DRUG PERMEABILITY ACROSS A PHOSPHOLIPID VESICLE BASED BARRIER

3. CHARACTERIZATION OF DRUG-MEMBRANE INTERACTIONS AND THE EFFECT OF AGITATION ON THE BARRIER INTEGRITY AND ON THE PERMEABILITY

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Abstract

Recently we reported on the development and structural characterization of a phospholipid vesicle based barrier useful for medium throughput screening of passive drug permeability. Here, we investigate the physical and functional integrity of the phospholipid vesicle based barriers to agitation by stirring or shaking, and whether agitation affects drug permeability of sulpiride, metoprolol and testosterone. In addition, three drugs (caffeine, naproxen and sulphasalazine) which were shown in a previous study to affect the electrical resistance of the barriers, were investigated for their influence on the permeability of a simultaneously applied hydrophilic marker (calcein), and on the thermotropic phase transition of the phospholipid bilayers using differential scanning calorimetry (DSC).

Electrical resistance measurements indicated that the barriers should withstand shaking speeds up to 150 rpm without losing their integrity, but significant release of phospholipids from the membrane barriers to the donor and acceptor chambers was observed under agitation ≥150 rpm. When using agitation up to 100 rpm no increase in permeability was observed for sulpiride, metoprolol and testosterone. The phospholipid vesicle-based barrier thus differ from other permeability models in that agitation does not lead to an increase in permeability, not even for highly lipophilic drugs such as testosterone. This is explained by the different morphology of the vesicle-based barrier which is containing a 100 μm thick layer of mostly aqueous compartments immobilised within a matrix of phospholipids vesicles.

Sulphasalazine and naproxen were shown to decrease the electrical resistance and increase the permeability of the hydrophilic marker calcein. The DSC experiments showed that these two drugs probably interact with the head groups of the phospholipids. In contrast, caffeine gave an increase in electrical resistance and a decrease in permeability of calcein. From the DSC experiments no signs of interaction of caffeine with the phospholipid bilayer could be observed.

Keywords: Artificial membrane, liposomes, phospholipids, phase transition, pH, Differential Scanning Calorimetry, DSC, shaking, stirring, membrane interactions, bilayer, drug permeability

1 Introduction

A novel medium-throughput screening method for prediction of passive drug permeability, constructed as a tight barrier of liposomes on a filter support was recently reported by us (Flaten, et al., 2006 a, 2006b). To the best of our knowledge, this is the first successful attempt to deposit membrane barriers without the use of an inert solvent such as hexadecane. The phospholipid vesicle based barriers were prepared from liposomes from egg phospholipids, deposited into/onto a filter support by use of centrifugation. The apparent permeability coefficients obtained from the phospholipid vesicle based model correlated well with literature data on human absorption *in vivo,* 18 out of 22 drug compounds were correctly classified according to their absorption ability *in vivo.* This new approach seems to model the *in vivo* absorption better than the bio-mimetic PAMPA model (Sugano et al., 2001) and equally well as the Caco-2 model (Yazdanian et al., 1998) and the double sink PAMPA (DS-PAMPA) model (Avdeef, 2003) in the prediction of passive diffusion of drug compounds (Flaten et al., 2006b).

The phospholipid vesicle based barriers are stable at –80 °C for up to two weeks and can stand a pH range from 2.0 to 8.0 without loosing their integrity (Flaten et al., 2006a). The pH-stability of the barriers make them suitable for further studies to provide insight into segmental absorption in the human gastrointestinal tract, it also allows pH-adjustments to solubilize poorly soluble compounds. The storage stability at the preferred temperature also provides the opportunity to produce larger batches of the barriers and thereby make the

laboratory work more efficient and the phospholipid vesicle based barrier more appropriate for medium to high throughput screening.

The structure of the phospholipid vesicle based barrier has been shown to be composed of liposomal or closed oligo- and/or multilamellar vesicular structures present in the pores as well as in a layer on top of the filter. By confocal laser scanning microscopy, described for this purpose by Nagahara et al. (Nagahara et al., 2004), we have shown that the pores are filled with liposomes and that no water channels through the barrier are present (Flaten et al., 2006a).

Diffusion across an artificial membrane consists of two parts; diffusion through the membrane and diffusion through the so-called unstirred water layer (UWL). If the thickness of the ULW is much larger than the thickness of the membrane, it can become rate limiting for diffusion of lipophilic drugs (Kansy et al., 2004). If this phenomenon is not taken into account, absorption parameters can be underestimated. The permeability of highly permeable drugs, such as testosterone, has been shown to depend on the stirring conditions in *in vitro* permeability assays (Karlsson and Artursson, 1991). So, if not compensating for the UWL-effect the permeability of lipophilic compounds in the UWL could significantly contribute to the resulting permeability values. Therefore we have performed permeability experiments with shaking and stirring to investigate the integrity of the barriers, as well as doing permeability experiments with three drug compounds, sulpiride, metoprolol and testosterone, at different shaking speeds to investigate the effect of shaking on the permeability properties in our model. The stability of the barrier in terms of electrical resistance and release of phosphatidylcholine to the donor and acceptor compartments was also investigated at different shaking and stirring speeds.

