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A characterisation study on the application of inverted lyotropic phases for subcutaneous drug release

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ABSTRACT

An experimental characterisation of lipid mixtures consisting of inverted hexagonal and inverted cubic phases composed of soybean phosphatidylcholine (SPC) and glycerol dioleate (GDO) was performed. The release of five chromophores of varying lipophilicity, used as model drugs, was investigated. Two experimental setups were applied: one based on maintaining sink condition, while a constant volume release medium was employed for the other. For neither setup, no correlation between the model drug lipophilicity and the polarity of the carrier matrix was found. However, the lipid phases showed a prolonged release, spanning weeks, of the model drugs, which exhibit lipophilicity values ranging by four orders of magnitude.

1. Introduction

For years lipid phases have been investigated for usage as drug delivery vehicles (Yuba et al., 2008; Sagalowicz et al., 2006; Mouritsen, 2005; Drummond and Fong, 1999; Thomas et al., 1991). Hitherto, such systems have mostly been limited to liposomes, which are injected directly into the blood stream (Han et al., 2007; Kwon et al., 2008). The idea with using these phases as drug carriers is to prolong the therapeutic concentration of the active compound within the body. An example for achieving this is using vegetable oils, which are injected into a large muscle. The drug diffuses out into the surrounding tissues, subsequently into the bloodstream, and a steady drug concentration can be maintained for approximately 20 days. Since most common drugs have a general hydrophobic character, they can be dissolved into oils and administered in this way. Instances where the drugs are not purely hydrophobic necessitate a delivery matrix exhibiting similar chemical properties. Lipid emulsions or liposomes are an interesting alternative to pure oils since they incorporate both hydrophilic and hydrophobic domains.

For decades, inverted phospholipid phases, which are stable in an aqueous environment, have been known to exist (Luzzati, 1968). One such interesting lipid system is composed of soybean phosphatidylcholine (SPC), diacylglycerol (DAG) and water. The phase diagram of this ternary system was first described by Orädd et al. (1995). It was found that all of the inverted phases can exist in equilibrium with pure water. The use of injectable lipids, which form semisolid drug receptacles when exposed to body fluids, is a potential alternative to the vegetable oil systems.

The aim of the present study is to investigate the use of the aforementioned SPC/GDO phase as a depot drug delivery system with respect to the incorporation of drugs with varying lipophilicity, release kinetics as well as structural integrity.

2. Materials and methods

2.1. Materials

Soybean PC (SPC) and glycerol dioleate (GDO) were a kind gift from Camurus AB shipped in the presence of dry ice, and stored in a −16 °C freezer upon arrival. Rhodamine 6G, fluoresceine, carboxy-fluoresceine, rhodamine 101 and 8-metoxypyrene-1,3,6-trisulphonic acid trisodium salt were purchased from Sigma and stored in the dark at +4 °C. *n*-Octanol of analytical grade (Riedel-De Haën, AG, Seelze-Hannover, Analytical grade) was used in the hydrophobicity studies. Analytical grade ethanol (95%) was used as solvent for the chromophores and batch solutions were prepared with concentrations of 16 ± 2 μM.

Abbreviations: CFl, carboxy-fluoresceine; Fl, fluoresceine; GDO, glycerol dioleate; MeOPy, 8-metoxypyrene-1,3,6-trisulphonic acid trisodium salt; Rh101, rhodamine 101; Rh6G, rhodamine 6G; SPC, soybean phosphatidylcholine; EtOH, ethanol.

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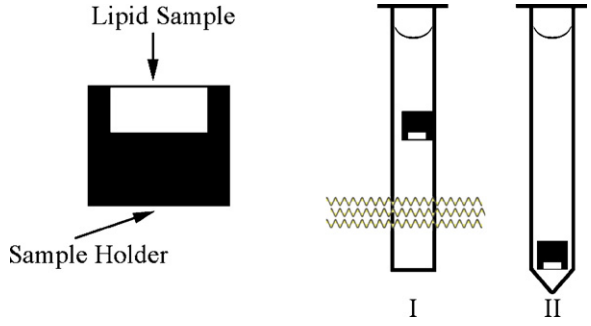


Fig. 1. A schematic of the plastic sample holder in which the lipid mixtures are placed, as well as the two experimental setups (I and II) used for *in vitro* release. Further details are given in Section 2.3.

