Newsletter

Volume 6, Number 2

November 2012

Phospholipid
Forschungszentrum/Research Center
Heidelberg

Newsletter Volume 6, Number 2

November 2012

Phospholipid
Forschungszentrum/Research Center
Heidelberg

Table of Contents

Introduction - Managing Director 3

Phospholipid Research Center News 4

Special Dosage Forms with Phospholipids:
Parenteral oil-in-water emulsions for parenteral nutrition 6

Special Lipids:
Cationic Lipids 9

Lipid-Analytics:
Thin Layer Chromatography 12

Contact 16

Phospholipid
Forschungszentrum e.V./
Research Center

Im Neuenheimer Feld 582
D-69120 Heidelberg
VR 3192

Phone +49 (0) 6221 / 588 8360
Fax +49 (0) 6221 / 651 5665
www.phospholipid-institute.com

Introduction

Phospholipids are at present not abundantly being used as excipients in oral pharmaceutical dosage forms. Sometimes they are present as minor component as wetting agent for tablet components. Oral phospholipids are, however, being used, as (supportive) therapeutic agent, especially in East Europe and China in treatment of liver disorders. Also, in disease related disorders which are characterized by a lack of phospholipids in diseased cells (e.g. Crohn´s disease), oral phospholipids are clinically explored as supplement therapy [1,2] .

In pharmaceutical technology oral phospholipids are considered for taste masking, retarded release, suppression of local side effects caused by oral NSAID (non-steroidal anti-inflammatory drugs) and oral absorption enhancers for poorly water soluble drugs. Since 50 % or more of NCE´s (new chemical entities) in the pipelines of pharmaceutical companies are poorly water soluble and are (because of their low water solubility) poorly absorbed, the oral absorption enhancement by means of solubilization by phospholipids may be a very interesting future role for phospholipid excipients.

Because of their lack of toxicity, biocompatibility and the fact that phospholipids are an essential component of bile which solubilizes poorly water soluble food components and poorly water soluble drugs to enable their absorption, the choice of phospholipids as oral excipient is a logical one.

In order to stimulate this area the Phospholipid Research Center invites researches all over the world to pay attention to the pharmaceutical technological use of oral phospholipids and to make corresponding research proposals for support by the Center.

PD Dr. Peter van Hoogevest
Managing Director
pvanhoogevest@phospholipid-institute.com

1. Stremmel W., Braun A., Hanemann A., Ehehalt R., Autschbach F., Karner M., Delayed Release Phosphatidylcholine in Chronic-active Ulcerative Colitis A Randomized, Double-blinded, Dose Finding Study. J Clin Gastroenterol 2010;(44):e101–e107.

2. Schneider H., Braun A., Fuellekrug J., Stremmel W., Ehehalt R., Lipid based therapy for ulcerative colitis - modulation of intestinal mucus membrane phospholipids as a tool to influence inflammation Int. J. Mol. Sci. 2010 (11): 4149-4164.

Phospholipid Research Center News

Phospholipid Research Center News

a) Attractive Membership

In order to make the membership of The Research Center even more attractive, all members were contacted to give permission to disclose their interests and capabilities in the phospholipid area to the attention of other members. This information will be placed in a password protected area of the web page. At this way members will be able to interact easily when they look for advice and cooperation options with other PL experts.

Also a further benefit for members was created. Members can apply for funding of workshops to encourage scientific discussions on selected topics with other experts in the phospholipid area: This tool is especially meant for academic members, but can also be considered when companies are involved. Application for funding has to be made by filling out a standard questionnaire, which is twice yearly evaluated by the Scientific Board.

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

b) Meeting of the Scientific Board, July 7, 2012 in Braunschweig

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. P. van Hoogevest (PRC)
Mr. Armin Wendel (PRC)
Dr. Jürgen Zirkel (PRC)

The next Board meeting will be on January 14, 2013 in Ludwigshafen am Rhein. Research proposals should be submitted before December 15, 2012.

c) Funded projects

The following projects are ongoing:

"Studies on the distribution of phospholipid stabilizer(s) in complex lipid nanoparticle dispersions"
Prof. Heike Bunjes, TU Braunschweig.

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

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

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

"Investigation of the retention of lipophilic drug compounds within liposomal drug carriers"
Dr. Stefan Hupfeld, University of Oslo / Prof. Brandl, University of Odense

"Interactions of the tumour-targeting vector peptide pHLIP with phospholipids"
Prof. Sandro Keller; Technical University Kaiserslautern.

