Newsletter

Volume 7, Number 1

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Phospholipid
Forschungszentrum/Research Center
Heidelberg

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Introduction

This year we will organize for the third time our symposium on Phospholipids in Pharmaceutical Research. The symposium will take place on September 16 and 17 in Heidelberg. You are invited to attend and to actively participate by means of e.g. a poster presentation. Furthermore, we would appreciate, when you could inform possibly interested colleagues on this event.

Focus of this symposium will be the properties and analysis of phospholipid excipients from pharmaceutical technological perspective. An in depth knowledge of these properties is an absolute prerequisite to use and rationally select the best phospholipid(s) for formulations and corresponding manufacturing procedure and conditions, taking their physic-chemical properties into consideration.

Phospholipids are excipients with an enormous scope of useful properties. For instance, upon mixing with water they may form, dependent on their chemical structure, e.g. lamellar, micellar or hexagonal phases. They can be used for preparation of liposomes, emulsions, micelles and mixed micelles and as wetting agent. The fatty acid composition, determines the phase transition temperature of the phospholipids. Above this temperature flexible structures are formed whereas below this temperature more robust structures are formed. When required, the formulations can have a neutral charge or can be given a preferred net charge by selecting neutral (zwitterionic), negatively or positively charged phospholipids, respectively. From production perspective, phospholipids form natural origin or synthetic phospholipids play a role in pharmaceutical technology.

This diversity allows the formulator to design the best possible formulation for their drug substance by selecting the appropriate phospholipids or phospholipid mixtures yielding the formulation with the desired properties in terms of e.g. compatibility with the drug substance, stability, tissue affinity and release characteristics etc.

Seminars devoted to this focus will be given by reputed specialists in phospholipid sciences. A detailed program you can find on our web page www.phospholipid-institute.com.

See you in Heidelberg!

PD Dr. Peter van Hoogevest
Managing Director
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Phospholipid Research Center News

a) Attractive Membership

In 2012 the membership of The Research Center was made more attractive by compiling a list of capabilities in phospholipid research of the members. At this way members will be able to interact easily when they look for advice and cooperation options with other phospholipid experts. Please note that we strive to get every active member registered in this file. The list of capabilities will be placed on a password protected area of the webpage.

Funding of workshops to encourage scientific discussions on selected topics among experts in the phospholipid area was another tool created: So far we received three proposals. We would like to encourage you to profit from this opportunity. Select an exciting, maybe even controversial workshop topic with exciting seminars from reputed experts in the field;

summarize the discussions in a proceeding and a significant contribution to the phospholipid research area has been made!

For more information on funding of workshops and research projects please visit our website: www.phospholipid-institute.com

b) Meeting of the Scientific Board, January 14, 2013 in Ludwigshafen am Rhein

Participants

Prof. Alfred Blume (Scientific Board)
Prof. Gert Fricker (Scientific Board)
Dr. Frank Martin (Scientific Board)
Prof. Christel Müller Goymann (Scientific Board)
Dr. Ralf-Olaf Quinkert (Scientific Board)
Dr. Herbert Rebmann (PRC)
PD Dr. Peter van Hoogevest (PRC)
Mr. Armin Wendel (PRC)
Dr. Jürgen Zirkel (PRC)

The next Board meeting will be on July 8, 2013 in Halle (Saale). Research proposals should be submitted before June 1, 2013. These proposals should fulfill the requirement described on the webpage.

c) Funded projects

The following projects are ongoing:

Investigation of cochleate formulation and cochleate-cell membrane interactions“
Prof. Alfred Fahr, University of Jena, FRG.

“Phospholipid/Tetraetherlipid based liposomes for oral administration of Hepatitis B Virus-derived lipopeptides for hepatocyte-specific drug delivery”
Prof. Gert Fricker, PD Dr. Walter Mier, Prof. Stephan Urban, University of Heidelberg, FRG.

“Zincphthalocyanine-containing multi-targeting liposomes for the treatment of solid tumors by photodynamic therapy”
Dr. Michal Heger, University of Amsterdam, The Netherlands.

“Interactions of the tumour-targeting vector peptide pHLIP with phospholipids”
Prof. Sandro Keller; University Kaiserslautern, FRG.

