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Research Article

Enzymatic Trans-esterification of Phospholipids to Aceto-phospholipid: A Good Surface Active Emulsifier and the Role of Phospholipid in Drug Delivery System

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ABSTRACT

Aceto-phospholipids were prepared enzymatically by incorporating the acetate group at sn-1 or sn-2 position with out acetylating the amine group of phospholipids. There are a sharp change in surface properties after acetylation. At the same time the emulsifying properties of aceto-phospholipids is also remarkable than original soy phospholipids. We have got the separate droplet size distribution pattern of water in oil type of emulsion in presence of different aceto phospholipids compare to original soy phospholipids. The emulsification properties of newly prepared aceto-phospholipids were explained from the HLB values. The biological activities of the aceto-phospholipid were also highlighted here using some reported articles.

Key words: Phospholipids, Aceto-phospholipids, Interesterification, Enzymatically, Emulsification, Lipase, Acetylation

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Introduction

Phospholipids have many applications in the food industry as natural emulsifier, stabilizer and antioxidants[1-3] as well as in cosmetic and pharmaceutical industry. The emulsifying properties of natural phospholipids need improvement because the emulsifying activity of the phospholipids component compete with each other [4.] By exchanging the fatty acid profiles asymmetrically in the phospholipid molecules, new physical properties of phospholipid can be achieved. These modified phospholipids can be used in liquid/membrane research or as new type of emulsifier in for food, cosmetic or medicinal substances [5]. The modification process for phospholipids may be split into three categories – those based on physical separation, those based on enzymatic modifications and those based on chemically catalysed modification.

Acetylated phospholipids exhibit improved fluid properties and improved water dispersability. Thus the oil-in-water emulsifying properties are enhanced and it is effective for a wide variety of food formulation. Acetylation occurs primarily on the amino group of phosphatidylethanolamine. There are several chemical processes reported to prepare aceto-phospholipid using acetic anhydride or acetic acid as acylating agent but generally, chemical methods suffer various drawbacks such as low yields, and decomposition of products that result in dark colored reaction products. A more common approach has been to carry out lipase-mediated transesterification of fully acetylated phospholipids with acyl donor such as fatty acids or a methyl ester[6-9]. Acetylation of phospholipids by enzymatic esterification process was also reported by Prasad et. al .[10] by overcoming the disadvantage of chemical method.

The aim of our study was to prepare aceto-phospholipids by the lipase catalyzed trans-esterification by using methyl acetate, ethyl acetate and butyl acetate as acetate donor and evaluate the surface-active properties of the aceto-phospholipids prepared in sight of its application to the health sciences.

Experimental Procedure

Materials

Crude soy phospholipids, used in the research work were supplied by Vippy Industries Ltd. (M. P. India). The enzyme lipozyme TLIM used, as biocatalyst were gift of NOVOZYME South Asia Pvt. Ltd, Bangalore, India. All others chemicals and reagents used for this purpose were purchased from S. D. Fine chemicals, India

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Methods

Modification of Soy Phospholipids by Acetates by Enzymatic Trans-esterification Reaction

To prepare the aceto phospholipids, phospholipids and acetates (methyl acetate, ethyl acetate and butyl acetate) were taken in 1:5 by the proportion of weight ratio. The enzyme- RMIM was used for trans-esterification reaction. The reaction was continued for 7 days. After complete removal of acetates the trans-esterified phospholipid was separated from the mixed methyl ester of the fatty acids displaced from soya phospholipid by several extractions with acetone. Acetone soluble portion contained methyl esters of these fatty acids displaced from soy phospholipids and trace amount of acetates. Acetone insoluble portion contained the aceto-phospholipids. The final separation of aceto-phospholipid was made by preparative thin layer chromatography on silica gel layer. For 8 days samples were collected at different time intervals (at 2^{nd}, 4^{th} 6^{th} day) for the kinetics study of trans-esterification reaction.

Preparation of oil-in-water type of emulsion:

Oil in water (o/w) type of emulsion was prepared by mixing different oils in water in the ratio 1:4 by stirring at 3000 rpm for one hour with different modified phospholipids (0.1% of total weight of materials) in different amount to stabilize the emulsion and the effects were determined. The emulsions were allowed to settle at a constant temperature without any further shaking. The emulsion height was measured after 24h. The emulsification index (E_{24}) was calculated by determining the percentage volume occupied by the emulsion after 24h.