"Hydrogenated Phospholipids as anti-metastatic agents"
Prof. Ulrich Massing, KTB Freiburg.

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

"Caco-2 cell studies on lipid-mediated intestinal adsorption of active substances"
Prof. Rolf Schubert, University of Freiburg.

„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.

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

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

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

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

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

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

Fischer S.M., Flaten G.E., Hagesæther E., Fricker G., Brandl M., In-vitro permeability of poorly water soluble drugs in the phospholipid vesicle-based permeation assay: the influence of nonionic surfactants J. Pharm. Pharmacol. 2011 63(8): 1022–1030.

Fischer S.M., Brandl M., Fricker G., Effect of the nonionic surfactant Poloxamer 188 on passive permeability of poorly soluble drugs across Caco-2 cell monolayers. Eur. J. Pharm. Biopharm. 2011 79: 416–422.

Hüsch J., Dutagaci B., Glaubitz C., Geppert T., Schneider G., Harms M., Müller-Goymann C.C., Fink L., Schmidt M.U., Setzer C., Zirkel J., Rebmann H., Schubert-Zsilavecz M., Abdel-Tawab M., Structural properties of so-called NSAID–phospholipid-complexes. Eur. J. Pharm. Sci. Sep 18 2011; 44(1-2):103-16.

Buckley A.T., Fischer S.M, Fricker G., Brandl M., In vitro models to evaluate the permeability of poorly soluble drug entities: Challenges and perspectives Eur. J. Pharm. Sci. 14 February 2012 45 (3), 235-250.

Basnet P., Hussain H., Tho I., Skalko-Basnet N., Liposomal Delivery System Enhances Anti-Inflammatory Properties of Curcumin. J. Pharm. Sci. February 2012 101(2):598-609.

Fischer S.M., Buckley S.T., Kirchmeyer W., Fricker G., Brandl M., Application of simulated intestinal fluid on the phospholipid vesicle-based drug permeation assay. Int. J. Pharm. 2012 422: 52– 58.

Special Dosage Forms with Phospholipids: Oil-in-water emulsions for parenteral nutrition

The intravenous use of phospholipids in form of liposomes in well known. Less known is that phospholipids are exclusively being used as emulsifier for oil-in-water emulsions for parenteral nutrition. Synthetic emulsifiers like Cremophor EL or Tween 80 are not very suitable for intravenous use because of their anaphylactic side effects. The rather large volumes of continuously administered emulsions for parenteral administration require the use of virtually non-toxic emulsifiers. Phospholipids meet these requirements. In early days soy lecithin of the development of such emulsions was used, nowadays egg lecithin is being used also or in combination with an minor amount of oleic acid (Table 1)[1,2].

Year	Company	Product	Event	Oil	Emulsifier
1951	Merck		Prototype	Olive/Sesame	Soy Lecithin (SL)
1957	Upjohn	Lipomul	FDA Approved	Cotton seed	SL/Pluronic
1962	Vitrum	Intralipid	Launch (Europe)	Soy	Egg Lecithin (EL)
1964	Upjohn	Lipomul	Withdrawal/Side effects/ Impurity gossypol in cotton seed oil	Cotton seed	SL
1964	B. Braun	Lipofundin	Launch	Soy	SL later EL
1978	Vitrum	Intralipid	FDA approved	Soy	Egg Lecithin
1979	Abbott	Liposyn	FDA Approved	Safflower/Soy	Egg Lecithin
1980-now			Various oils; addition amino acids		

Table 1: Development history of oil-in-water emulsions for parenteral nutrition

The oils concentration in such formulation ranges from 10 to 30 %, whereas the emulsifier/phospholipid concentration is 0.8 – 1.2 % w/v.

The emulsions are prepared by dispersing the phospholipids Egg Lecithin E80, optionally with a minor amount of sodium oleate or oleic acid in the aqueous phase and mixing the aqueous phase with the oil phase to make a crude oil in water emulsion. Then the emulsion is several times passed through a high pressure homogenizer to achieve a particle size of about 250 nm diameter.