2.2. Preparation of samples

All lipid samples were prepared according to the same formula. SPC was freeze dried, in batches ranging from approximately 0.500–1.500 g, sealed and stored in a freezer at −16 °C. So as to minimize water condensation, each batch was allowed to warm up to room temperature before exposure to air, preceding mixing. SPC was placed into a glass vial and an amount of GDO was added so as to obtain the desired lipid mass ratios. To each SPC/GDO mixture enough EtOH/chromophore solution was added to produce 5–10 wt% ethanol content. The resulting chromophore concentration ranged between approximately 0.03 and 0.06 wt%. A Teflon coated screw cap was firmly positioned over the glass vials and then they were alternatively placed on a shake table and vortexed, until a homogenous solution was produced.

2.3. Experimental setup for in vitro release studies

The sample holders were made of polyvinylchloride plastic and specifically designed for the purpose of drug release into an aqueous solution. A schematic of the plastic sample holder in which the lipid mixtures are placed, as well as the two experimental setups (I and II) used for *in vitro* release is shown in Fig. 1. The exposed area of the lipid mixture was well defined with a diameter of 8 mm. The depth of the drilled wells was 2 mm creating a maximum sample volume of approximately 0.1 ml. In experimental setup I, the cuvette is sealed from the atmosphere with paraffin. Here, the sample holder is mechanically fixated against the wall of a cuvette, with enough space from the bottom so as to allow the absorption of the water to be measured below the sample holder. The total volume of water is approximately 8 ml. In experimental setup II, the sample holder rests on the bottom of a conically shaped test tube, which is covered with a plastic cork. The total amount of water is approximately 12 ml. Sample volumes (4 ml) are removed at specific times and an equal volume of fresh release medium is added. The samples were kept at 23 ± 2 °C. The absorbance and thus the concentrations of the removed samples (volumes) are measured separately. The glass test tubes had thus a lipid:water ratio of 1:120, by volume, and the plastic cuvettes held an aforementioned ratio of 1:80.

2.4. Determination of hydrophobicity

In order to determine the partition coefficient between water and *n*-octanol, the model drugs were dissolved in de-ionized water and the concentrations were measured with vis-absorption spectroscopy. These aqueous solutions were added to an equal volume of *n*-octanol. Equilibration was reached by means of mixing on a tilting table for 3 days at 23 ± 2 °C. The concentrations of the model drugs in the water phase were then inferred from measuring the absorbance. The hydrophobicity index (=$^{10}\log P$) was calculated from the concentration ratios according to (Lahnstein et al., 2008):

$$^{10}\log P = {}^{10}\log\left\{\frac{[X]_{\text{n-oct}}}{[X]_{\text{water}}}\right\}$$

2.5. Polarized microscopy

The cross-polarized microscope investigations were performed by using a Zeiss Axiolab binocular microscope, for bright field use. Lipid samples were placed on glass plates, as droplets, by glass Pasteur pipettes, and glass cover plates were put on top of the samples. A part of a cover plate was positioned underneath one side of the cover plate covering the sample. This was done to create a wedge shape of the cover plate, which facilitated the hydration of the lipid sample when a drop of water was placed on the side of the sample: the capillary effect pulled in the water towards the sample underneath the cover plate.

The white light shown from underneath the sample was linearly polarized, and a linear polarizer was set at right angle immediately in front of the eyepiece. Any isotropic lipid structures (such as a cubic phase or an isotropic liquid) would appear dark in the microscope, whereas any anisotropic structure (such as a lamellar or a hexagonal phase) would be evident as bright white crystals.