In preliminary studies some of the drug compounds used in the validation of the phospholipid vesicle based barrier affected the electrical resistance of the barrier (Flaten et al., 2006b).

Sulphasalazine and naproxen decreased the electrical resistance whereas caffeine had the opposite effect resulting in an increase in electrical resistance. In the present study the influence of these three drugs on the permeability of the hydrophilic marker calcein has therefore been investigated. An examination of possible interactions between the drugs and the phospholipid bilayer by use of differential scanning calorimetry (DSC) has also been performed.

2 Experimental section

2.1 Materials

Egg phosphatidylcholine, Lipoid E-80, was obtained from Lipoid, Germany. Caffeine, metoprolol tartrate, sulphasalazine, sulpiride, testosterone, naproxen, calcein and 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC) were purchased from Sigma-Aldrich Co, St. Louis, USA. Filter inserts (small filter holders which fits into the 24well titer plate) (Transwell-Clear, d = 6.5 mm) and plates were purchased from Corning Inc, Corning, USA, the filters removed and replaced by mixed cellulose ester filters (0.65 µm pore size) from Millipore, Billerica, USA.

2.2 Methods

2.2.1 Preparation of the barrier

The phospholipid vesicle based barriers were prepared according to the procedure reported by us (Flaten, et al., 2006b) with storage up to two weeks at – 80 °C as described (Flaten, et al., 2006a). In brief, liposomes with two different sizes were deposited on a filter support in consecutive steps, first the smaller liposomes and then the larger, by use of centrifugation. Freeze-thaw cycling was then used to promote liposome fusion to produce a tight barrier.

2.2.2 Investigation of the stability of the phospholipid vesicle based barriers during agitation and the influence on the permeability of drugs

2.2.2.1 Investigation of the stability of the barriers during shaking at different speeds by electrical resistance measurements

Permeability experiments were simulated at different shaking speeds to show the influence of the agitation on the electrical resistance of the barriers. The experiments were performed as described earlier (Flaten et al., 2006b) using phosphate buffer (pH 7.4) in both the donor and acceptor compartment with the exceptions that the system was shaken in an orbital shaker (Unimax 2010, Heidolph Instruments, Schwabach, Germany) at 50, 100, 150 or 200 rpm, respectively, and that the filter inserts were not moved from one well to the next during the experiments. A schematic drawing of the experimental set-up used in the permeation studies is given earlier (Fig. 2, Flaten et al., 2006b)

2.2.2.2 Determination of the amount of phospholipid in the donor and acceptor chambers after agitation

Permeability experiments were simulated by loading the filter inserts with phosphate buffer (pH 7.4), and placing them in the acceptor chambers containing the same type of buffer as described earlier (Flaten, et al., 2006b). The only exception was that the filter inserts were not moved to fresh wells at certain intervals but left in the same acceptor wells for 5 h. Different shaking and stirring speeds were applied in the different experiments (see Figure 2 in section 3.1.1.2). The 5 mm stirring bars were placed in the acceptor chamber and a Telemodul C from Variomag, USA was used to control the stirring speed (see Figure A in the Supplementary material).

Samples from the donor and the acceptor chambers were dissolved in sodium cholate solution (0.83 mg/ml). Quantification of phosphatidylcholine was carried out as described earlier (Grohganz, et al., 2003) with the following adaptations: 50 µl of the samples and 250 µl of the phospholipid B-enzymatic colorimetric reagent were mixed in a 96-well titer plate and incubated for 45 min in the titer plate reader at 37 °C, with shaking for the first 5 min. The UV-absorption at 492 nm was measured and evaluated against standard solutions of egg phospholipids and cholate. The results are the mean of 12 filter inserts done on two different days.

2.2.2.3 The permeability of sulpiride, metoprolol and testosterone at different shaking speeds

Permeability experiments were performed with three drugs (sulpiride, metoprolol and testosterone) at different shaking speeds to investigate how agitation would affect the permeability of the drugs studied. The permeation experiments were performed with solutions of sulpiride, metoprolol and testosterone as described earlier (Flaten et al., 2006b) except that the system was shaken in an orbital shaker at 50, 100, 150 or 200 rpm, respectively, during the experiments.

