Chemical (Pittsburgh, PA, USA) and high-quality aminopolyethylene glycols (methoxypolyoxyethylene amine, mean masses of 350 and 750) were a gift from Dr. Etienne Schacht (University of Ghent). The internal standard tetraethylene glycol dimethyl ether (molecular mass 222.28) was chosen because of having a similar chemical structure but no common ions with PEGs and it was obtained from Sigma–Aldrich (Gillingham, UK). HPLC grade acetonitrile was from Rathburn (Walkerburn, UK), ammonium acetate from Riedel-de Haen (Seelze, Germany). Water was deionized and purified using a Milli-Q system from Millipore (Bedford, MA, USA). Balanced salt solution (BSS; BSS Plus) was used as a buffer solution and was obtained from Alcon (Fort Worth, TX, USA).

2.2. Preparation of standard solutions

Stock solutions of the PEGs, aminoPEGs and their combination (each 100 μM) were prepared in BSS Plus and buffered at pH 7.4 with 0.5 M NaOH. Standard solutions (0.1, 0.25, 0.5, 1.0 and 3 μM) and control solution (0.2 μM) were prepared by diluting stock solutions with the same buffer. Computational apparent pK_a value of aminoPEG (methoxypolyoxyethylene amine, $CH_3O[CH_2CH_2O]_nCH_2CH_2NH_2$) is 8.75±0.10 calculated by ACDLABS ACD/pK_a program (Version 4.56/26 April 2000). Therefore, aminoPEG is expected to be positively charged at physiological pH. The I.S. (tetraethylene glycol dimethyl ether) was dissolved to 10 μM in BSS Plus solution. Both the stock and the I.S. solutions were stored in a cold place until use.

2.3. Sample preparation

Corneal epithelial cells were grown on filters as described previously [11]. Stock solutions (100 μM) were added to the apical side of the cell monolayer (donor) and PEG-free buffer was added to opposite, basolateral side. Samples from the apical and basolateral side of 6-well plates were collected for analysis. The samples from the apical side were diluted before determination of the initial concentration. The samples from the basolateral side were taken every 30 min from time points 0 to 240 min and replaced with fresh buffer. The samples were stored at −20 °C until analysed by HPLC–ESI-MS. A 20-μl volume of I.S. (10 μM) was added to 100 μl of each sample before centrifugation at 13 000 rpm for 5 min (Biofuge Fresco, Heraeus Instruments, Germany) and before application to HPLC.

2.4. Liquid chromatography

The HPLC system consisted of a Rheos 4000 pump (Flux Instruments, Danderyd, Sweden) and a Rheodyne 7725 injector with a 20-μl loop (Cotati, CA, USA). The HPLC gradient was 2–31% acetonitrile (containing 10 m*M* ammonium acetate, pH 6.62) in 4.5 min and the run was performed over 6 min. The composition was returned to the initial conditions for 1.5 min before the next injection. The flow-rate was 200 μl/min and the reversed-phase column was a Xterra C_{18}, 2.5 μm, 20×2.1 mm (Waters, Milford, MA, USA).

2.5. Mass spectrometry

All HPLC–ESI-MS measurements were performed with a LCQ quadrupole ion trap mass spectrometer equipped with an ESI ion source (Finnigan, San Jose, CA, USA) and positive ion spectra were obtained. The electrospray was stabilized using nitrogen sheath gas (flow 90) and the spray needle voltage was 3.5 kV. The inlet capillary was heated to 200 °C and the tube lens offset was set to 15 V. The parameters used in the mass spectrometer were optimized manually and tuning was done using standard solution (1 μ*M*). The response was measured by comparing the intensity of peaks. The full scan mass spectra from *m*/*z* 190 to 1000 were measured. Collection time of the ions was 500 ms in the trap and two scans were summed. During the analysis an automated divert valve was installed, and the eluent was diverted to waste for 1.5 min to remove salts and other impurities. The concentrations of the samples were determined using peak areas of the chromatograms and the standard curve.