“Hydrogenated Phospholipids as anti-metastatic agents”
Prof. Ulrich Massing, KTB Univeristy Freiburg. FRG.

„A Lipid Based Drug Delivery System for CNS Applications“
Dr. Valerie Reichel, University of Heidelberg, FRG.

„Mechanistic Action and Adverse Event of therapy with delayed release - Phosphatidylcholine in a genetic mouse model of ulcerative colitis“
Prof. Wolfgang Stremmel, University Clinic of Heidelberg, FRG.

"Characterization and optimization of omega-3 intravenous phospholipid stabilized diglyceride emulsions for the treatment of organ ischemia"
Prof. Richard Deckelbaum; Columbia University, USA.

"Distribution of phospholipid based drug carriers into organs and tumors – monitoring by mass spectrometry imaging"
Prof. Carsten Hopf; University Mannheim, FRG.

"Oral anticancer-indirubins: Solubility enhancement, permeability and bioavailability assessment of phospholipid containing (SMEDDS) formulations"
Dr. Anne Mahringer, University Heidelberg, FRG; Prof. Eisenbrand University Kaiserslautern, FRG and Prof. Brandl, University Odense, Denmark.

"Impact of Phospholipid Oxidation on Biophysical Properties of Membranes"
Prof. Motomu Tanaka; University Heidelberg, FRG

The Board approved the following projects as requested or after revision.:

Phospholipid Based Solid Dispersions for Enhanced Oral Absorption of Poorly Soluble Drugs.
Prof. Annette Bauer-Brandl, University Odense, Denmark

Investigation of the interaction of liposomal formulation containing poorly water soluble drugs with human plasma.
Prof. Alfred Fahr, University Jena, FRG.

d) Publications

Following publications based on projects supported by the PRC were recently issued:

Bonechi C, Martini S, Ciani L, Lamponi S, Rebmann H, Rossi C., Ristori S 2012. Using Liposomes as Carriers for Polyphenolic Compounds: The Case of Trans-Resveratrol. PLoS ONE 7(8):e41438.

Huesch J, Gerbeth K, Fricker G, Rebmann H, Zsilavecz M, Tawab M 2012. Effect of Phospholipid-based Formulations on solubility, permeability and absorption of Boswellia serrata Extract. J Nat Prod 75 (10):1675–1682.

De Kroon AIPM, van Gulik TM, Heger M 2012. Development of interstitially targeted liposomes with Zn-phthalocyanine for application in photodynamic therapy. Free Rad Biol Med 53:122.

Special Dosage Forms with Phospholipids: Mixed Micelles for Intravenous Administration

Mixed micelles comprising phospholipids and bile salts, pose, besides oil-in-water emulsions, using phospholipids as emulsifier, and liposomes, alternative phospholipid-based formulation options to solubilize poorly water soluble drug substances for intravenous administration. In these formulations the phospholipid is added to the bile salt to eliminate its hemolytic properties[1]. Minimum weight ratios of bile salt to phospholipid of 0.7-0.8 are needed to obtain clear solutions[2].

Mixed micellar formulations can be prepared by dissolving the phospholipid component in an aqueous

solution of the bile salt, followed by dissolving of the drug substance. Alternatively a blend of phospholipid and bile salt, with or without drug substance can be-manufactured by drying from organic solvent or aqueous solutions, followed by hydration of the dry blend. The aqueous mixed micellar formulation is then sterilized by means of sterile filtration and when needed lyophilized.

This type of formulation was for the first mentioned in the literature in 1909 by B. Moore[3]. In 1916, H. Wieland mentioned the use of mixed micelles to solubilize poorly water soluble drugs[4]. Nattermann patented in 1965 mixed micelles containing a phospholipid with essential fatty acids[5], whereas in 1976 Hoffmann La Roche patented a mixed micellar formulation for diazepam[6]. Since then mixed micelles formulations are used in several injectable products to solubilize poorly water soluble drug substances or vitamins for intravenous administration (Table 1). There are also injectable mixed micellar products on the market with phospholipids comprising essential fatty acids as active. They are used for e.g. removal of tissue fat (liposuction), lowering of blood cholesterol levels (lipid regulation) and liver protection. In all products a high quality, parenteral grade, soy phosphatidylcholine is being used as essential phospholipid component in the mixed micelles and sodium glycocholate or sodium deoxycholate as the bile salt component.