The lipid particles have then about the same size as the natural lipid carrying particles in the blood, the chylomicrons. After filtration through a 5 or 10 µm pore size filter, the emulsion is sterilized in a rotating autoclave (to maintain a homogenous heat distribution in the container) and filled under nitrogen in glass vials or infusion bags [3,4]. The quality control of such formulations is described in the United States Pharmacopeia.
Nowadays such emulsions contain several types of oil like soy bean oil, olive oil, medium chain triglycerides and, fish oil [5,6]. The composition is optimized to provide the patient, beside the necessary calories, essential vitamins, amino acids and fatty acids.

Interestingly the emulsions can also be used as intravenous carrier for drugs which are poorly water soluble and possesses a high solubility in the oil [7]. A classical example of such a product is Propofol a well-known narcotic for operations. Other examples are provided in Table 2 [7,8,9,10].

Product	Drug Substance	Indication	Introduction Year	Producer
Diazemuls	Diazepam	Sedative & muscle relaxant	1983	Pharmacia
Limethason	Dexamethasone palmitate	Rheumatoid arthritis	1988	Mitsubishi
Liple	Alprostadil (Prostaglandin E1)	Vasodilator, platelet inhibitor	1988	Mitsubishi
Diprivan / Disoprivan	Propofol	Anesthetic	1989	AstraZeneca
Etomidat® -Lipuro	Etomidate	Anesthetic	1990	B. Braun
Vitalipid	Vitamin A, D2, E, K1	Parenteral nutrition	1991	Fresenius Kabi
Ropion	Flurbiprofen axetil	Non-steroidal analgesic	1992	Kaken Pharm.
Cleviprex	Clevidipine butyrate	Dihydropyridine Ca channel blocker, Antihypertensive	2008	The Medicine Company

Table 2: Examples of parenteral oil in water emulsions containing drug substances and egg phospholipids as emulsifier

It can be concluded that phospholipids are the golden standard for emulsions for parenteral nutrition.
Egg phospholipids are the emulsifiers of choice as they are:

- of natural origin
- non-irritant
- non-toxic
- compatible with the other ingredients
- preventing the coalescence of the oil/drug droplets
- enabling the production of large quantities of lipid emulsions at an acceptable price

Moreover the resulting oil in water emulsions are suitable solubilizers for poorly water soluble drugs with high oil solubility, may increase the bioavailability of poorly water soluble drugs and may stabilize sensitive drugs and ingredients.

If you would like to know more about these emulsions, please contact the Phospholipid Research Center.

PD Dr. P. van Hoogevest

References:

1. Adolph M., Clin. Nutr. 2001 20(4): 11-14.

2. USP 33, First Supplement to the USP-NF Reissue (2010), Official Monographs, NF 28 / Egg Phospholipids, R851-R852.

3. USP 32 Official Monographs – Lipid Injectable Emulsions, 2793-2794 and First Supplement, 4070-4071.

4. USP 33, First Supplement to the USP-NF Reissue (2010), <729> Globule Size Distribution in Lipid Injectable Emulsions and <788> Particulate Matter in Injections, R336-R341.

5. Grotte G., Jacobson S., Wretlind A., in Total parenteral nutrition, ed. J.E. Fischer, Boston, (1976).
6. Adolph M., Aktuel Ernaehr Med 2007 32(1):22-29 (review).

7. Stevens J., Mims P., Coles N., Lipid Emulsions as Drug Delivery Systems. Business Briefing: Pharmatech 2003 1-4.
8. Lucks J.S., Mueller W.W., Krankenhauspharmazie 1994 15: 52-57.

9. Schmitt J., Parenterale Fettemulsionen als Arzneistoffträger. in Pharmazeutische Technologie: modern Arzneiformen Eds. Müller R.H., Hildebrand G.E., Stuttgart (1998).

10. Cada D.J., Levien T.L., Baker D.E., Clevidipine Butyrate Injectable Emulsion. Hospital Pharmacy, 2008 43(11): 903–912.

Special Lipids: Cationic lipids

DOTAP (1,2-dioleoyloxy-3-trimethylammonium-propane (chloride salt)), DOGS (dioctadecyl-amidoglycylspermine), and DC-Chol (3ß-[*N-(N',N'*-dimethylaminoethane)-carbamoyl]cholesterol hydro-chloride) are examples of cationic lipids (Fig. 1).