3. Results and discussion

3.1. Chromatography

In this study PEGs and aminoPEGs were separated and measured effectively using the combination of HPLC and ESI-MS. Chromatographic separation of 34 PEG oligomers (neutral and charged) was

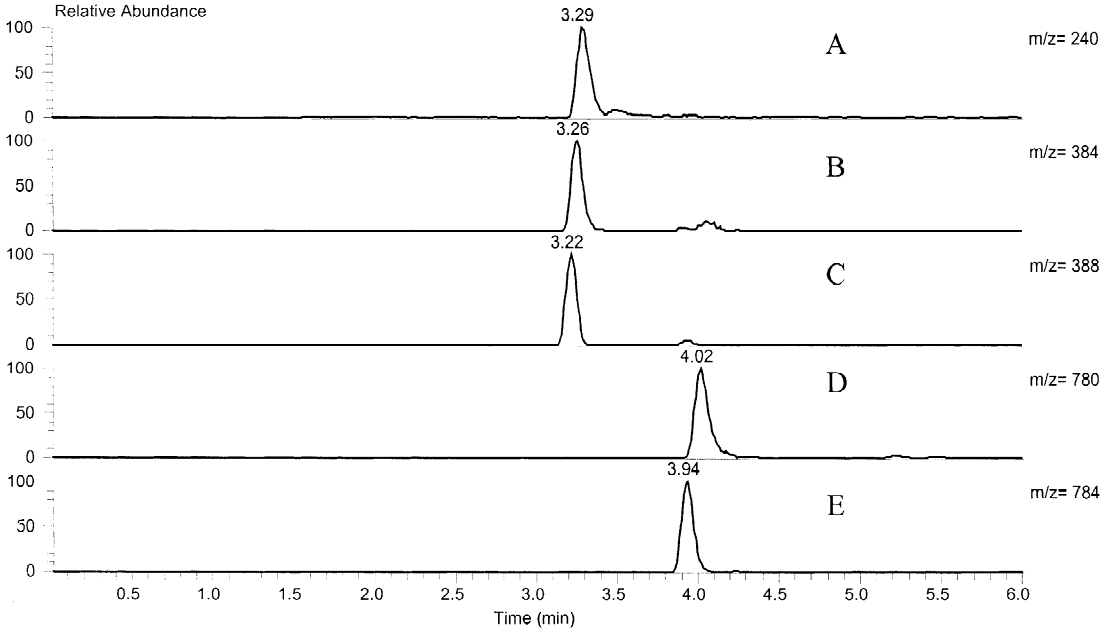

Fig. 1. Extracted ion chromatogram of PEG oligomers by HPLC–ESI-MS. Mixture of (A) internal standard 240 $(M+NH_4)^+$, (B) aminoPEG 384 $(M+H)^+$, (C) PEG 388 $(M+NH_4)^+$, (D) aminoPEG 780 $(M+H)^+$ and (E) PEG 784 $(M+NH_4)^+$ in the standard solution. Conditions: the reversed-phase column was a Xterra C_{18}, 2.5 μm, 20×2.1 mm. HPLC gradient was 2–31% ACN (containing 10 m*M* ammonium acetate, pH 6.62) in 4.5 min and the composition was returned to the initial conditions for 1.5 min, at a flow-rate of 200 μl/min.

