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Development of a stable isotope dilution LC–MS assay for the quantitation of multiple polyethylene glycol (PEG) homologues to be used in permeability studies

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ABSTRACT

A new quantitation method based on a multiple stable isotope dilution assay (SIDA) was developed for polyethylene glycol (PEG) homologues from PEG mixtures with average molecular weights (MW) of 400, 1500, 3000 and 4000 Da in urine. Seven [$^{13}C_4{}^2H_4$] and two [$^{13}C_8{}^2H_8$]PEG homologues were synthesized and served as labelled internal standards for SIDA. PEG oligomers were resolved by reversed phase high performance liquid chromatography (RP-HPLC) coupled to mass spectrometry (MS) in multiple ion (MI) scan modus. Very low limits of detection (LODs) in a range of 0.4–12 ng/mL were achieved for the single homologues. Higher PEG homologues showed increased LODs and LOQs and less effective recovery (77–87%) than PEG with lower molecular masses (95–121%). Precision (relative standard deviation) varied between 3 and 13% and showed no dependence of the chain length. The method was successfully applied to human and mice urine samples. Beside an accurate quantitation of single PEG homologues it was possible to show an alteration in the MW distribution in urine samples compared to the dosed PEG solutions. The highest MW, with which a PEG can pass the intestinal wall (so called "cut off") for humans appeared to be higher than for mice.

1. Introduction

Polyethylene glycols (PEG) are synthetic polymers of ethylene oxide with the general molecular formula $H(OCH_2CH_2)_nOH$, where *n* is the number of ethylene oxide units. They are often used in cosmetic and pharmaceutical products as basis for ointments or as carrier for active agents. The widespread use of PEG is based on their high water solubility, nontoxicity as well as their chemical and microbiological inertness. Furthermore they are available as mixtures of differently sized homologues in broad ranges of molecular weight (MW) distribution and are rapidly excreted into urine. In particular the latter two properties render them suitable markers for assessing intestinal barrier functions, e.g. in studies of inflammatory bowel diseases (IDB). According to the common hypothesis of trans-epithelial penetration in sound biological membranes a general decrease of intestinal permeability can be observed with an increase of PEG molecular weight detectable in urine. To study membrane permeability, Chadwick et al. [1] were the first to report data on PEG 400 as a marker and described its excretion depending on the dose. Later on, the PEG spectrum was extended to mixtures with higher MW ranging between 1000 and 10,000 Da [2–5]. With this extension a decrease of permeability with increasing MW as well as differences in permeability of high MW PEG between healthy persons and patients with IDB, e.g. Crohnís disease, were observed [3]. Whereas PEG 400 is readily volatile after derivatization and can be analyzed by gas chromatography (GC) coupled to flame ionization detection (FID) [1], new analytical methods mostly based on reversed phase high performance liquid chromatography (RP-HPLC) for the higher PEG were developed. PEG are very simply structured molecules, which do not possess any chromophores and thus ultra violet or fluorescence detection are not suitable for their analysis. Escott et al. [6] circumvented this shortcoming by using low UV wavelength detection, but in practice evaporative light scattering detection (ELSD) or refractive index (RI) detection are more frequently applied [2,3,5,7]. Lately, detection by mass spectroscopy (MS) found its way into PEG analysis. Using MS technology, short measurement periods with ultra HPLC (UHPLC) or complete waiving of separation in flow injection analysis (FIA) MS are favored,

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without loss of accuracy. However, with the latter method only short-chained PEG (PEG 300, PEG 400) [8–10] and branched 40 kDa PEG [11] have been examined in biological or pharmaceutical samples. At present, electrospray ionization (ESI) with detection of the protonated molecules or the sodium, ammonium or potassium adducts have been applied most commonly. There are also reports on the successful use of in-source collision-induced dissociation (in-source CID) of protonated high molecular PEG and methoxyl-PEG (8 and 40 kDa) [11,12] but this detection method lacks information about single PEG homologues. However, ESI is highly susceptible to ionization suppression or enhancement and, therefore, accurate quantitation needs either (a) tedious matrix calibration or (b) standard addition or (c) isotope-labelled internal standards, the latter of which have become the reference methods e.g. in clinical or sections of food analysis [13,14]. Therefore the purpose of this study was the development of a stable isotope dilution assay (SIDA) as quantitation method for selected PEG homologues out of mixtures with average MW of 400, 1500, 3000 and 4000 Da and an adequate sample preparation for human and murine urine.

2. Materials and method

2.1. Chemicals and reagents

Polyethylene glycol mixtures with nominal molecular masses (M_r) of M_r 400, M_r 1500, M_r 3000 and M_r 4000 Da were obtained from Merck (Darmstadt, Germany). The M_r number corresponds to the average molecular mass of the mixtures and subscript numbers indicate the repetition of ethylene oxide units, e.g. PEG 400 consists of 10 homologues (PEG_6–PEG_{15}) with a Gaussian distribution and a mean M_r of 400 Da (mass range: 282–678 Da). The other mixtures are characterized as follows: PEG 1500 (PEG_{20}–PEG_{45}; mass range: 899–2000 Da), PEG 3000 (PEG_{51}–PEG_{90}; mass range: 2264–3982 Da) and PEG 4000 (PEG_{75}–PEG_{115}; mass range: 3322–5084 Da). PEG mixtures were the same for permeability studies and analytical procedures. Sodium hydride (60% as dispersion in mineral oil), sodium iodide, lithium aluminum deuteride and D_4-methanol were from Sigma Aldrich (Steinheim, Germany). [$^{13}C_2$]bromoacetic acid was obtained from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA) and all solvents were from Merck (Darmstadt, Germany) and were of analytical reagent grade.