At the end of the permeation experiment, samples (200 µL) from each acceptor compartment were transferred into 96-well UV transparent plates (Costar) and the concentration of drug was measured spectrophotometrically (Spectramax 190; Molecular devices, Molecular Device Corporation, California, USA) at wavelengths of 250 nm for sulpiride and testosterone and 275 nm for metoprolol, respectively (Flaten et al., 2006b). The electrical resistance across the lipid barriers was measured (Millicell-ERS, Millipore, USA) immediately after completion of the permeation studies. A value of 119 ohms resulting from the filters was subtracted from the observed resistance to account for that resulting from the lipid barrier alone. The experiments

were performed at least in triplicate with six filter inserts in each parallel for every compound. For the experiment performed at 200 rpm only the electrical resistance across the barrier is given.

2.2.3 Influence of drug substances on the phospholipid vesicle based barrier

2.2.3.1 Permeability experiment with a hydrophilic marker together with drugs known to influence on the electrical resistance of the barriers

Permeability experiments with calcein together with sulphasalazine, naproxen and caffeine, respectively, were performed to determine whether these three drugs interact with the barrier in a way that also affects the permeability of a hydrophilic marker. The concentrations used were 110 mM, 3.9 mM, 8.6 mM and 4.4 mM for calcein, caffeine, naproxen and sulphasalazine, respectively. The concentrations were chosen to be appropriate with respect to the detection method used.

2.2.3.2 Differential scanning calorimetry (DSC) to study possible interactions between drug compounds and the lipid bilayers

1,2-Dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC) (20% (w/v)) was dispersed in phosphate buffer (pH 7.4) and in solutions of caffeine (7.8 mM (C1) and 3.9 mM (C2)), sulphasalazine (4.8 mM (C1) and 2.4 mM (C2)), naproxen (9.6 mM (C1) and 4.8 mM (C2)), testosterone (0.1 mM (C1) and 0.05 mM (C2)) and metoprolol (6.4 mM (C1) and 3.2 mM (C2)). The dispersions were produced by mixing the phospholipids with the different solutions in a Retsch MM200 ball mill (F.Kurt Retsch GmbH & Co, Germany) at 25 s^{-1} for 3 min. The dispersions were stored at room temperature over night and shaken in the ball mill (25 s^{-1} for 3 min) again before weighing out three samples from each dispersion. Samples were stored at room temperature until analysis.

DSC-experiments were carried out using a Perkin-Elmer Pyris 1 DCS differential scanning calorimeter (Perkin-Elmer Analytical Instruments, Norwalk, USA) and Pyris software for Windows NT. DSC runs were performed in an atmosphere of flowing nitrogen gas using standard aluminum sample pans. The DSC was calibrated with indium and tin from Perkin-Elmer (P/N 0319-0033 and 0319-0034). For each sample three consecutive re-heats within the instrument, with 30 min waiting time between each run was performed. All the experiments were carried out at a heating rate of 5 °C/min.

3 Results and discussions

3.1.1 Influence of agitation on the permeability of drugs and the physical stability of the barrier

In the most used permeability models, the Caco-2 model and the PAMPA model, the adherent ”fixed” layer of water, the so-called unstirred water layer (UWL) has been found to significantly influence the permeability values (Adson et al., 1995; Kansy et al., 2004; Karlsson and Artursson, 1991). Especially if drug diffusion in water is slow, i.e. in the case of poorly water-soluble drugs, this can become rate limiting for the permeation process. Means to decrease or even minimize this effect have involved e.g. shaking or stirring of the permeation assay setup (Adson et al., 1995; Avdeef et al., 2004; Karlsson and Artursson, 1991). The phospholipid vesicle based method has so far been run without agitation, so the aim of this part of the study was therefore to investigate the effect of agitation. One advantage with shaking or stirring is that the assay time can be decreased significantly.

3.1.1.1 Stability of the barriers during shaking at different speeds

To investigate if the barriers were stable to agitation in an orbital shaker, they were shaken at different speeds for 5 hours whereafter the electrical resistance across the barriers was measured. The results are shown in Figure 1. The barriers seem to stand shaking up to a speed of 200 rpm. However, when increasing the shaking speed further a drastic decrease in resistance was observed, indicating that the barriers have lost their integrity to some extent.

Figure 1

3.1.1.2 Amount of phospholipid released to the donor and acceptor chambers at agitation

Because of the decrease in electrical resistance at the higher shaking speeds we investigated whether this is due to a release of membrane constituents, e.g. liposomes from the barrier, into the acceptor and/or donor chamber. This could interfere with the UV measurements for some of the drugs tested as well as affect the integrity of the barriers. The phospholipid B-enzymatic colorimetric enzyme test (Grohganz et al., 2003) was used to quantify the amount of phosphatidylcholine in the acceptor and donor chambers. In these experiments we also used stirring as a means of agitation to examine if this is a more gentle form of agitation for our model as compared to shaking.

In order to collect enough phosphatidylcholine to be able to quantify the amount of phosphatidylcholine in the acceptor chambers the experiments were done without moving the filter inserts to fresh wells during the experiments. The amount of phosphatidylcholine found in the donor and acceptor chambers after 5 hours at the different agitation conditions are given in Figure 2.