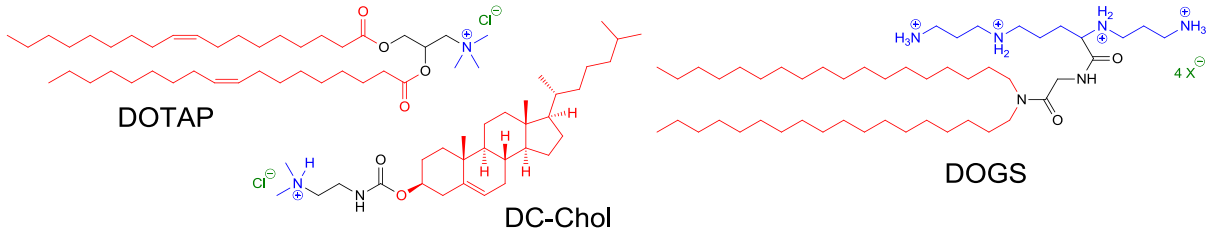

Fig. 1: Structures of cationic lipids.

Cationic lipids are not phospholipids – because of a phosphoric acid ester moiety, phospholipids either bear a negative charge (PG, PI, PS) or are neutral (PC, PE) at pH 7.0. The positive charge of cationic lipids is a result of the presence of amino groups in the molecule, which are either quaternized or proto-nated at pH 7.0[1].

These cationic lipids do not occur in nature, but are synthetic products. A great variety of different struc-tures have been synthesized so far[2]. However, they share main structural features with natural phospho-lipids: a hydrophobic tail is connected via a linker to a hydrophilic, in this case positively charged, head group. The hydrophobic tail can consist of fatty acids, other long-chain alkyl residues, or cholesterol (e.g. in DC-Chol)[3]. Various residues have been used as positively charged head groups. In DOTAP and its ether analogue DOTMA (1,2-*bis((Z)*-octadec-9-enyloxy)-3-trimethylammonium-propane (chloride salt)), which have a strong structural similarity to naturally occurring phospholipids, the aliphatic chains are attached to 2,3-dihydroxy-*N,N,N*-trimethyl-1-propanammonium chloride, the positive charge re-sulting from the trimethylammonium group as in cho-line. In DC-Chol, an *N,N*-dimethyl-1,2-ethanediamine is attached to cholesterol via a carbamate linkage. The positive charge in this case comes from a proto-nated tertiary amine. In other lipids, spermine or de-rivatives thereof are bound via a peptide bond to the lipophilic part, thus yielding a molecule with multiple (potentially) positively charged sites. Examples are DOGS and DOSPA (*N,N*-dimethyl-*N*-[2-(sperminecarboxamido)ethyl]-2,3-bis(dioleyloxy)-1-propaniminium pentahydrochloride)[1].

The major use and potential of cationic lipids is in transfection and gene delivery[4] There are different ways of introducing genetic information in form of plasmid DNA (pDNA) into a cell, one of which is the use of liposomes. This procedure has been termed lipofection[5] Liposomes have some advantages over other gene delivery systems, especially viruses, which are potentially hazardous. Liposomes are attractive transfection vehicles, because they can encapsulate water soluble, hydrophilic, compounds (like DNA), as well as amphiphilic and lipophilic actives, have low immunogenicity and cytotoxicity, and can be produced on a large scale[3]. However, there are problems arising from the fact that both the pDNA and the phospholipids bear negative charges. With cationic lipids, it is possible to form cationic vesicles, which bind the negatively charged DNA to form so-called lipoplexes[2,3,6]. Binding of DNA can have a strong effect on the topology of the vesicle, and the mode of binding is not always clear[7,8]. In most cases, cationic lipids are combined with phospholipids (helper lipids), preferably lipids with fusogenic properties like DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine), to form cationic liposomes which are more effective in gene delivery[3,6]. Transfection efficiency seems to be highest when the resulting complexes have a small net positive charge. The lipoplexes can then interact with the negatively charged plasma membrane, be internalized by endocytosis, and release their DNA load into the cytosol[3]. All different structural features of the cationic lipids, like fatty acid chain length and saturation, have a strong influence on their efficiency. Multivalent polar head groups (like spermine) can be more effective due to more efficient binding and packing of the DNA, but other effects may also play a role like their buffer capacity[1]. Entrapment of DNA into liposomes can protect the DNA from degradation by DNA-ases[3]. Lipoplexes may show cytotoxicity to some extent[9]. Liver damage by DOTAP/Cholesterol liposomes could be efficiently suppressed in mice by incorporation of all-trans retinoic acid (ATRA) into the liposomes[10].