Table1. Injectable products comprising mixed micelles of phospholipids and bile salts

Product	Drug Substance/Active	Company	Indication
Rimadyl	Carprofen	Vericore/Pfizer	NSAID (vet. use)
Rycarfa	Carprofen	Krka	NSAID (vet. use)
Valium MM	Diazepam	Roche	Anxiolytic
Essentiale	PPC*	Sanofi Aventis	Liver therapy
Phosphogliv	PPC	Phs	Liver protection
Lipodissolve	PPC	Pharmastandard	Liposuction
LipoJection	PPC	Amedis	Liposuction
Lipostabil	PPC	Nattermann	Lipid regulation
Plaquex	PPC	Biorica	Liposuction
Cernevit	Vitamin blend	Baxter-Clintec	Vitamin supplement
Konakion MM	Vitamin K	Roche	Vitamin supplement

)* polyene phosphatidylcholine

In the literature, the exploration of mixed micelles for other drugs like teniposide[7], taxol[8], amphotericin B[9], cyclosporine[10], tetrazepam[11], lorazepam[11.12] clonazepam[11.13], and indomethacin[14] is described. Pharmaceutical industry is, considering the claimed high percentage of lipophilic drugs in NCE pipelines

and the low number of products using mixed micellar formulations, probably not prioritizing mixed micelles for development of solubilizing formulations. The publication of preclinical safety assessment of mixed micelles[15] and safety assessment in humans[16] should, however actually encourage the use of mixed micelles in pharmaceutical development projects. These publications also underscore the complete acceptance of natural phospholipids for parenteral administration.

In conclusion, mixed micelles, comprising the key excipients soy bean phosphatidylcholine and bile salt, present a very attractive solubilizing formulation technology because:

- the excipients are used in marketed products in the EU and USA, suggesting adequate stability and acceptance by regulatory authorities,
- the excipients are biocompatible and are natural components present e.g. in blood,
- the excipients are available in parenteral quality,
- they can be produced using relatively simple tech nologies,
-.they have no risk for anaphylactic reactions compared to synthetic solubilizers like Tween, Cremophor and Solutol.

The only disadvantage of mixed micelles may be the possible change of particle size as results of dilution with infusion media like 0.9 % NaCl or 5 % glucose, due to formation of larger micelles and eventually liposomes. Dilution protocols have therefore be standardized and prescribed.

Interestingly, the mixed micelles are also suitable for the oral administration to children (Konakion MM). This means that the same formulation can be explored for oral as well as parenteral administration, allowing a. very efficient pharmaceutical development of dosage forms.

Therefore, the mixed micelle technology can be considered as a “sleeping beauty”, which deserved to be kissed awake by “formulation princes”.

PD Dr. P. van Hoogevest

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Special Lipids: Sphingomyelins

Sphingomyelin, first isolated by Thudichum in 1884 from brain tissue[1], is a polar, amphiphilic, zwitterionic phospholipid containing choline. Its structure is, however, significantly different from phosphatidylcholine: sphingomyelin is not a glyceride, but contains a sphingoid base. This base comprises a long-chain amino alcohol, which is either a mostly monounsaturated diol, i.e. sphingosine; or the saturated analogue, i.e. sphinganine (Fig. 1).

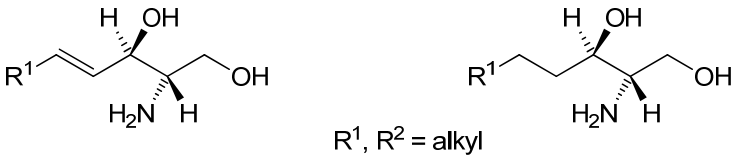

Figure 1. The sphingoid bases sphingosine (left) and sphinganine (right).

In order to get the sphingomyelin structure, a polar phosphocholine head group is attached to the respective primary hydroxyl group and one fatty acid is attached *via* an amide bond to the amino group of the sphingoid base, while the secondary, in the case of sphingosine allylic, hydroxyl group is free and not esterified. The resulting molecular structures of sphingomyelin, either containing sphingosine or sphinganine, are provided in Fig. 2.