2.6. In vitro release studies

The amorphous lipid phases were placed in plastic sample holders, which were immediately immersed in vials containing de-ionized water with approximately 1 wt% sodium azide. The release of chromophore from the lipid mixtures was conducted according to two methods. The reason for investigating two methods was to assure that sink condition was maintained throughout the release experiments. One was by using cuvettes as vials and fixating the sample holders with the lipid phase facing down (see Fig. 1). This was to prevent the lipid samples, which are less dense than the water release medium, to dislocate from the sample holders and rise to the surface. In order to minimize water from evaporating, the openings of the cuvettes were coated with Teflon tape and sealed with paraffin. The other method, which represents “true” sink condition, measures the release by placing the sample holders inside glass test tubes with conical bottoms. The lipid sample holders were not fixated, but forced to lie with the exposed lipid phase facing down, as illustrated in Fig. 1. At specific time intervals, 4 ml samples were removed and immediately replaced with an equal volume of fresh release medium. Before each sample removal the water phase in both methods was mixed by repeatedly turning the vials upside down 3 times by hand. This very gentle mixing of the water phase ensured a minimization of the mechanical shear on the surface of the lipid sample, which other methods of mixing had proven detrimental to the homogeneity of the lipid water interface. The removed volumes were placed inside quartz cuvettes and the absorbance was measured. For the case where the sample holders were fixated inside the cuvettes, the absorbance was measured through the cuvettes, beneath the sample holders.

The absorption was converted into concentration values by means of the following molar absorptivities [$\varepsilon(\lambda_{max})$]:

$\varepsilon_{Rh6G}(\lambda_{max}) = 64\,400\ \text{mol}^{-1}\ \text{dm}^3\ \text{cm}^{-1}$, at 530 nm;
$\varepsilon_{Fl}(\lambda_{max}) = 13\,500\ \text{mol}^{-1}\ \text{dm}^3\ \text{cm}^{-1}$, at 500 nm;
$\varepsilon_{CFl}(\lambda_{max}) = 21\,300\ \text{mol}^{-1}\ \text{dm}^3\ \text{cm}^{-1}$, at 495 nm;
$\varepsilon_{Rh101}(\lambda_{max}) = 37\,400\ \text{mol}^{-1}\ \text{dm}^3\ \text{cm}^{-1}$, at 575 nm;
$\varepsilon_{MeOPy}(\lambda_{max}) = 7000\ \text{mol}^{-1}\ \text{dm}^3\ \text{cm}^{-1}$, at 404 nm.

Molar absorbtivity values were obtained by the following procedure: ethanol solutions were prepared with known concentrations

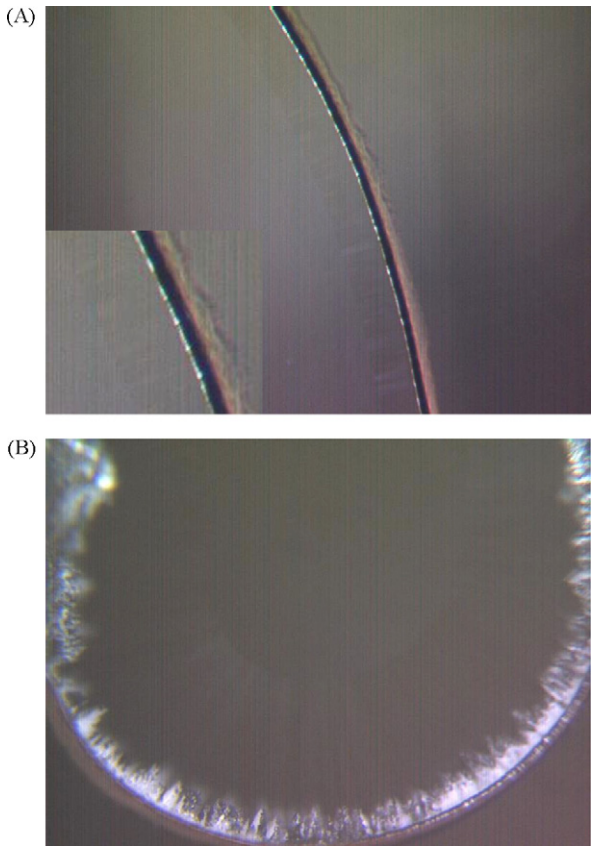


Fig. 2. (A) A picture of a 45/55 (wt%/wt%), SPC/GDO lipid mixture, containing 5–10 wt% ethanol, in contact with air, as seen through a cross-polarized microscope. The creation of the halo is due to a growth of liquid crystalline phases, induced by the presence of water. (B) A picture of a 45/55 (wt%/wt%), SPC/GDO lipid mixture, containing 5–10 wt% ethanol, in contact with liquid water, as seen through a cross-polarized microscope.

of each dye. The solutions were diluted 1000 times in water and the absorbance was measured. The molar absorbtivity of each dye was calculated from Lambert–Beer's law.