Figure 2.

From Figure 2 it can be seen that no or only little phosphatidylcholine seems to be released from the barrier to both the donor and the acceptor chambers up to a shaking speed of 100 rpm. For shaking speeds of 150 rpm and above and for all the stirring speeds examined (the lower speed limit of the stirring equipment was 130 rpm) more than 5% of the total amount of phospholipid in the membrane (3.3 mg (Flaten et al., 2006a)) was found to be released into the donor and/or acceptor chamber.

Although the electrical resistance measurements indicated that the barriers stand shaking up to 200 rpm without loosing their integrity, the quantification of the amount of phosphatidylcholine released from the barrier showed that as much as 5% of lipid is released into the acceptor and donor chambers already when shaking at 150 rpm and stirring at 130 rpm.

3.1.1.3 Influence of shaking on the apparent permeability of sulpiride, metoprolol and testosterone

Permeability experiments with sulpiride, metoprolol and testosterone at shaking speeds from 0 to 150 rpm were performed to examine if shaking would affect the permeability of these drugs. The compounds were chosen to represent the extreme points of fraction absorbed in humans after oral administration (F_a), and to cover a wide range of hydrophilic/lipophilic properties. The results from the permeability studies are shown in Figure 3.

Figure 3.

Both the apparent permeability and the mass balance for testosterone (Figure 3A) are unchanged when using shaking up to 100 rpm, but a large increase in mass balance was observed with increasing shaking speed. At 150 rpm the mass balance appeared to be larger than unity which only can be explained by turbidity due to the release of phosphatidylcholine vesicles from the barriers into the donor and acceptor chambers. The observed elevated apparent permeability may thus be looked at as an artefact.

The permeability values for metoprolol were not increasing with increasing shaking speed up to 100 rpm (Figure 3B). At 150 rpm again an increase in apparent permeability was observed along with a mass balance larger than unity which points towards turbidity in the acceptor chamber as a consequence of release of phosphatidylcholin. In contrast, for sulpiride neither increase in permeability, nor change in mass balance was observed indicating that the membrane retention is not dependent on the shaking speed. The reason that the quantification of sulpiride is not affected to the same extent by the turbidity is that sulpiride has a higher specific absorbance compared to the other two drugs. From Figure 2 it is apparent that with shaking at 150 and 200 rpm more than 5% of the total amount of phospholipid was released from the barriers. For testosterone both the permeability and mass balance values at 150 rpm are affected by contamination from the membrane since the UV measurements of testosterone were performed at a wavelength (250 nm) and an absorbance level where turbidity would have a significant effect. Liposomes in the chambers would thus be the main reason for the unrealistically high values for the mass balance and, at least partly, for the apparent increase in permeability of testosterone at higher shaking speeds.

It has been shown in both Caco-2 and PAMPA models that agitation during the permeability experiments can decrease the UWL and thereby increase the apparent permeability of lipophilic drug compounds (Adson et al., 1995; Avdeef et al., 2004; Karlsson and Artursson,

1991). It has been claimed that slight shaking using plate shakers cannot reduce the thickness of the UWL sufficiently to match the *in vivo* situation (Avdeef et al., 2004), but there are other reports showing that already at 150 rpm the thickness can be considerably reduced down to 220 µm when using an orbital shaker (Adson et al., 1995). In that study the amount of testosterone that entered the acceptor chamber was clearly increased when increasing the shaking from 25 rpm to 50 or 100 rpm. This was not observed in our shaking experiments using testosterone. In another study (Avdeef et al., 2004) in which stirring with stir disks in the donor chamber was used, it was shown that the permeability of metoprolol across the UWL increased with increasing stirring speed. An increase in permeability of metoprolol was not observed in our experiments with shaking up to 100 rpm.

Our previous studies have shown that the phospholipid vesicle based model gives reasonable permeability values even without agitation and that it is possible to predict the permeability of highly lipophilic compounds like testosterone and caffeine in a satisfactory manner (Flaten et al., 2006b). Thus, we conclude that our model is not affected by the UWL, an important difference compared to the PAMPA and Caco-2 models. The reason for this difference is that the phospholipid vesicle based model is neither a monolayer of cells nor a continuous layer of phospholipid in an organic solvent as the PAMPA model. The phospholipid vesicle based barriers consist of liposome filled pores together with a layer of liposomes on top of the filter (Flaten et al., 2006a). Inside the liposomes there is a water phase, and this water will not be affected by the shaking or stirring of the system. The structure of our barriers is in other words not a lipophilic layer but a spongy structure. Even when reducing the unstirred water layer on both sides of the barrier, the drugs would still have to pass through the water phase trapped within the barrier, so the effect of decreasing the unstirred water layer is obviously less important for the overall permeability values. Furthermore, during the permeability studies the filter inserts are moved to fresh wells at certain time intervals, and this movement

is expected to contribute to movements in the donor and acceptor phases and thereby lead to a reduction of the UWL. The membrane is also thicker (>100 µm) than those used in other permeability models, so the ratio between the UWL on both sides and the liposome barrier is smaller than for the other models.