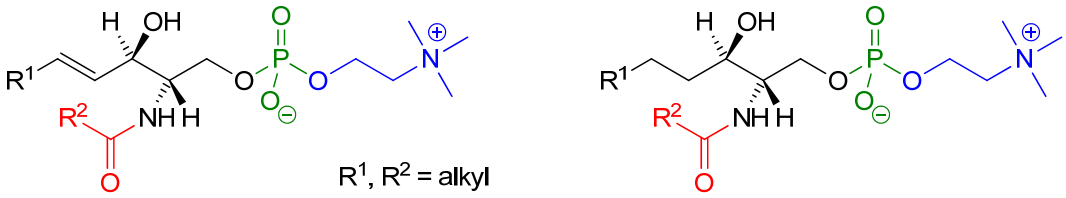

Figure 2. Sphingomyelin with sphingosine (left) and sphinganine backbone (right).

N-Acylated sphingoid bases without polar head group are called ceramides (Fig. 3).

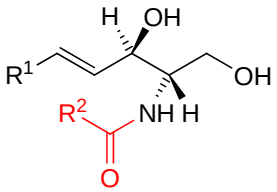

Figure 3. Structure of a ceramide.

Mammalian sphingomyelin usually contains sphingosines with 18 or 20 carbon atoms[2]. The fatty acids with chain lengths of 14–30 carbon atoms are mostly saturated. A widely used nomenclature for the sphingoid bases is d18:1 (the diol sphingosine with 18 carbon atoms and one double bond), d18:0 (sphinganine, the saturated diol) or t18:0 (phytosphingosine, a saturated triol of mainly plant origin). Naturally occurring sphingomyelin has the D-*erythro*-(2*S*,3*R*) configuration[3].

Because of the low reactivity of the amide bond compared to ester bonds, sphingomyelin is not hydrolysed as easily as ester phospholipids. Thus, it is stable to mild alkaline hydrolysis, a characteristic that many analytical and purification methods take advantage of, because it simplifies the separation of sphingomyelin from hydrolysable polar lipids[4]. Sphingomyelins are cylindrical molecules similar to phosphatidylcholines and form bilayers in aqueous systems[5]. Because of their very long acyl chains and high degree of saturation, their transition temperatures are relatively high, mostly distinctly above 30 °C.

The biosynthesis of sphingomyelin proceeds *via* the phosphorylcholine transfer from CDP choline to the primary hydroxyl group of a ceramide[6] or, predominantly, by the phosphorylcholine transfer from a phosphatidylcholine to a ceramide mediated by a ceramide cholinephosphotransferase[7,8]. Sphingomyelin is not found in plants or microorganisms, but is ubiquitous in human and animal cell membranes, its content usually being lower than the PC content. It is not evenly distributed in the cells: the plasma membrane has the highest content of sphingomyelin (mostly located in its outer leaflet[5]), followed by the Golgi apparatus and the endoplasmic reticulum. The membranes of the nucleus and mitochondria only contain very little sphingomyelin[9]. The proportion of sphingomyelin increases in almost all organs during aging[9,10]. Because of the higher degree of saturation of sphingomyelin compared to phosphatidylcholine, this leads to higher membrane rigidity.

Many strategies have been developed for the chemical synthesis of sphingolipids[11]. Chemical synthesis can be used to produce these substances, which are not readily isolated from natural sources. However, synthesis remains challenging and tedious because of their high structural complexity. Important natural sources for sphingomyelin are the lipid fractions of milk and egg. Chicken egg yolk contains ca. 10 % phospholipids of which ca. 2.5 % is sphingomyelin and ca. 60–73 % is phosphatidylcholine. In egg yolk sphingomyelin, the predominant fatty acid is palmitic acid (ca. 84 %) together with stearic and nervonic acid (C24:1)[12]. Bovine milk contains ca. 0.1–1 % of phospholipids, of which ca. 19–37 % is phosphatidylcholine and 18–34 % is sphingomyelin. They are mainly present in the milk fat globule membrane[13]. More than 80 % of its fatty acids are saturated: the main fatty acid species are palmitic, behenic (C22:0), tricosanoic (C23:0) and lignoceric acid (C24:0), making the milk sphingomyelin fraction more complex. The main sphingoid base species are d18:1 (64 %) and d16:1 (23 %) besides d18:0, d17:1 and d16:0[14]. In comparison, human milk mainly contains the fatty acids C24:0, C24:1, and C22:0 (each around 17 %) besides C16:0 (16 %), C18:0 (12 %), C20:0 (9 %), and C23:0 (4 %). The main sphingosine species is d18:1 (62 %) and the overall sphingoid base pattern is more heterogeneous[4].