3. Results

3.1. Phase characterisation by crossed polarized microscopy

Three lipid compositions of SPC, GDO and ethanol, in an amorphous liquid state, have been investigated following the addition of water by means of crossed polarized microscopy. Initially, a drop of liquid lipid mixture was placed between a slide and a cover slip and for the sample containing the largest amount of SPC, a halo rapidly formed at the lipid air interface (*cf.* Fig. 2). This process visualized the formation of a non-isotropic phase due to the pronounced hygroscopic property of this composition, leading to an uptake of water from the atmosphere.

A second set of crossed polarized microscopy experiments were performed, where water was immediately added and absorbed by the lipid samples. Here a halo was formed, but at a much faster rate. In addition, the presence of water surrounding the lipid droplet induced a swelling of the depolarizing phase towards the centre of the droplet (see Fig. 2). The progression rate of the depolarizing phase for these mixtures was observed to increase, with higher concentrations of SPC, which is due to the highly hydrophilic nature of the SPC lipid. This is compatible with the concept of injecting a liquid into water, whereby a phase transition occurs and forms a semisolid. This corresponds to the two-phase region composed

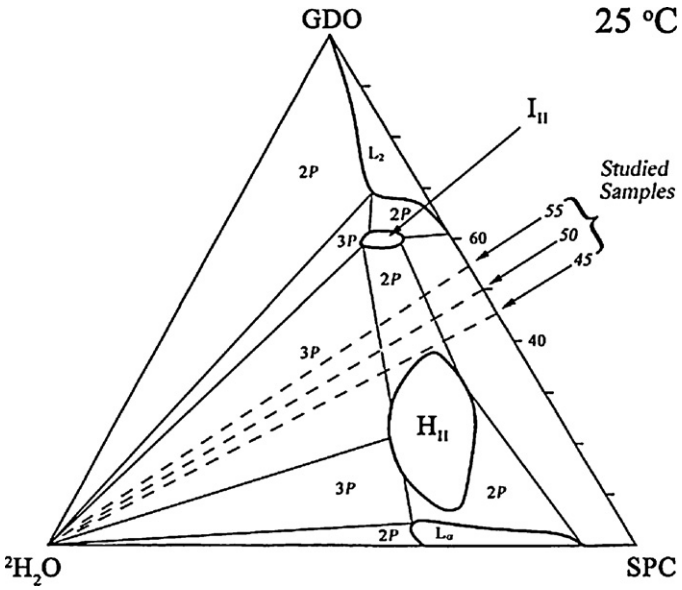


Fig. 3. Phase diagram of the system soybean phosphatidylcholine (SPC), diacylglycerol (GDO) and deuterated water, amended from Orädd et al. (1995). The studied three compositions are indicated on the binary SPC–GDO axis by dashed lines. The phases regions indicated by I_{II} and H_{II} refer to a reversed hexagonal phase and a reversed cubic liquid crystalline phase, respectively.

of a cubic liquid crystalline phase (I_{II}) and a reversed hexagonal phase (H_{II}) at the onset of thermodynamic equilibrium (*cf.* Fig. 3). The observed optical textures are compatible with the presence of these phases.

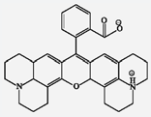
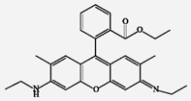
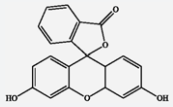
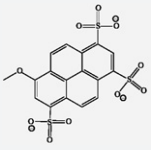
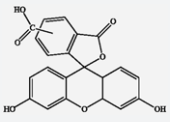
3.2. Lipophilicity studies of model drugs

The solubility ratios of the model drugs were investigated and the obtained values of $^{10}\log P$ are presented in Table 1. The values of $^{10}\log P$ were determined as is described in Section 2. The concentrations of the model drugs were in the mM range. The $^{10}\log P$ values of the test group are evenly spread over a wide range, $-2.45 \leq {}^{10}\log P \leq 2.17$, which actually means that lipophilicity varies by four orders of magnitude. One can note the MeOPy is not the most hydrophilic compound, despite its large number of permanent charges. Furthermore, the uncharged substance, CFl, with the lowest molecular mass, is also the most water soluble. Among the substances which are lipophilic, the most extreme carries two opposite charges. This compound also has the highest molecular mass.