Thus, the phospholipid vesicle based barrier differs from the other models in that agitation within a range that does not destroy the barrier does not lead to an increased permeability, not even for the highly lipophilic drug testosterone. Obviously the UWL does not represent a major diffusion barrier, and neither the lag phase observed in the earlier studies (Flaten et al., 2006b) (data not shown) nor the experiment time is changed by shaking.

3.2 Influence of drug substances on the phospholipid vesicle based barrier

Preliminary studies have shown that some drug compounds seem to influence the electrical resistance across the barrier during the permeability experiment (see Figure 4). When caffeine was present the electrical resistance increased dramatically, whereas with sulphasalazine and naproxen a decrease was observed. No significant influence on the electrical resistance was observed with the 19 other drugs examined (Flaten, et al., 2006b). To the best of our knowledge such effects have not been observed with the cell based or PAMPA models. Permeability experiments with the hydrophilic marker calcein together with sulphasalazine, naproxen and caffeine, respectively, were performed to study if the drugs interact with the membrane in a way that could influence the permeability of a calcein. DSC experiments to study possible interactions between the drug compounds and the lipid bilayers were also performed using DPPC vesicles as a model. The reason for this is that the egg phospholipid does not go through a phase transition at ambient temperature. Several reports have been published on interactions between drug compounds and lipid membrane bilayers as revealed

by DSC. NSAIDS, cytostatics and antimicotics for example have been reported to interfere with phase transitions (Ambrosini et al., 1998; Beni et al., 2006; Kyrikou et al., 2004).

In addition to the three drugs known to interfere with the barriers also metoprolol and testosterone, that did not show any effect on the resistance, were included in the study. Two different concentrations of the drugs were used, C1 being the concentrations used in the permeability experiment at which the effects on the electrical resistance were observed, and C2 being half the concentration of C1 to allow detection of any concentration effects.

Figure 4.

3.2.1 Permeability experiments with calcein together with drugs known to influence the electrical resistance across the barriers

The results from the permeability experiments with calcein under the influence of caffeine, naproxen and sulphasalazine are shown in Figure 4. The permeability of calcein increased to a large extent when sulphasalazine was present and decreased when caffeine was present in the donor compartment. For naproxen there was also observed an increase in permeability of calcein, but not to the same extent as for sulphasalazine.

3.2.2 Differential scanning calorimetry (DSC) to examine possible interactions between drug compounds and the lipid bilayers

DSC has found wide application as a sensitive and fast method to study the thermotropic phase transition of biological membranes and model lipid membranes, including the influence of membrane proteins. This technique has also shown to be appropriate to detect interactions between drug compounds and phospholipid bilayers (Mcelhaney, 1982; Skalko et al., 1996). Phospholipid bilayers undergo a thermally induced phase transition from a highly ordered crystalline phase (gel phase, $L_{\beta'}$) at lower temperature where the hydrocarbon chains mainly

adopt extended and rigid all-*trans* conformations and the polar head groups are relatively immobile, to a more disordered liquid-crystalline phase (fluid phase, L_α) at higher temperature where the carbon chains are more flexible and can adopt also *gauche* conformations. In between these two states there is an intermediate state (ripple phase, $P_{\beta'}$). The so-called pretransition ($L_{\beta'} \rightarrow P_{\beta'}$) of synthetic phospholipids such as dipalmitoylphosphatidylcholine (DPPC) has been shown to be due to structural changes in the lamellar lattice (Taylor and Morris, 1994), whereas the main transition ($P_{\beta'} \rightarrow L_\alpha$) represents the "melting" of the hydrocarbon chains. Interactions between any drug molecule and the bilayer would interfere with both these phase transitions.

DSC measurements were therefore performed to observe any interactions between model liposomes made of DPPC and the drugs. Heating scans of DPPC in excess buffer showed two characteristic peaks in the temperature range from 20 to 55 °C which correspond to the pretransition and the main transition, respectively. The onset temperatures and the ΔH-values obtained from DSC measurements for the pre- and main transitions are given in Figure 5 and 7 respectively. In addition, the half-with of the main transition peak ($\Delta T_{m1/2}$) is given in Figure 6.

Figure 5.

Figure 6.