Cationic lipids and liposomes seem promising in topical gene therapy and vaccine targeting[11,12]. DOTAP liposomes have been shown to be effective in the delivery of pDNA through the stratum corneum in an ex-vivo model after topical application[13]. DOTAP has also been formulated in Flexosomes[14] loaded with low-molecular weight heparin. The positively charged Flexosomes had a much higher entrapment efficiency than their neutral or negatively charged analogues because of favorable electrostatic interactions with the negatively charged heparin, and were effective for heparin delivery into the skin[15]. Cationic liposomes may also be useful for nasal vaccination[16], both as delivery systems and effective adjuvants, as has been studied with influenza[17]. In this case, a cationic sphingolipid (*N*-palmitoyl D-erythro-sphingosyl-carbamoyl-spermine) gave superior results compared to DOTAP.

A relatively new approach in the field of gene therapy is the use of cationic liposomes as delivery vehicles for siRNA (small interfering RNA) for gene silencing[18]. Apart from liposomes, also other vesicle types have been formulated, e.g. cationic solid lipid nanoparticles (cSLNs)[19] and nanoemulsions[20,21] for the delivery of antisense oligonucleotides.

If you would like to know more about cationic lipids, please contact the Phospholipid Research Center.

Dr. Christoph Heidecke

1. Balazs D.A., Godbey WT., Liposomes for Use in Gene Delivery, J. Drug Del. 2011 2011: Article ID 326497, 12 pages.

2. Tros de Ilarduya C., Sun Y., Düzgüneş N., Gene delivery by lipoplexes and polyplexes, Eur. J. Pharm. Sci. 2010 40(3):159–170.

3. Cao A., Briane D., Coudert R., Cationic Liposomes as Transmembrane Carriers of Nucleic Acids, in: Advances in Planar Lipid Bilayers and Liposomes, Ed.: Leitmannova Liu A., Vol. 4, Elsevier, Amsterdam, 135–190, 2006.

4. McNeil S.E., Perrie Y., Gene delivery using cationic liposomes, Expert Opin. Ther. Pat. 2006 16(10):1371–1382.

5. Felgner P.L., Gadek T.R., Holm M., Roman R., Chan H.W., Wenz M., Northrop J.P., Ringold G.M., Danielsen M., Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure, P. Natl. Acad. Sci. USA1987 84(21):7413–7417.

6. Ulrich A.S., Biophysical Aspects of Using Liposomes as Delivery Vehicles, Bioscience Rep. 2002 22(2):129–150.

7. Rädler J.O., Koltover I., Salditt T., Safinya C.R., Structure of DNA–Cationic Liposome Complexes: DNA Intercalation in Multilamellar Membranes in Distinct Interhelical Packing Regimes, Science 1997 275(5301):810–814.

8. Safinya C.R., Structures of lipid–DNA complexes: supramolecular assembly and gene delivery, Curr. Opin. Struc. Biol. 2001 11:440–448.

9. Nguyen L.T., Atobe K., Barichello J.M., Ishida T., Kiwada H., Complex Formation with Plasmid DNA Increases the Cytotoxicity of Cationic Liposomes, Biol. Pharm. Bull. 2007 30(4):751–757.

10. Charoensit P., Kawakami S., Higuchi Y., Hashida M., Incorporation of all-trans retinoic acid into lipoplexes inhibits nuclear factor B activation mediated liver injury induced by lipoplexes in mice, J. Gene Med. 2008 10(1):61–69.

11. Geusens B., Strobbe T., Bracke S., Dynoodt P., Sanders N., Van Gele M., Lambert J., Lipid-mediated gene delivery to the skin, Eur. J. Pharm. Sci. 2011 43(4):199–211.

12. González-Rodríguez M.L., Rabasco A.M., Charged liposomes as carriers to enhance the permeation through the skin, Expert Opin. Drug Deliv. 2011 8(7):857–871.

13. Birchall J.C., Marichal C., Campbell L., Alwan A., Hadgraft J., Gumbleton M., Gene expression in an intact ex-vivo skin tissue model following percutaneous delivery of cationic liposome–plasmid DNA complexes, Int. J. Pharm. 2000 197(1–2):233–238.

14. Cevc G., Blume G., Lipid vesicles penetrate into intact skin owing to the transdermal osmotic gradients and hydration force, Biochim. Biophys. Acta 1992 1104(1):226–232.