3.3. Release experiments

The release of the model drugs, Rh101, Rh6G, CFl, Fl and MeOPy from the lipid composition 45/55 (wt%/wt%) of SPC/GDO was investigated by using the methods I and II described above. The concentration of the model drugs in method I was always within an accurately measurable range. Method II was used in order to compare method I with a setup that has a better approximated sink condition. The intention was to correlate the release kinetics/profile of the substances with their inherent lipophilicity. The three compositions are indicated on the binary SPC–GDO axis by dashed lines in the phase diagram (*cf.* Fig. 3). When *ca.* 20 wt% of water is present, the compositions enter a two-phase region, which is composed of a reversed cubic liquid crystalline phase (I_{II}) and a reversed hexagonal phase (H_{II}). With an increased SPC fraction, the amount of the H_{II} phase increases. Beyond the two-phase region, *i.e.* at a water content $\geq$ 30 wt%, a three-phase region (3*P*) exists,

Table 1
The chemical structures, $^{10}\log P$ values and molecular masses of the model drugs.

Chemical structure	Abbreviation	$^{10}\log P$	M_W (Da)
	Rh101[a]	2.17	491
	Rh6G[b]	1.13	479
	Fl[c]	0.63	332
	MeOPy[d]	−2.00	538
	CFl[e]	−2.45	376

[a] Rhodamine 101 (Rh101).
[b] Rhodamine 6G (Rh6G).
[c] Fluoresceine (Fl).
[d] 8-Metoxypyrene-1,3,6-trisulphonic acid trisodium salt (MeOPy).
[e] Carboxy-fluoresceine (CFl).

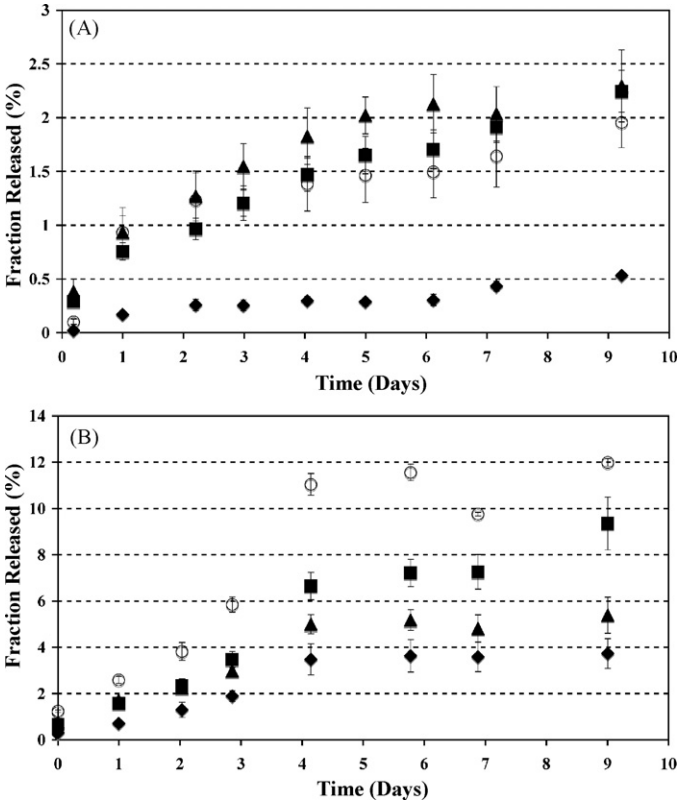


Fig. 4. (A) The released fractions of four model drugs from a lipid mixture of SPC/GDO at a ratio: 45/55 (wt%/wt%). The model drugs are R6G (♦), Fl (■), CFl (▲) and Rh101 (○). The method used (II) is that of removal and addition, so as to retain sink condition. The cumulative amount of released substance is calculated by taking into account the removed amount at each moment of measurement. (B) The released fractions of four model drugs from a lipid mixture of SPC/GDO at a ratio: 45/55 (wt%/wt%). The method used (I) is that of continuous release into a sealed cuvette (*cf.* Fig. 1).

where the reversed lipid phases are in equilibrium with an excess of water.