The pretransition is generally acknowledged to be a consequence of fluid lipid line defects, i.e. formation of periodic ripples in the membrane surface that appear between the pretransition temperature (T_{pre}) and the main transition temperature (T_m) (Beni et al., 2006; Heimburg, 2000; Janiak et al., 1976). The pretransition is less cooperative than the main transition. The occurrence of the pretransition is dependent on the type of head group of the phospholipids in the bilayer, it also seems to be dependent on the lamellarity of the liposomes

(Heimburg, 2000). The shift in the T_{pre} towards lower temperatures upon addition of drug compounds indicates a molecular interaction between the head group of the phospholipid and the drugs. This interaction can result in higher mobility in the head group region (Beni et al., 2006). From Figure 5 it can be seen that two drugs, sulphasalazine and naproxen, show a decrease in both T_{pre} and ΔH_{pre} compared both to the rest of the compounds as well as to the control (DPPC in buffer). Metoprolol also appears to cause a shift in T_{pre}, but not to the same extent as sulphasalazine and naproxen. The higher the concentration of drugs the larger is the observed influence in terms of shift in T_{pre} and ΔH_{pre} to lower values. Sulphasalazine and naproxen thus seem to interact with the head groups of the phospholipids in the bilayer, this might be the reason for the lowering of the electrical resistance across the barriers observed during the permeation experiment as well as for the change in calcein permeability as shown in Figure 4. Katz et al. (Katz et al., 2006) reported no interaction between a bilayer made of dimyristoylphosphatidylcholine (DMPC) with polydiacetylene (PDA) and naproxen, however, they did not observe the pretransition.

The main transition occurs when the bilayer shifts from the gel state to the fluid state (Brandl, 2001; Huang and Li, 1999; Ladbrooke and Chapman, 1969). Changes in T_m when adding a drug compound indicate an effect on the molecular motion of the lipid chain (Beni et al., 2006). A shift in T_m to lower values indicates a fluidization of the bilayer, i.e. a decrease in the order parameter (Skalko et al., 1996). The stronger this interaction is the larger is the shift in transition temperature to lower values. The sharpness of the transition peak is a measure of the intermolecular cooperativity between phospholipid molecules in a bilayer during phase transition, a narrow peek indicates complete cooperativity. The cooperativity of a gel to liquid-crystalline phase transition can be semiquantitatively evaluated from the DSC-trace by

calculating the temperature width at half height of the transition peak ($\Delta T_{m1/2}$) (Mcelhaney, 1982).

When looking at Figure 6 the same clear trend cannot be seen for the main transition as for the pretransition, metoprolol however appears to cause a decrease in T_m. This is in agreement with the literature where metoprolol is reported to interact with the lipid chains of the phospholipids (Katz et al, 2006). The samples with the highest concentrations (C1) of sulphasalazine and naproxen also produced a slight decrease in T_m. These three drugs also resulted in a broadening of the main transition peak. The observed increase in half-width of the peak indicates a decrease in cooperativity. Sulphasalazine and naproxen have shown to influence the electrical resistance of the barrier, but this is not the case for metoprolol.

For the DPPC with caffeine no obvious changes in neither pre- nor main-transition were detected. Thus, the observed increase in electrical resistance across the barrier when caffeine is present is not reflected by a change in the thermotropic phase transition of the bilayer.

For both caffeine and metoprolol it is obvious that drug-induced changes in electrical resistance as well as in permeability towards hydrophilic compounds not in all cases go in parallel with alterations of the thermotropic phase transition. A reason for the observed discrepancy between the DSC experiments and changes in electrical resistance and permeability in the case of caffeine could be that the system studied in the DSC experiments was prepared from DPPC in drug/buffer solution. Bilayers of this phospholipid are in the gel state at room temperature, while the bilayers in our barriers are in the fluid state at the same temperature. Unfortunately, the DSC instrument used in this study is not sensitive enough for studies of the observed drug interaction with the bilayers over a broader concentration range. To investigate this in more detail, further studies with a more sensitive DSC instrument is needed. The use of other techniques such as fluorescence anisotropy experiments, electron

spin resonance (ESR) and nuclear magnetic resonance (NMR) also appear promising (Johansson et al., 1993).

4 Conclusions

In this study the physical and functional stability of the phospholipid vesicle based barriers to agitation, and the agitation effect on drug permeability measurements have been investigated. In addition, the drugs which showed unexpected effects on the bilayers in our earlier studies were investigated in terms of their effect on the permeability of a simultaneously applied hydrophilic marker. Whether this was related to possible interactions of the drugs with the phospholipid bilayers was also investigated using DSC.

It was found that the phospholipid vesicle based barriers differ from other permeation models in that gentle agitation (up to 100 rpm) to reduce the UWL does not lead to increased permeability, not even for highly lipophilic drugs such as testosterone. Thus, in contrast to the Caco-2 and the PAMPA models the permeability results obtained without shaking already correlate well with the fraction absorbed in humans and shaking does not seem to improve the prediction obtained using this model.