2.2. Urine samples

Human and murine urine samples were provided from the Chairs of Nutritional Medicine of the Technische Universität München as part of the human study approved by the Ethics Committee of the Faculty of Medicine of the Technische Universität München (File 5499/12) and the animal experiments performed with permission from the district government of Upper Bavaria (Regierung von Oberbayern, reference number AZ 55.2.-1-54-2532-116-11), respectively. Human subjects ingested a mixture of PEG 400 (1 mg), PEG 1500 (200 mg), PEG 3000 (4 g) and PEG 4000 (4 g), whereas 12 weeks old BL/6J mice were subjected to dosing of PEG 1500 (3 mg) and PEG 3000 (10 mg). The sampling intervals for urine of humans and mice were 24 h and 14 h, respectively.

2.3. Purification of unlabelled PEG homologues by preparative HPLC-UV

Single PEG homologues were isolated from the PEG 400, 1500, 3000 and 4000 mixtures. Therefore, a preparative HPLC-UV system equipped with UV detector (Saphire 600 Variable Wavelength Detector; ECOM spol.s r.o. Americka, Praha, Czech Republic), pumps (HD 2-400; BESTA-Technologie für Chromatographie GmbH, Wilhelmsfeld, Germany), sample injector (FCV-20AH2; Shimadzu, Kyoto, Japan), degasser (FLOM GASTORR VG-25 4; Fischer Analytics GmbH, Bingen am Rhein, Germany) valve unit (KRONLAB high Speed Valve; YMC Europa GmbH, Dinslaken, Germany) and PrepCon 5 software (YMC Europa GmbH, Dinslaken, Germany) was used. The wavelength was set to 190 nm and the mixtures were separated on a semi-preparative HPLC column (YMC, ProC18, 150 × 10 mm I.D., 5 μm) with water (A) and acetonitrile (B) at flow rates of 2 mL/min (PEG 400 and 1500) or 1 mL/min (PEG 3000 and 4000) and different gradients. For PEG 400, the starting conditions of 10% B was held for 1 min followed by raising the content of B to 24% within 19 min, which was held for 3 min. Thereafter, the gradient was brought within 1 min to the starting conditions and the column was equilibrated for 7 min. PEG 1500 were eluted stepwise with 27% B for the first 13 min and 30% (B) until 24 min, followed by 7 min of equilibration. A mixture of PEG 3000 and 4000 was separated into fractions under isocratic conditions at 35% B. After solvent removal and lyophilisation each fraction was rechromatographed with 35% B and separated in purer sub fractions. This procedure was repeated until only one PEG homologous remained in the fraction. Acetonitrile was evaporated and the remaining water content was removed by freeze drying.

2.4. Synthesis of ^2H- and ^{13}C-labelled standards

The synthesis of labelled PEG homologous was carried out following the approach of Abello et al. [15] for the synthesis of labelled PEG monomethyl ether with some significant modifications for the synthesis of PEG. Accordingly, [$^{13}C_2$]bromoacetic acid (3 equiv), sodium iodide (0.01 equiv) and sodium hydride (as 60% dispersion in mineral oil; 10 equiv) were added to a solution of the PEG homologous in dichloromethane (1 mL/10 μmol). After stirring for 48 h at 50 °C the reaction vessel was cooled to 5 °C and $LiAlD_4$ (7 equiv) was added. After 3 h the $LiAlD_4$ excess was quenched with methanol. The obtained suspension was centrifuged (5 min, 14,000 rpm) and the residue was extracted twice with 1 mL DCM. The combined organic phases were washed to neutral pH with water, then brought to dryness by means of a mild stream of nitrogen and the residue was dissolved in water/acetonitrile (70/30; v/v) for purification with the preparative HPLC-UV at the same conditions as mentioned above. For labelled standards in the mass ranges of PEG 3000 and 4000 the synthesis and purification was repeated.

2.5. Quantitative nuclear magnetic resonance (qNMR) spectroscopy

Isolated PEG homologues were subjected to qualitative and quantitative ^1H NMR spectroscopy. Lyophilisates were dissolved in 600 μL D_4-methanol (Sigma Aldrich, Steinheim, Germany), transferred into NMR tubes (5 × 178 mm; Bruker BioSpin Corporation, Fällanden, Switzerland) and analyzed with a 500 MHz Avance III NMR spectroscope (Bruker BioSpin Corporation, Fällanden, Switzerland). An external calibration with L-tyrosine and integration of the two protons at 7.1 ppm was carried out for quantitative ^1H NMR, detailed data are reported elsewhere [16], [17]. PEG homologues were quantitated by the signal of 4 protons at 3.58 ppm. Furthermore the ^1H NMR of [$^{13}C_4{}^2H_4$]PEG_{30} was recorded.

2.6. LC–MS method

2.6.1. Liquid chromatography (LC)

PEG oligomers were partially resolved by a liquid chromatography system consisting of pumps (LC-10Advp), sample injector (SILHTC) column oven (CTO-10Svp) and controller (SCL-10Avp), all components of the LC equipment were from Shimadzu