The obtained results are summarised in Fig. 4, in which the release profile of MeOPy is omitted. This is due to the fact that the concentrations of MeOPy never reached a high enough level in the release media, so as to be measurable with the implemented absorption techniques.

It is also apparent that the choice of release method had an effect on the release profile of the model drugs, as well as on the detectable fraction of drug released. A much smoother profile was observed when sink condition was maintained. However, that method introduced a greater degree of noise to each data point, as indicated by the greater width of the error bars seen in Fig. 4A. This was because the removal and replenishment of the release medium, as well as the separate measurements performed apart from the ongoing release, added uncertainties. As for the higher concentrations observed when measuring according to method I, it is most probable that these values did not reflect the true concentration inside the whole cuvette. The experimental setup used in method I created a very small gap, in the range of tenths of millimetres, between the sample holder and the cuvette wall. This made it very difficult to attain a perfectly mixed release medium inside the cuvette. In fact, following approximately 10 days from the start of the release experiments, it was observed, by ocular inspection, that there existed a slightly higher concentration of model drug in the release medium below the sample holder than above it.

It can be observed from Fig. 4 that, in general and quite independent of the method used, the release of the model drugs followed one initial form of kinetics (during approximately 5 days), and displayed a slower rate afterwards.

An internal comparison between the model drugs revealed that the compound Rh6G exhibited the lowest ability to be released from the used lipid matrix. This was especially pronounced in the method where sink condition was maintained (*i.e.* method II). This is notable, considering the fact that its lipophilicity is not particularly deviant from the rest of the model drugs. In fact, the internal variation of the release data for the substances does not seem to be related to their solubility (unpublished data).

Concerning the lipophilicity of the studied substances with respect to their release properties, it is tempting to imply a correlation between the release profile and the lipid composition. Therefore it is pertinent to investigate the influence of varying the lipid ratios in the release system. Since SPC is zwitterionic, while GDO is non-ionic, an increased ratio of SPC leads to a more polar lipid mixture. In the presence of water this corresponds to an increased fraction of the inverted hexagonal phase (H_{II}) *versus* the inverted cubic (I_{II}) one, as is evident from the phase diagram. Three lipid compositions were studied, which are indicated in the ternary phase diagram displayed in Fig. 3. The following SPC/GDO ratios were selected: 45/55, 50/50 and 55/45 (wt%/wt%). Two sets of these mixtures were prepared, with the inclusion of two model drugs, Rh101 and Rh6G, respectively. The release profiles from these lipid samples, obtained by using method I, are displayed in Fig. 5. Despite the fact that the lipophilicity of the two drugs differs by a factor of 10 (*cf.* Table 1), the internal variation of the release kinetics, for each substance, is larger than the variation between the two compounds.