Despite the apparent structural similarity of sphingomyelin and phosphatidylcholine, there are distinct physico-chemical differences between the two phospholipids. In the interface region (the link between polar head group and hydrophobic chains), sphingomyelin, with its hydroxyl and amide groups, bears hydrogen bond donors as well as acceptors, whereas the phosphatidylcholine ester bonds can only serve as hydrogen acceptors. This strongly influences the lateral interactions between the molecules in a membrane[5]. Typically, sphingomyelin contains saturated fatty acid chains in contrast to phosphatidylcholine, which very often has a saturated chain in the *sn*-1 and an unsaturated in the *sn*-2 position. This, in combination with a mismatch in chain lengths between the sphingosine base and the fatty acid attached, has consequences for the lateral packing in membranes and leads to the formation of cholesterol-rich, rigid, detergent-resistant microdomains (so-called lipid rafts)[5,15]. It was shown experimentally, that cholesterol has a 5- to 12-fold higher affinity to sphingomyelin than to phosphatidylcholine[16] and can form stoichiometric complexes with sphingomyelin[17]. Lipid rafts are important regions for the anchoring of certain membrane proteins[18]. Sphingomyelin, together with cholesterol, also seems to have a strong effect on the fusogenic properties of membranes[19].

In *in vitro* experiments it could be shown that vesicles were more stable against micellation by bile salts the more sphingomyelin they contained. Mixed micelles exhibited a lower cytotoxicity against human erythrocytes and were less damaging to Caco-2 cells when they contained higher fractions of sphingomyelin[20]. Liposomes based on sphingomyelin ("sphingosomes")[21] in some cases have been found to have superior clinical efficacy compared to phosphatidylcholine liposomes[22]. Whereas single applications of sphingomyelin liposomes showed no adverse effects[23], the effects of chronic injections are discussed controversially[24,25]. Chronic injection of sphingomyelin liposomes into mice caused enlargement of liver and spleen, an effect that was not observed with phosphatidylcholine liposomes[26].

Apart from its function as a membrane constituent, sphingomyelin plays an important role in cell signalling as part of the sphingomyelin cycle[2,27]. With regards to that, also nutritional benefits of sphingomyelin have been postulated, such as tumour suppression[28] and reduced cholesterol absorption[29,30].

Dr. C. D. Heidecke

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Lipid Analytics: Molecular Weight Distribution in Pegylated Phospholipids

Pegylated phospholipids are a special class of polar lipids used for the production of so called stealth liposomes. These liposomes show, after inravenous administration, a significantly better stability in the blood circulation than liposomes without pegylated phospholipids. One example for a pharmaceutical product on the market is Doxil™.

The most common pegylated phospholipid is DSPE-PEG 2000 (pegylated- distearoyl- phosphatidyl-ethanolamine). This is a phosphatidyl ethanolamine (PE) with two stearic acids bound to the glycerol and with a polyethylene glycol monomethyl ether chain linked to the amino group of the PE. This linkage is a substituted carbamate or urethane:
$-CH_2-NH-CO-O-(CH_2CH_2-O)_n-CH_3$:

The mean molecular mass of the polyethylene glycol monomethyl ether (MPEG) is about 2000 and the mean molecular mass of the complete molecule is about 2800. But due to the production process of the MPEG; this material is a mixture of different MPEGs who differ in the number of ethylene glycol units.

For structure elucidation and for quality control reasons it is important to have methods to measure this molecular weight distribution. One way to do this is mass spectroscopy. Fig. 1 shows a typical mass spectrum of DSPE-PEG 2000.