Determination of Fatty Acid Composition by Gas-Liquid Chromatography

Methyl esters of the component fatty acids of phospholipids were prepared by the method of Litchfield [11] and analysed by GLC analysis. The methyl esters of fatty acids were analyzed on a Hewlett-Packard gas chromatograph (HP 5890A). (Palo Alta CA), equipped with a flame ionization detector (FID) by Gas Liquid Chromatography. The analysis was done with a polar column. The polar column consist of chromosorb-WHP coated with DEGS (6′ X 1/8″ id) and the non polar column consist of chromosorb – WHP coated with SE30 (2 m length, od 1/8”). But the amount of incorporation of acetate (-$COCH_3$-) group in the phospholipid molecule could not determine by GLC analysis.

Test for amino-acid group in the modified phospholipids

One drop of sample (modified phospholipids) is poured on a piece of filter. Then a circle is drawn around the spot with a soft pencil and allowed the spot to dry thoroughly. The 0.6% ninhydrin solution (in acetone) is sprayed on the filter paper. After 20 minutes a violet blue colour is appeared on the spot of sample.

Measurement of Saponification value and amount of fatty acid collected from product

Saponification value and amount of fatty acid collected from original and different aceto-phospholipids prepared by enzymatic and chemical catalytic trans-esterification reaction of phospholipids and different acetates were measured by AOCS method.

Measurement of droplet size of w/o and o/w type of emulsion

The viscosity of emulsion was measured by Oswald viscometer (viscometer const. 1.12 and 0.3094) and the particle size was measured by Malvern Zetasizer 1000HS.

Measurement of Interfacial Tension

Interfacial tension of solvent (chloroform) as well as the chloroform solution of the original and the different modified phospholipids against water was determined by well-known drop weight method [12].

Results and Discussion

These modified phospholipids had the significant change in surface active properties like effectiveness of interfacial tension reduction (γ_{CMC}), critical micelle concentration (CMC), maximum surface excess concentration (Γ_{max}), minimum area/molecule at the interface (A_{min}). The change in fatty acid composition of the modified product and original phospholipids were given in table-**1**. Finally the presence of acetate group in modified product EPE1 was confirmed by NMR spectrum (Fig-1).

Table-1: Fatty acid composition of original and different acetylated phospholipid (by enzyme (*RM IM*) catalysed and chemical catalyzed reaction)

Sample	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$
PL	27.0	1.9	14.1	52.9	3.3
BPE1	16.1	2.3	18.9	58.7	3.4

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EPE1	17.9	2.5	18.3	57.9	3.3
MPE1	18:1	2.5	18.3	56.9	3.4

BPE1 = Phospholipid modified by butyl acetate by *RM IM* enzyme
EPE1 = Phospholipid modified by ethyl acetate by *RM IM* enzyme
MPE1 = Phospholipid modified by methyl acetate by *RM IM* enzyme
Reaction Temperature = 55-60^{0}C
Reaction Time = 7 days
Reaction Condition = By magnetic stirring at 600 rpm