Sulphasalazine and naproxen are reducing the electrical resistance and increasing the permeability of the barriers for simultaneously applied calcein. In the DSC experiments these compounds also show an influence on the thermotropic phase transition of DPPC model membranes, which is interpreted as an interaction with the head groups of the phospholipids. In contrast, the presence of caffeine results in an increase in electrical resistance and a decrease in permeability of calcein but in the DSC experiments no influence on the phase transition of DPPC model bilayers could be detected.

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Supplementary material available

Photos of the filter inserts and plates, as well as the stirring equipment used in experiments are shown in Figure A. Supplementary material is available at http://www.sciencedirect.com.

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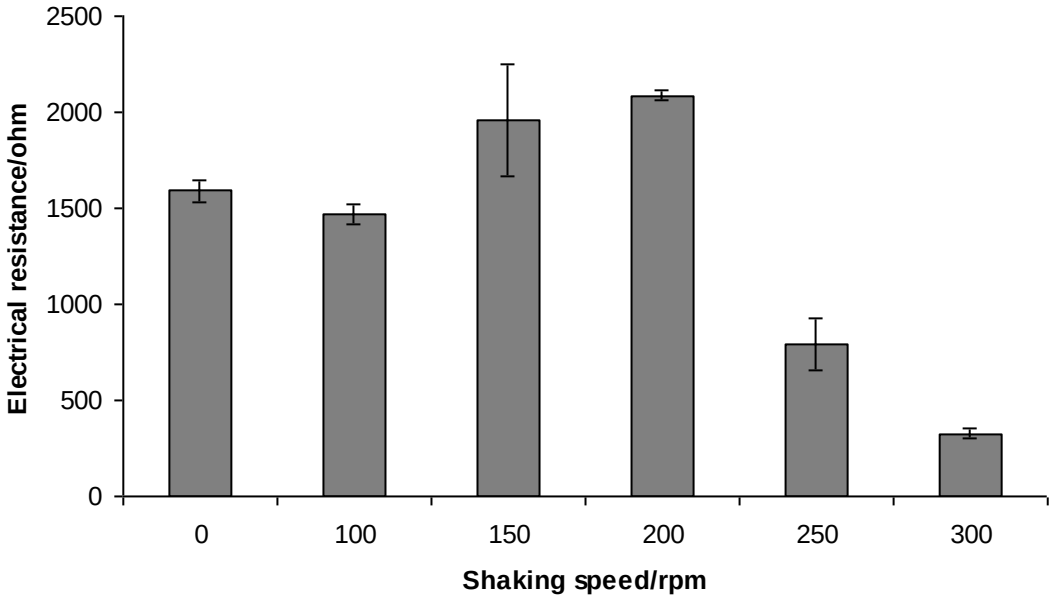


Figure 1. The electrical resistance measured across the barriers after 5 hours of shaking at different speeds (n=3).