15. Song Y.-K., Kim C.-K., Topical delivery of low-molecular-weight heparin with surface-charged flexible liposomes, Biomat. 2006 27(2):271–280.

16. Heurtault B., Frisch B., Pons F., Liposomes as delivery systems for nasal vaccination: strategies and outcomes, Expert Opin. Drug Deliv. 2010 7(7):829–844.

17. Even-Or O., Joseph A., Itskovitz-Cooper N., Samira S., Rochlin E., Eliyahu H., Goldwaser I., Balasingam S., Mann A.J., Lambkin-Williams R., Kedar E., Barenholz Y., A new intranasal influenza vaccine based on a novel polycationic lipid-ceramide carbamoyl-spermine (CCS). II. Studies in mice and ferrets and mechanism of adjuvanticity, Vaccine 2011 29(13):2474–2486.

18. Thakur A., Fitzpatrick S., Zaman A., Kugathasan K., Muirhead B., Hortelano G., Sheardown H., Strategies for ocular siRNA delivery: Potential and limitations of non-viral nanocarriers, J. Biol. Eng. 2012 6:7.

19. Jin S.-E., Kim C.-K., Long-term Stable Cationic Solid Lipid Nanoparticles for the Enhanced Intracellular Delivery of SMAD3 Antisense Oligonucleotides in Activated Murine Macrophages, J. Pharm. Pharm.. Sci. 2012 15(3): 467–482.

20. Bruxel F., Cojean S., Bochot A., Teixeira H., Bories C., Loiseau P.-M., Fattal E., Cationic nanoemulsion as a delivery system for oligonucleotides targeting malarial topoisomerase II, Int. J. Pharm. 2011 416(2):402–409.

21. Hagigit T., Abdulrazik M., Valamanesh F., Behar-Cohen F., Benita S., Ocular antisense oligonucleotide delivery by cationic nanoemulsion for improved treatment of ocular neovascularization: An in-vivo study in rats and mice, J. Control. Rel. 2012 160(2):225–231.

Lipid Analytics: Thin-Layer Chromatography

TLC (**T**hin **L**ayer **C**hromatography) is a widely used technique in phospholipid analytics. It uses the same principle of separation as other chromatographic techniques. The analyte is part of a two phase system in which one phase is stationary and the other one is mobile.

In the case of capillary gas chromatography the stationary phase is the inner layer of the capillary column and consists of highly viscous liquids like polyethylene glycols or polysiloxanes. The mobile phase is gaseous and moves through the capillary column to transport the analytes. The separation of the different analytes takes place at the surface boundary between the two phases.

In liquid chromatography the stationary phase is usually a highly porous solid material like silica gel which is flowed through by the mobile phase. Again, the place of separation is the surface boundary between the solid and the liquid phase. Now it depends only on several geometric circumstances how to call this separation technique. If you have placed your silica gel into a glass column and you are using rather low pressure to force the liquid phase through the column, then you are doing low pressure chromatography or flash chromatography. Replace your glass column with a tube of stainless steel filled with stationary phase with smaller particle size (3 to 10 µm) and apply high pressure, then you have a HPLC set-up. A further step is called UPLC (Ultra Performance Liquid Chromatography) where smaller particles (< 3 µm), smaller column diameters, and higher pressures are used.

But chromatography is not limited to the geometric shape of a cylinder. If you place the stationary phase on a plane surface, then the resulting analytical method is called thin layer chromatography (TLC). The most common TLC plates are aluminium foils covered with a layer of about 200 µm of silica gel with a particle diameter of 10 – 12 µm. But there are of course lots of other possibilities with regard of the supporting material (glass, plastic) and the stationary phase (aluminium oxide, modified silica gels, cellulose, polymers, etc.).

A main difference to the other chromatographic technics is the way the mobile phase is transported through the stationary phase. In the case of TLC the mobile phase moves through the thin layer of stationary phase by means of capillary forces. So the speed of chromatography depends strongly on the particle size of the stationary phase and the viscosity of the mobile phase.

A second important difference between TLC and the other chromatographic techniques is the way the separated substances are detected. GC and HPLC application are using detectors which are generating throughout the complete separation process a time dependent signal. In the case of TLC the separation and the detection process are temporally separated but not locally. Because the stationary phase is an open system it is very easy to detect the different substances directly on the TLC-plate. If you have coloured substances you only need your eyes as a detector. For all the other colourless substances there are countless procedures of visualization using reagents and dyes often specific for the type of compound.