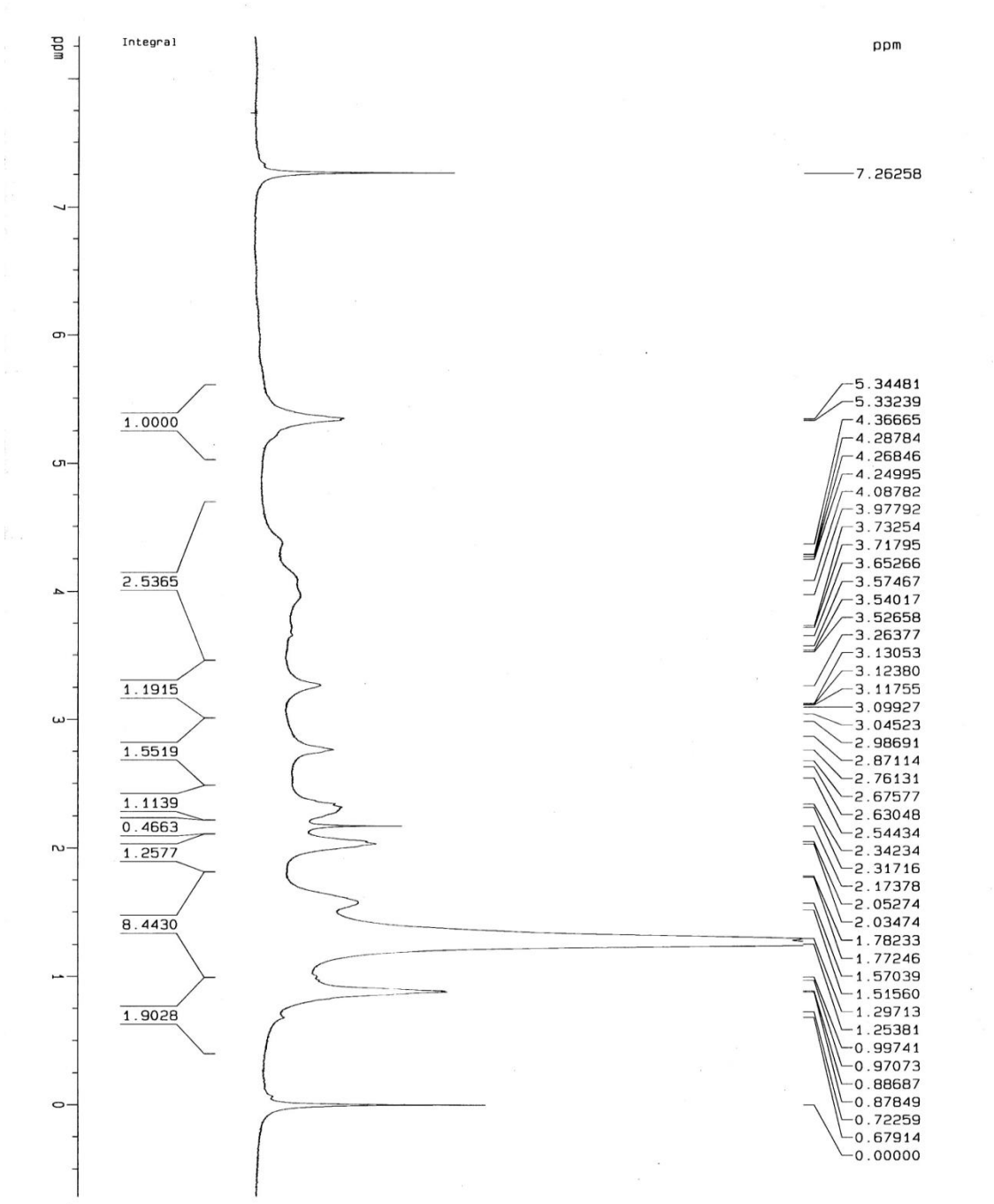

Fig.-1 Proton NMR Spectrum of EPE1

The trans-esterified soy phospholipids containing noticeable quantities of the acetate group were examined for interfacial tension values (table-2) against water at 27^0C. The interfacial tension measurement definitely showed that incorporation of acetate into soy phospholipids increased the interfacial tension against water. The interfacial tension of chloroform/water at 27^0C is 33 mN/m. For the presence of original soy phospholipids (0.1% solution) this value was reduced to 13.2 mN/m. The saponification values of original and modified phospholipiuds have also been listed in table-2. This value is also higher than original phospholipids. Acetate group has been incorporated in soy phospholipid by replacing the long chain fatty acids from the original soy phospholipids. For this there was a change in saponification value. Other surface properties and thermodynamic properties were listed in table-2. These values were calculated form the graphical presentation of log of molar concentration vs. interfacial tension of original and different aceto-phospholipids The CMC values depend on the hydrocarbon chain length of the surface active molecule. In enzymatically modified product EPE1 hydrocarbon chain length from sn-1 position decreased as a result of the acetate group consequently the CMC value was decreased. The surface excess concentration (Γ_{max}) in mol/cm^2 and minimum area/molecule (A_{min}) in Å^2 were calculated[13] Gibbs Adsorption Equation.