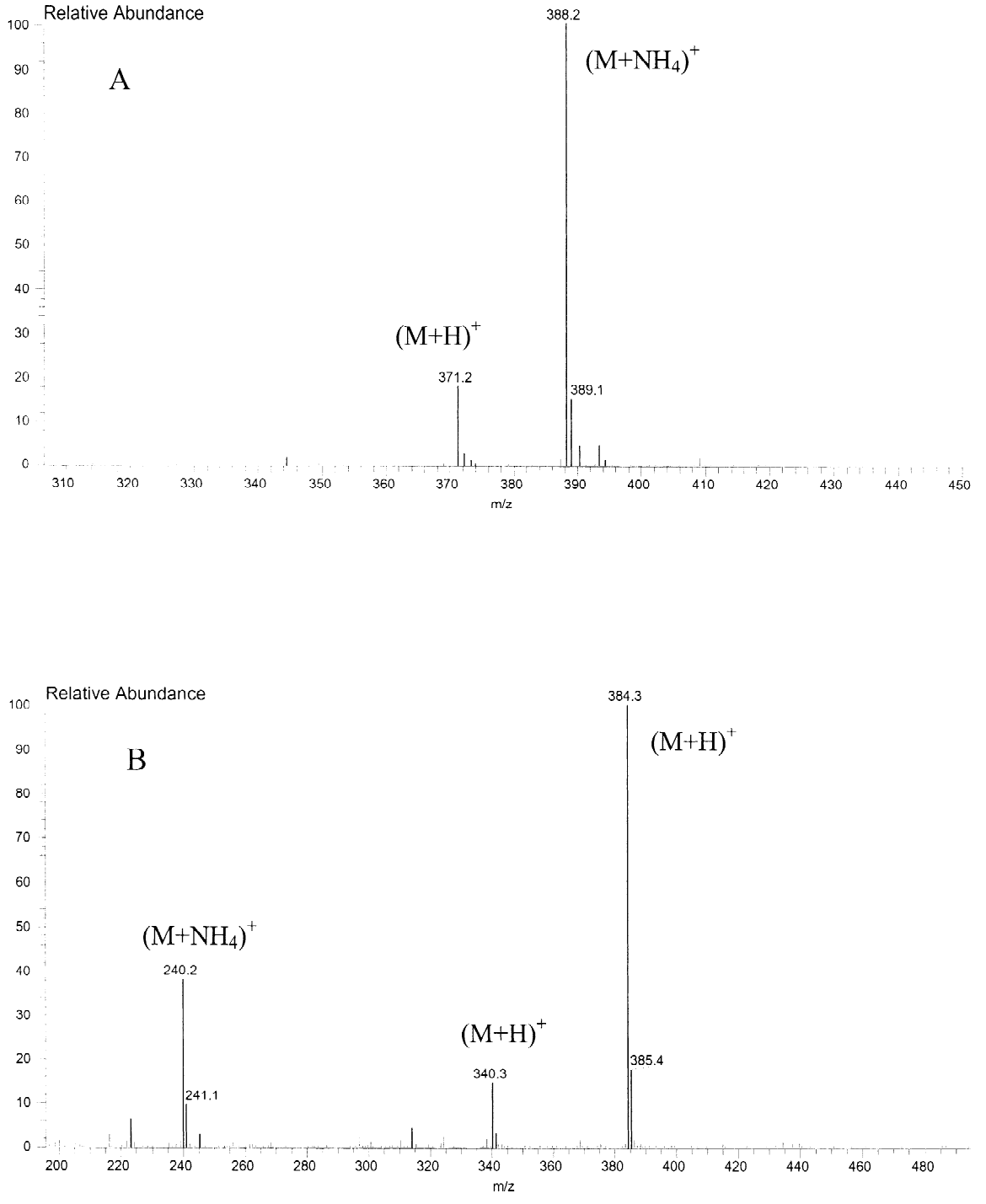

Fig. 2. HPLC–ESI-MS spectrum of neutral PEG, charged aminoPEG and internal standard. (A) Mass spectrum of PEG ion $(M+NH_4)^+$ at m/z 388 and $(M+H)^+$ at m/z 371. (B) Mass spectrum of two different aminoPEG oligomer ions $(M+H)^+$ at m/z 340 and 384. The internal standard shows $(M+NH_4)^+$ ion at m/z 240.

Table 1
Within-day LC–ESI-MS repeatability of PEGs and aminoPEGs

	LC–ESI-MS (0.2 μ*M*)													*n*
PEG	344	388	432	476	520	564	608	652	696	740	784	828	872	
RSD (%)	10.7	18.3	45.5	9.7	5.4	6.7	5.3	6.0	3.4	8.6	7.2	6.4	6.0	6
Mean	0.232	0.223	0.210	0.228	0.249	0.249	0.250	0.244	0.233	0.248	0.226	0.230	0.224	
NH_2PEG	340	384	428	472	516	560	604	648	692	736	780	824	868	
RSD (%)	5.9	5.9	3.5	6.5	10.4	16.4	5.5	4.6	4.1	8.9	9.4	12.9	19.7	6
Mean	0.207	0.203	0.209	0.199	0.199	0.208	0.204	0.210	0.204	0.201	0.201	0.214	0.224	

achieved in 5 min with fast gradient elution and short reversed-phase column (Fig. 1). I.S. (*m*/*z* 240) and two representatives of neutral PEGs (*m*/*z* 388 and 784) and aminoPEGs (*m*/*z* 384 and 780) were selected to show the separation. As seen in Fig. 1 measurements on mixtures of the oligomers showed a good response although PEGs and aminoPEGs were close to each other. The effect of mobile phase was tested by using acetonitrile and methanol. Acetonitrile–water solution offered the best sensitivity and enabled fast analysis (data not shown). Number of analyzed samples per day was 140–150. After samples with a high concentration of PEGs the column was contaminated and had to be washed with eluent before injecting the next sample. The problems with contamination were minimized by analysing the low concentration samples first, i.e. analysis of samples were performed from time points 0 to 240 min.