The principal procedure of TLC-analytics can be divided into four parts:

First: Prepare solutions of your samples and standard substances and apply them onto the plate as small spots or bands.

Second: Develop the plate by placing it into a developing chamber where the mobile phase can move upwards through the stationary phase.

Third: Visualize the different compounds by using reagents, dyes etc.

Fourth: Evaluate the visualized spots of your sample and the standard substances. This evaluation can be done either visual or by means of technical equipment.

TLC-Analytics with Phospholipids can be divided into two main parts, analysis of polar components and analysis of non-polar components. Part of the “polar components group” are all the phosphorus containing lipids like phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidic acid (PA), sphingomyelin (SM) and many others together with the corresponding lyso-compounds. These compounds can be separated on silica gel TLC-plates by using solvent mixtures of chloroform, methanol and water, sometimes together with small amounts of ammonia. A typical composition consists of chloroform/methanol/water in a volumetric ratio of 65/25/4. The samples are dissolved in the solvent mixture hexane/2-propanol/water at a concentration of 2 % or 4 % (20 or 40 mg/ml) and typically 20 µl of the sample solution is applied onto the plate. Standard substances are usually applied in much smaller concentrations depending on the expected concentration of the specific compound. Fig. 1 shows the polar components of egg lecithin. On rows #3 and #4 the sample solution was applied (2 %), on rows #1,2,5,6 standard solutions for lyso-phosphatidylcholine (LPC), egg sphingomyelin (ESM), and on rows #2,5 standard solutions for phosphatidylethanolamine (PE) were applied.

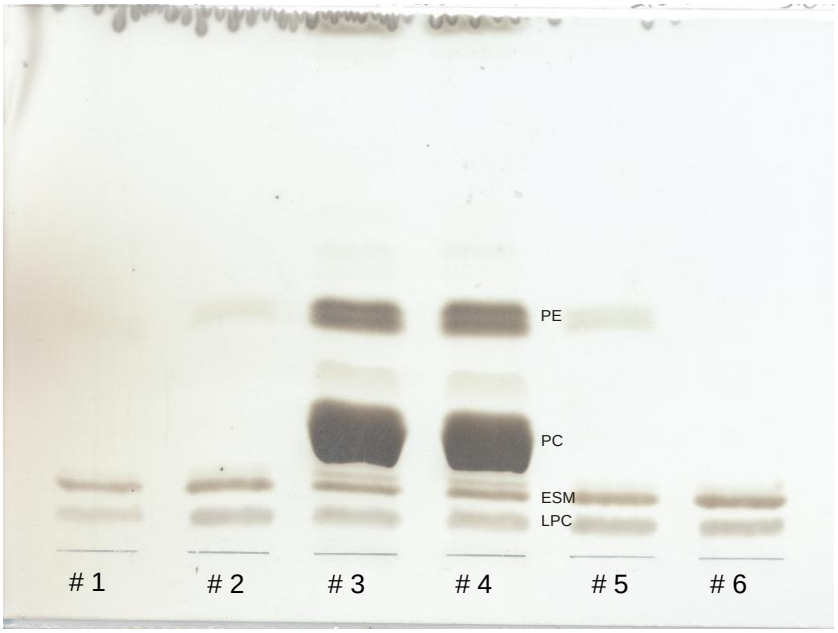

Fig.1: Polar components of an egg lecithin by TLC

The “non-polar components group” consists of the typical concomitant components of phospholipids like free fatty acids, mono- di- and triglycerides, tocopherol, sterols and many others. On silica gel these compounds can be separated using a mobile phase like hexane/diethyl ether/acetic acid (70/30/1). Under these conditions only the non-polar components move over the TLC-plate, all the polar components stay on the starting line and do not interfere with the non-polar ones. This is a great advantage to HPLC methods where it is not acceptable that parts of the sample will stay permanently on the column. HPLC columns are too expensive to use them only once, while TLC-plates are especially made for single use.

Fig 2 gives an example of the non-polar components present in egg lecithin. On this plate cholesterol, free fatty acids (FFA), and triglycerides (TG) were applied as standard solutions besides the sample solution on rows #3 and #4.