Table-2:Surface Properties and thermodynamic properties of different acetylated phospholipid

Sample	I. F. (Nm/m)	γ_{CMC} (Nm/m)	CMC	Γ_{min} X 10^{-10} (Moles/cm^2)	A_{min} (Å)^2	$-\Delta G^0_{mic}$ (KJ/mol)	Sap. Value (%)	Fatty acid collected %(w/w)
PL	**13.2**	**24.2**	**5.16 X 10^{-7}**	**1.6**	**87.3**	**36**	**197.0**	**70.2**
EPE1	**23.5**	**21.6**	**2.86x10^{-6}**	**1.09**	**152**	**31.8**	**254.0**	**47.2**

γ_{CMC} = interfacial tension at critical micellization concentration,
Γ_{min} = surface excess concentration of surface active molecule,
A_{min} = minimum area / molecule,
CMC = critical micellization concentration
ΔG^0mic = standard free energy change of micellization
I. F. = Interfacial Tension
Sap. Value = Saponification value
PL = Soy phospholipids
EPE1 = Phospholipid enzymatically modified by ethyl acetate
(All measurements were done at 27^0C. At 27^0C the value of chloroform/water interfacial tension is 33.5 dynes/cm)

Aceto-phospholipids have a pronounced effect on the emulsification process. The behavior of oil-in water types of emulsions was studied in presence of aceto-phospholipids and original phospholipids. We have got the different types of droplet distribution of oil-in-water emulsion could be seen in presence of original and EPE1. Fig-3 shows the droplet distribution pattern of sunflower oil-in-water and coconut-oil-in-water in presence of original phospholipids. This distribution pattern was very broad. Slightly narrow distribution pattern was observed for the enzymatically prepared aceto-phospholipids (EPE1) and very narrow distribution for chemically prepared aceto-phospholipids (presented in Fig.-4 and Fig.-5) appears that sufficient hydrocarbon chain length of emulsifier is necessary to make oil-in-water-type of emulsion of small size distribution. The value of emulsifying index (E24) of enzymatically prepared and chemically prepared aceto-phospholipid with original phospholipid is shown in Fig.-6 which reveals stabilization the oil-in-water emulsion.

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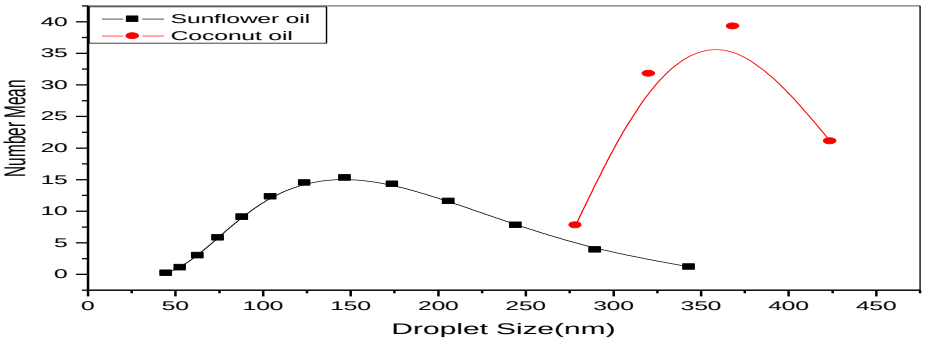

Fig.-4: Droplet size distribution of different type of oil in water emulsion stabilised by soya phospholipids

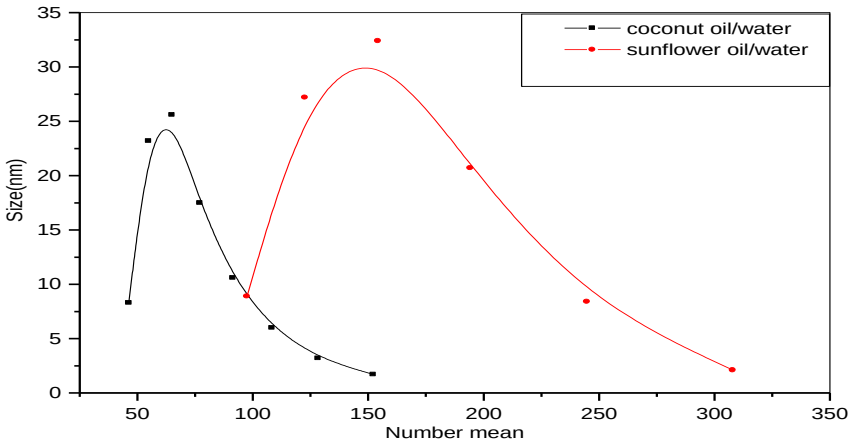

Fig.-5: Droplet size distribution of different type of oil in water emulsion stabilised by acetophospholipids(EPE1)