3.2. Mass spectrometry

The mass spectrometer parameters were optimized manually. The signal intensities were tripled by using a tube lens with a value of 15 V instead of 40 V. Furthermore, the intensities were doubled by using a temperature of 200 °C for the heated inlet capillary instead of 225 °C. The ionization conditions were chosen so that the intensity of single charged PEG molecular ions were greater than the intensity of multiple charged molecules. Concentrations of the PEG and aminoPEG samples were determined using single charged molecules. The full scan mass spectra from *m*/*z* 190 to 1000 were measured with no fragmentation. Normally, neutral PEGs were seen as sodium adducts [10,12]. In this study neutral PEG molecules (*m*/*z* 256–960) represented ammonium adducts $(M+NH_4)^+$ (Fig. 2A) and aminoPEGs (*m*/*z* 252–956) were seen as protonated molecules $(M+H)^+$ (Fig. 2B). Intensity of the PEGs with high- and low-molecular masses were poor due to the molecular-mass distribution. Salts and other impurities did not have any effects on the results of the measurements, most of the impurities had been diverted to waste. Typically, the PEGs were detected in basolateral samples after 30–60 min. The calibration curve was generated for 34 PEG oligomers by a concentration range of 0.1–3 μ*M* PEGs (five point curve, three replicate standards) and R^2 values ranged between 0.9469 and 0.9985. The reproducibility of the analysis method was tested by multiple injections of 0.2 μ*M* quality control sample ($n=6$) (Table 1).

3.3. Transport across corneal epithelial (HCE) cell culture

The HPLC–ESI-MS method was used to evaluate the paracellular permeability of neutral and charged PEGs through a corneal epithelial cell culture. When the PEGs were applied to the apical side of the cell culture, linear fluxes were observed for all oligomers to the basolateral side of the cell culture. Fig. 3 shows the time courses of the neutral PEGs (*m*/*z* 388 and 784) and aminoPEGs (*m*/*z* 384 and 780). The current results shows that permeation is size dependent with PEG molecules, low-mass oligomers permeated the pores faster than high-mass ones. This well-known size dependent result is suitable for calculating the number and size of the corneal epithelial cellular pores. The result is also useful for evaluating the combined effects of charge and size on the paracellular permeation of PEGs. Positive charge appears to slow down paracellular permeation

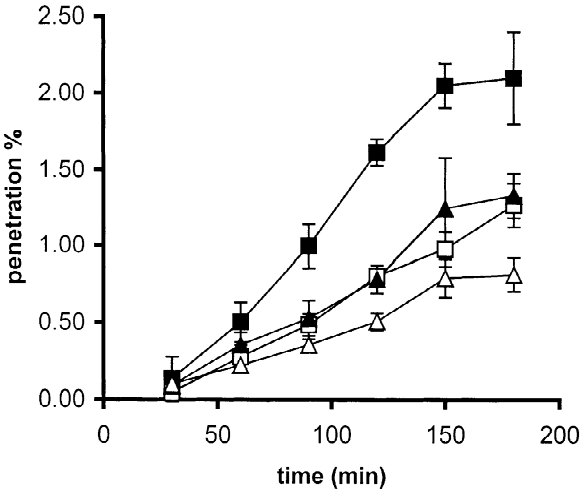

Fig. 3. Time course of neutral PEG and charged aminoPEG oligomer penetrations across corneal epithelial cell culture. Figure shows the effect of size and charge on the permeation of PEGs. The samples from the basolateral side of 6-well plates were taken every 30 min and were analysed by HPLC–ESI-MS. The time profiles of the neutral PEGs m/z 388 (■), m/z 784 (□) and positively charged aminoPEGs 384 m/z (▲), 780 m/z (△) are presented, ($n=2$ with aminoPEGs and $n=3$ with PEGs, average±SD).

of PEGs in the corneal epithelial culture. This result is in line with earlier report with tripeptides [4], but in contrast with the published protonated amines data [13]. Molecular size and charge may have combined effects on paracellular permeability.

4. Conclusion

The new HPLC–ESI-MS method allows fast analysis and accurate determination of neutral PEGs and aminoPEGs simultaneously. It is suitable for rapid high throughput routine analysis and this method can be applied in paracellular permeability studies with biomembranes.

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