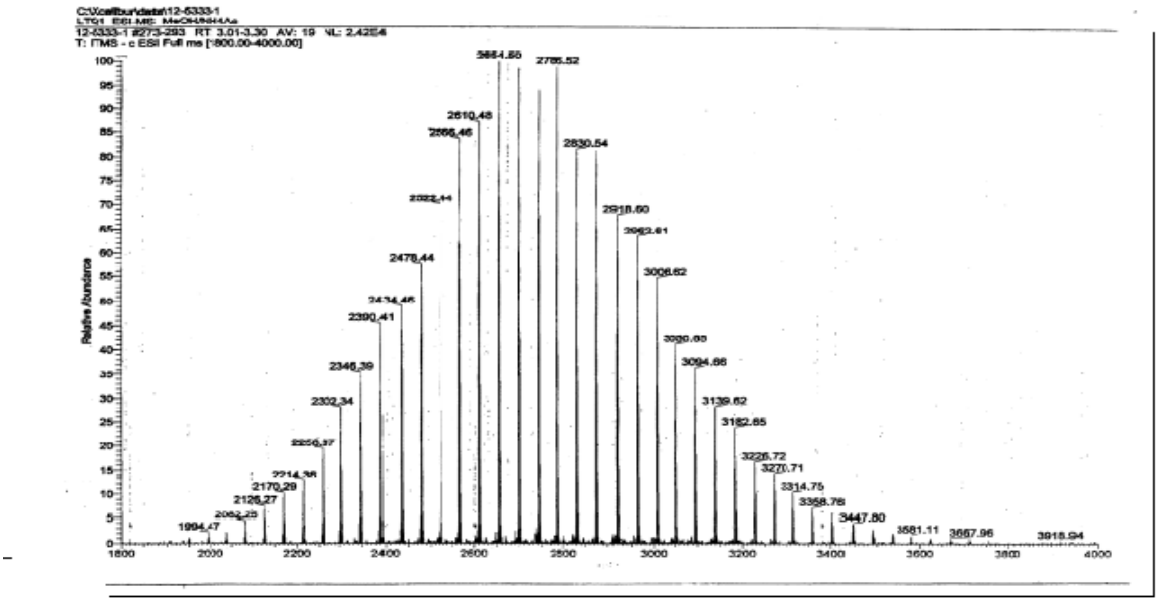

Fig. 1: Mass spectrum of DSPE-PEG 2000

All the signals with a characteristic distance of 44 mass units (-O-CH_2CH_2-) represent one of the many different DSPE-PEG species with a maximum at about 2800. Due to technical restrictions of mass spectroscopy this gives a more qualitative view of the molecular weight distribution. To get a quantitative measure of the distribution or better the polydispersity of the material a HPLC method provides more precise results.

To achieve a sufficient good separation between the different polyethylene glycol species reversed phase HPLC is the best choice. Unfortunately this doesn't work with the complete DSPE-PEG 2000 molecules because the very non-polar stearic acid moiety leads to a high retention under the conditions where the MPEGs are separated. So the first step is to hydrolize the DSPE-PEG 2000 under basic conditions to obtain the free MPEG. This mixture is then separated by a gradient elution with water/acetonitril on a C18 column and ELSD (Evaporative Light Scattering Detector) detection. Fig. 2 shows a chromatogram where all the different MPEG species are separated for integration and quantitative analysis. In this figure, e.g. MPEG-30 refers to a MPEG specie with thirty ethylene glycol units.