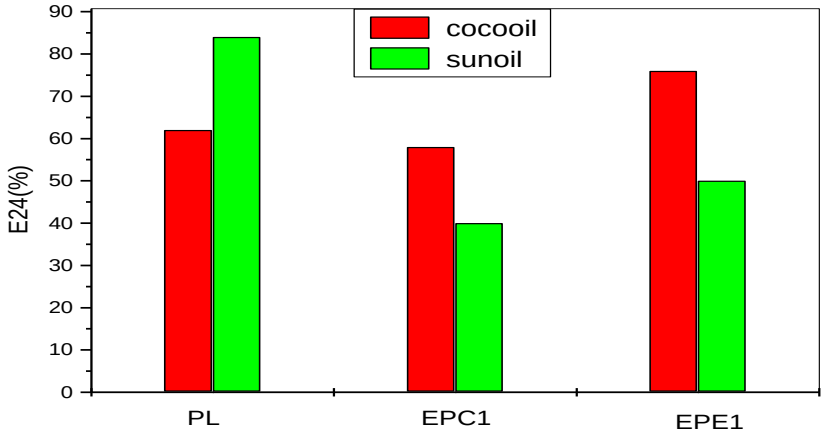

Fig.-7: Emulsifing index (E24) of different oil in water type of emulsion in presence of original and different acetophospholipis EPC1(0.12%) and EPE1(0.24%)

Important Application of Phospholipids in Health Sciences

The human body uses phospholipids as emulsifiers. Phospholipids are basic substances to maintain life activity. They are widely distributed in humans, animals, plants, and so on. Phospholipids are indispensable components of all cellular and sub-cellular membranes, they can arrange as bilayer membranes due its emulsifying properties. In addition to assembling the membrane, phospholipids are also used to assemble the circulating lipoproteins, the main task of which is to transport lipophilic triglycerides and

cholesterols through the hydrophilic blood. Together with cholesterols and bile acids, they form mixed micelles in the gallbladder to promote the absorption of fat-soluble substances. The human body also uses phospholipids as the surface-active wetting agents in the pleura and alveoli of lung, pericardium, joints, etc. Different kinds of phospholipids have some general properties, but they also own their unique physiological functions (Table 3).

Table 3: Physiological Properties of major Phospholipids

Phosphatidylcholine (PC),	An important substrate of synthesis of the neurotransmitter acetylcholine, and having the function of nourishing the brain and improving intelligence [
Phosphatidylethanolamine (PE),	Playing an important role in membrane fusion
Phosphatidylserine (PS),	a. Improving function of nerve cells, regulating the conduction of nerve impulse, and enhancing the memory function of brain [29]. b. The common feature of apoptosis [30]. c. The main acidic phospholipids of membranes of platelets, and involved in the clotting process
Cardiolipin (CL)	A phospholipid of unusual structure is localized almost exclusively to inner mitochondrial membrane, is particularly rich in unsaturated fatty acids. This phospholipid plays an important role in mitochondrial bioenergetics by affecting the activity of key proteins of mitochondrial inner membrane
Phosphatidylinositol (PI)	The precursor of second messenger, which plays a very important role in the process of transmission of messages in neural system
Sphingomyelins (SM)	Along with cholesterol, constituting the key components of the stable, detergent-resistant nanodomains in membranes, called functional lipid rafts which have been identified as the important membrane structure of signal transduction, protein transport and sorting of membrane components [

Drug delivery systems (DDS) is the prime system of therapeutics. In a suitable dosage and mode of administration to achieve the best therapeutic effect is the research objective of DDS. As main components of cellular membrane, phospholipids have excellent biocompatibility and for their amphiphilic structures, it is being a good DDS. Phospholipids have a propensity to form liposomes, which can be employed as the drug carriers[14]. Phospholipids have good emulsifying property which can stabilize the emulsions. In addition, phospholipids as surface-active wetting agents which can coat on the surface of crystals to enhance the hydrophility of hydrophobic drugs. The above properties are successfully employed in the DDS design. Phospholipids based DDS have been found promising for better and effective delivery of drugs and providing much appropriate systematic drug delivery. In recent years, a variety of phospholipid-related formulations, such as Doxil®, Cleviprex®, Valium® and Silybin Phytosome™, have been used in clinic, and achieving good results. Phospholipid takes an important role in the development of liposomal drug delivery system. Liposomes are vesicles prepared with phospholipids as the main substance, the structure of which is similar to cellular membrane. Liposomes as carriers of therapeutic drugs have attracted attention more than 40 years. As a DDS, liposomes have many advantages as follows: delivering both hydrophilic and lipophilic drugs (Fig. 6), possessing targeting, controlled release properties, cell affinity, tissue compatibility, reducing drug toxicity and improving drug stability.