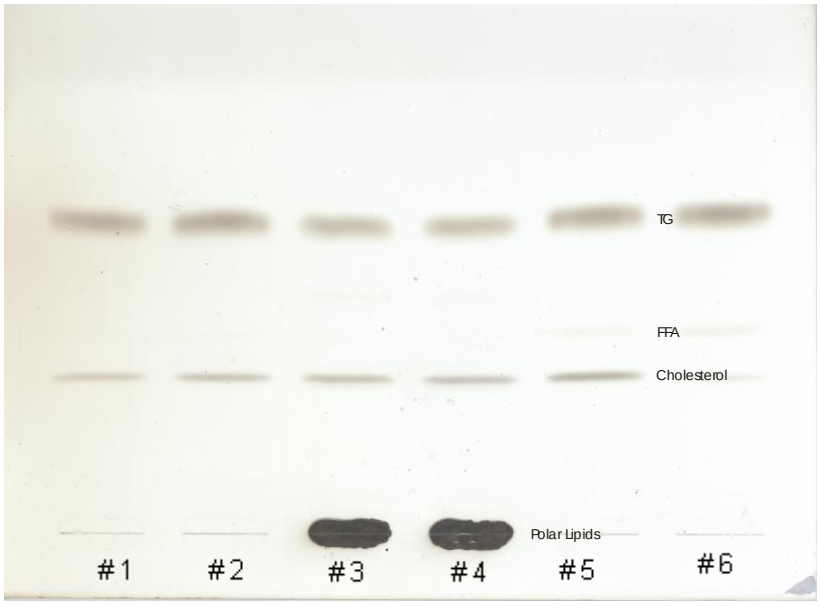

Fig.2: Non-polar components of an egg lecithin by TLC

For visualization or detection of the separated compounds often a so called “copper sulphate reagent” is used. This is a 10 % solution of $CuSO_4$ in diluted phosphoric acid (about 7 %) and the TLC plates are immersed for about 2 s, dried in hot air and then heated to 170 °C for 10 min. After this treatment nearly all lipids show brown to black spots and size and intensity of the spots can easily be evaluated with the naked eye or with photometric devices. But there are of course many more reagents for visualization of lipid compound. For example “ninhydrin reagent” is a specific for compounds containing amino groups ($-NH_2$) like phosphatidylethanolamines or phosphatidylserines, and the “Dittmer-Lester-reagent” is a specific one for phosphor containing compounds.

A recommended reference book is “Dünnschichtchromatographie, Reagenzien und Nachweismethoden, Band 1a” by Hellmut Jork, Werner Funk, Walter Fischer, Hans Wimmer, VCH Weinheim. It gives a good overview of TLC techniques and especially a broad spectrum of applications with many different reagents for visualization.

In many cases the visual evaluation of the TLC-plates provides a sufficient accuracy for routine analysis. If a better performance is desired, then so called HPTLC (High Performance Thin Layer Chromatography) can be used. The differences to the regular TLC technique can be exemplified on different aspects. First the HPTLC-plates are usually made of silica gel with smaller particle size and glass is used as supporting

plate. The particle size has an influence on the selectivity of the separations. The smaller the particles, the better two substances can be separated. Second the samples and standard solutions are no more applied by hand, but with the help of a mechanical device which provides a higher precision in volume and application geometry. Third the evaluation of the plates is fully automated by means of a photometric device, sometimes called a densitometer. The size and intensity of the spots are evaluated by measuring the remission or transmission at specific wavelength in the UV-VIS region. By moving the plate while measuring the photometric characteristics of the spots which are stained by means of e.g. ninhydrin, a chromatogram can be recorded showing a signal as a function of the location of the spots on the plate. These signals or peaks can be integrated and with standards of known concentration calibration function can be calculated.

In many cases it is worthwhile to think about TLC as an alternative to HPLC, especially in the field of phospholipids. TLC methods are well established and provide reliable, fast and cost-efficient analytical results for identity, purity, and content.

If you would like to know more about TLC and HPTLC, please contact the Phospholipid Research Center.

Dr. Ralf-Olaf Quinkert

Contact

Phospholipid Research Center
Im Neuenheimer Feld 582
69120 Heidelberg
Germany

Phone: +49 (0)6221 / 588 8360
Fax: +49 (0)6221 / 651 5665
E-Mail: info@phospholipid-institute.com

Web: www.phospholipid-institute.com