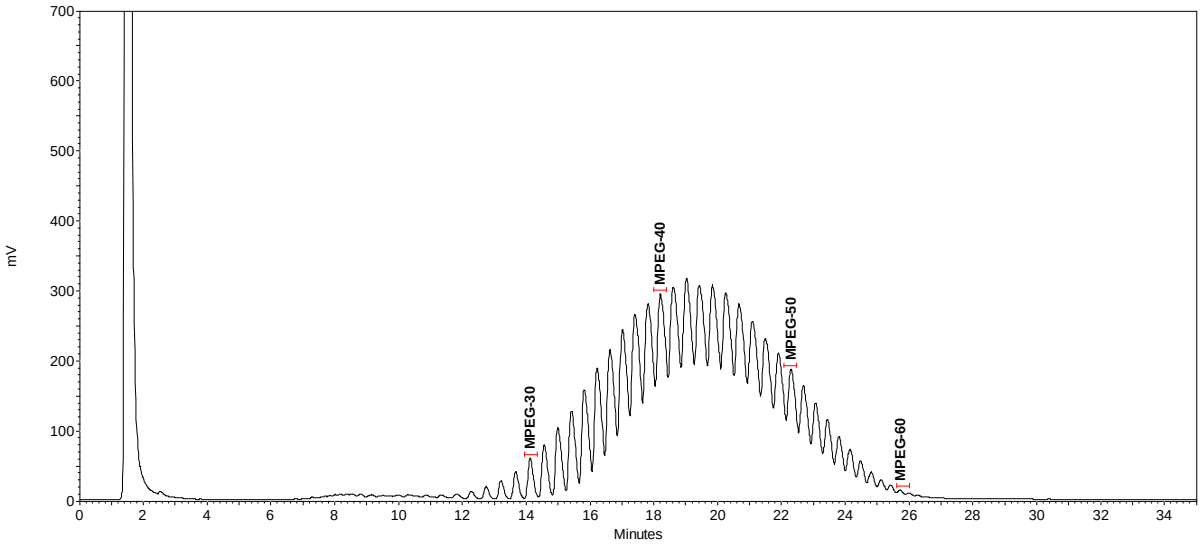

Fig. 2: HPLC-ELSD chromatogram of different MPEG species

To identify the individual peaks a standard material is needed, but in the region of MPEGs with 30 to 60 ethylene glycol units no individual substances are availlable. Commercial availlable is a pure MPEG with 12 ethylene glycol units and together with MPEG material of different mean molecular weights of 500, 1000, and 2000 a standard mix covering the complete range can be used (Fig. 3).

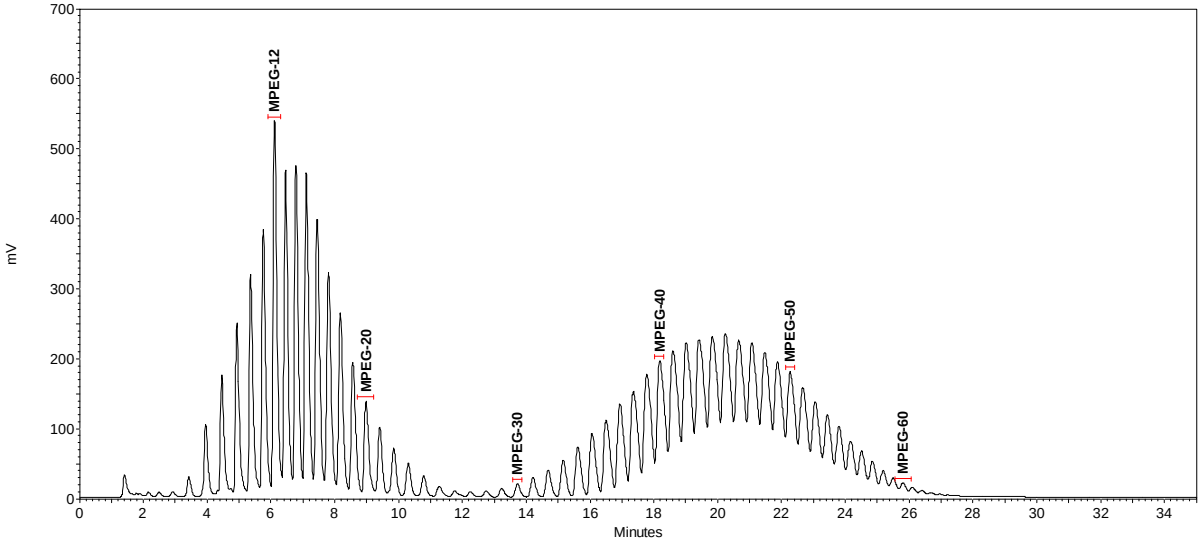

Fig. 3: HPLC-ELSD chromatogram of MPEG standard mixture

With the help of this mixture of standards all individuel MPEG peaks can be identified simply by counting the peaks starting at the unequivocally identified MPEG-12 peak. The relative peak area of each individual MPEG peak can be interpreted as the mass fraction of this compound and the weight average molecular weight (M_W) can be calculated. By using the individual molecular weights for calculation the number average molecular weight (M_N) can be calculated too. A measure for the dispersity of the material is then the quotient M_W/M_N called the polydispersity index (PDI). For a pure homogeneous substance the PDI is unity, but for materials with a molecular weight distribution the PDI is always greater than 1. Typical values for MPEG 2000 are about 1.016 to 1.020.

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Contact

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