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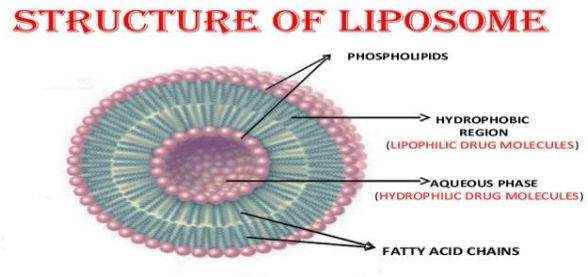

Fig. 7: Structure of liposome

During the researches, the conventional structures of the liposomes have some changes, which have brought out a series of new type liposomes, such as long-circulating liposomes, stimuli-responsive liposomes, cationic liposomes and ligand-targeted liposomes. Liposomes can serve as the carriers of antitumor drugs, antifungal drugs, analgesic drugs, gene therapeutics and vaccines, and there have been some liposomal formulations industrialized which are listed in Table 4.

Table 4: Approved liposomal formulations

Product	Phospholipids	Drug	Year approved
Ambisome	HSPC and DSPG	Amphotericin B	1990 (Europe)
Doxil	HSPC and DSPE-PEG_{2000}	Doxorubicin	1995 1999 2003 (Europe, Canada) 2007
DaunoXome	DSPC	Daunorubicin	1996 (Europe) 1996 (USA) 1997 (USA), 2000
Myocet™	EPC	Doxorubicin	2000 (Europe)
DepoDur™	DOPC and DPPG	Morphine sulfate	2004
DepoCyt	DOPC and DPPG	Cytosine 1999 Arabinoside	1999
Lipo-Dox	DSPC and DSPE-PEG_{2000}	Doxorubicin	2001 (Taiwan)
Marqibo	ESM	Vincristine	2012 (USA)

Phospholipids as zwitterionic surfactants can be used as emulsifying agent of O/W type emulsions, and due to their biological and non-toxic characteristics, they can be used as emulsifiers for intravenous injection. The O/W type emulsions are mainly composed of two parts: the oil core and the emulsifying agents on the surface. Unlike liposomes, fat emulsions are suitable for large-scale industrial production and relatively stable below 25 C for long term. More importantly, a large quantity of lipophilic drugs can be dissolved in the hydrophobic core of emulsions [15].

Table 6: Representative list of currently marketed drug containing injectable emulsions

Product	**Drug**	**Market**	**Emusifier**
Cleviprex®	Clevidipine Butyrate	USA	EP
Diazemuls®	Diazepam	Europe, Canada, Australia	EP
Diazepam-Lipuro®	Diazepam	Europe, Canada, Australia	EL
Diprivan®	Propofol	Worldwide	EL
Etomidat-Lipuro®	Etomidate	Germany	EL
Fluosol-DA®	Perfluorodecalin	Worldwide	EP and pluronic F68
Perflurotripropylamine Liple®	Alprostadil (PGE1)	Japan	EP
Limethason®	Dexamethasone Palmitate	Japan, Germany	EL
Lipo-NSAID®	Flurbiprofen axetil	Japan	EL
Stesolid®	Diazepam	Europe	EP

Vitalipid®	Vitamins A, D2, E, K1	Europe	EL

EL: egg lecithin EP: egg phospholipid.

Conclusion

Aceto soy phospholipids were prepared by incorporating the acetate group by enzymatic trans-esterification using methyl acetate. Our study showed that the newly prepared aceto phospholipids act as better emulsifiers in the oil-in-water emulsion than original soy phospholipids. The surface active and thermodynamic properties of the aceto-soy phospholipids are different from those of the original soy phospholipids. As different phospholipids take a major role in a drug delivery system, our modified aceto-phospholipid being a good emulsifying and surface active properties can take a new role in DDS. Our future work is on searching the role of aceto-phospholipid in Drug Delivery System.

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