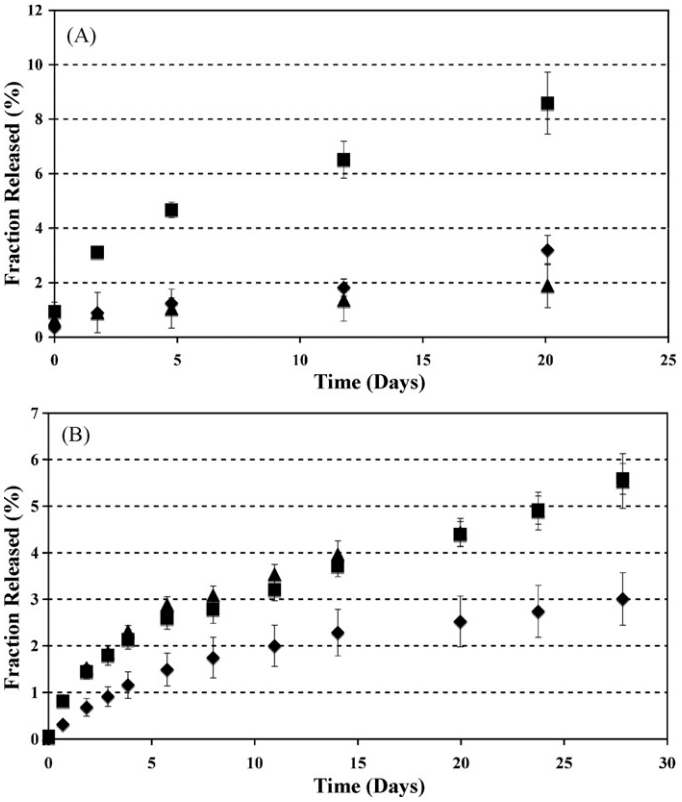


Fig. 5. Release of the model drugs Rh101 (A) and Rh6G (B) from three different lipid compositions of SPC and GDO. Method I is used, so as to maintain the sink condition. The compositions, given in wt%, are SPC/GDO as follows: 45/55 (♦), 50/50 (■) and 55/45 (▲).

Thus, the lipophilicity criterion, as defined here, is insufficient for predicting the release properties. Therefore, in order to use different lipid compositions' relative polarity as a method of controlling the release rate, each potential drug needs to be investigated for each lipid composition.

4. Discussion and conclusions

This study strongly supports the supposition that the investigated lipid mixtures have properties, *in vitro*, which make them usable as an injectable liquid, *i.e.* by forming a semisolid matrix in an aqueous environment. Based on studies with crossed polarized microscopy and an existing ternary phase diagram, it was deduced that this matrix is composed of a mixture of an inverted hexagonal phase and an inverted cubic phase.

By observing the release of model drugs of different lipophilicity from the studied lipid phases, it is suggested that these phases can be used as drug delivery matrices for drugs that are intended for slow-release administration. After 10 days, no more than 3% of the total amount of any investigated model drug had been released when method II was used. An equivalent maximum value for method I is 14%, but as previously discussed in Section 3.3, this value is probably quite a bit higher than the true fraction. It is reasonable to believe that the concentrations in method I should, in fact, be lower than those in method II, since it has a "true" sink condition.

A significant observation is that the release profile includes no indication of a burst effect, which is quite common for *ordinary* cubic phases when used for drug delivery (Lee et al., 2009; Boyd et al., 2006). Both of these findings can possibly be explained by noting the fact that the transport, and subsequent release of the drug molecule, takes place through a semisolid, which implies a restricted freedom of motion for anything trapped inside. Right at the moment of administration, the phase transition starts, as is evident from the advent of the halo, described in Section 3.1. This fast phase change at the lipid/water boundary most probably prevents any release burst, as would occur from a liquid drug depot.

The polar properties of the model drugs, within the range of molecular mass studied here, seem to have little or no impact on the release kinetics. It has also been shown that a lipophilicity span of four orders of magnitude has negligible influence on the model drug release profile. This highlights the fact that the release mechanism is very complex. In conjunction with the drug release, at least three simultaneous processes are occurring, albeit on different timescales. One is the hydration process, which probably never reaches an end point, on the timescale of the release experiments. The second one is the transportation of the ethanol out of the lipid phase. The ethanol has a much greater affinity to an aqueous environment than to the lipid matrix, hence it leaves the lipid system virtually completely within the first hours of the release experiment. The third main process is the release of the model drugs themselves. As previously described, for all model drugs in this study, two release kinetic processes appear to exist on different timescales. During approximately the first 5 days, the release is somewhat faster than in the remaining timescale. This fact, independent of the lipophilicity of the model drugs, leads to the conclusion that the lipid matrix in this study can be used as a drug carrier for prolonged release. The question of controlling the release rate by varying the lipid composition, proved to be unanswerable when only considering the lipophilicity, as described in this work. Nonetheless, the examined lipid matrix has interesting physico-chemical properties for subcutaneous administration, although the release profiles of any potential drug need to be individually investigated with respect to the lipid matrix composition.

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