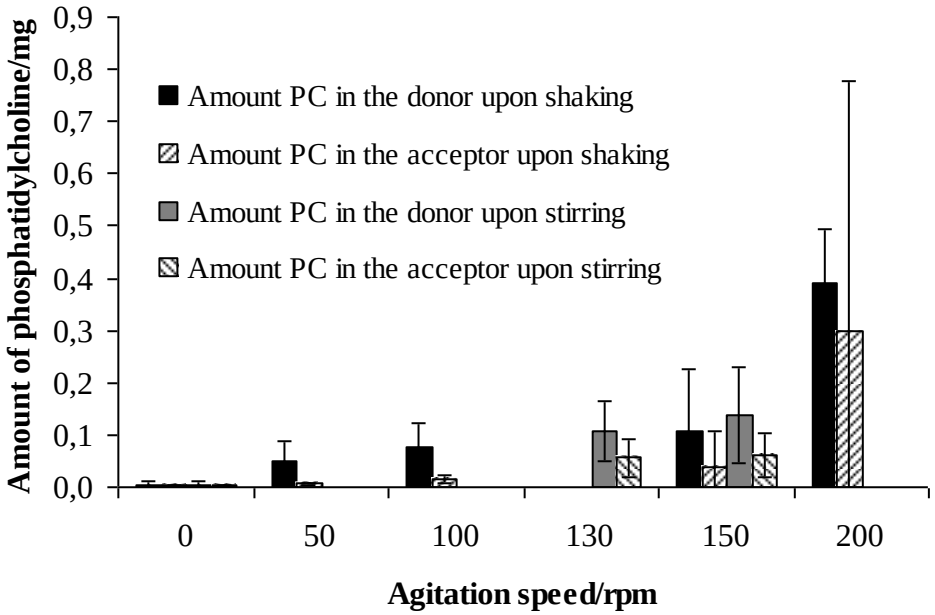
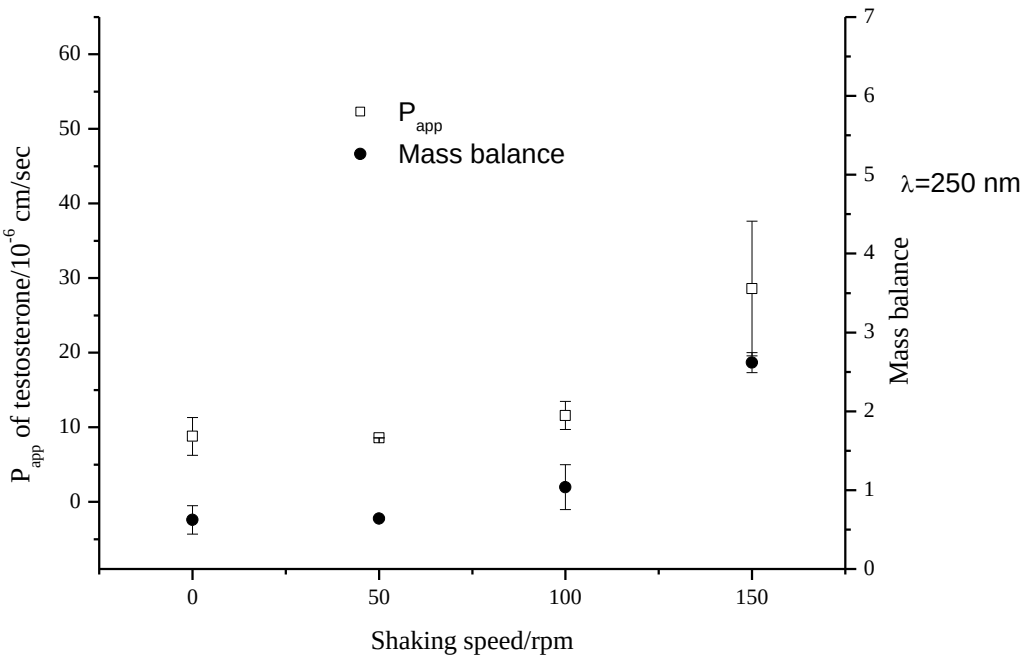
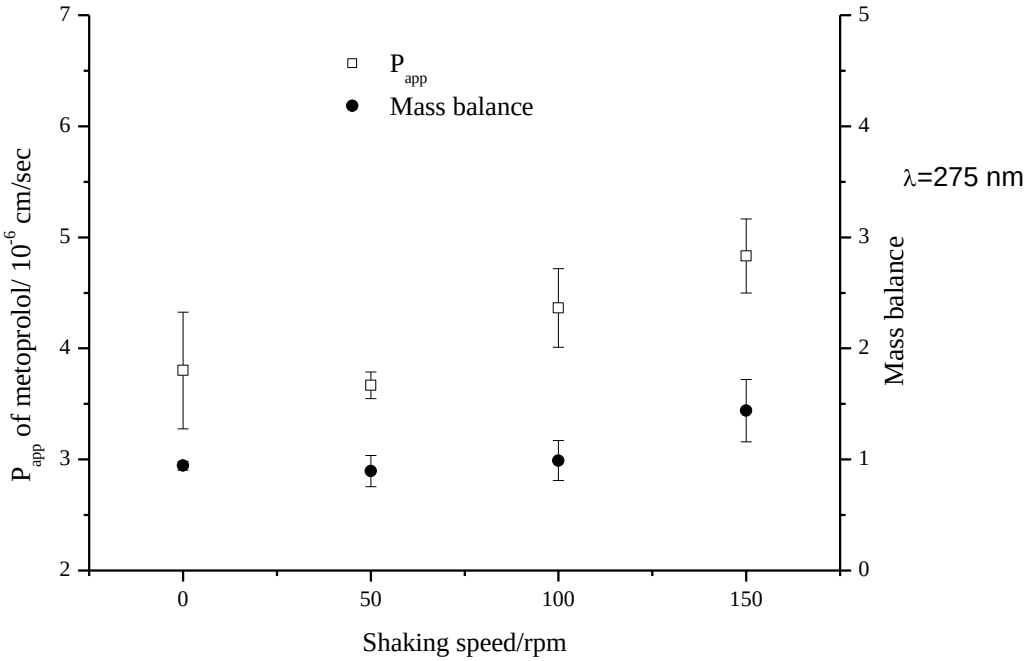


Figure 2. Amount of egg phosphatidylcholine found in donor and acceptor chambers with different agitation conditions. A stirring speed of 130 rpm was the lowest possible.

A


P app
Mass balance
λ=250 nm
P app of testosterone/10-6 cm/sec
Mass balance
Shaking speed/rpm
0
50
100
150
0
10
20
30
40
50
60
0
1
2
3
4
5
6
7

B


P app
Mass balance
λ=275 nm
P app of metoprolol/ 10-6 cm/sec
Mass balance
Shaking speed/rpm
0
50
100
150
2
3
4
5
6
7
0
1
2
3
4
5

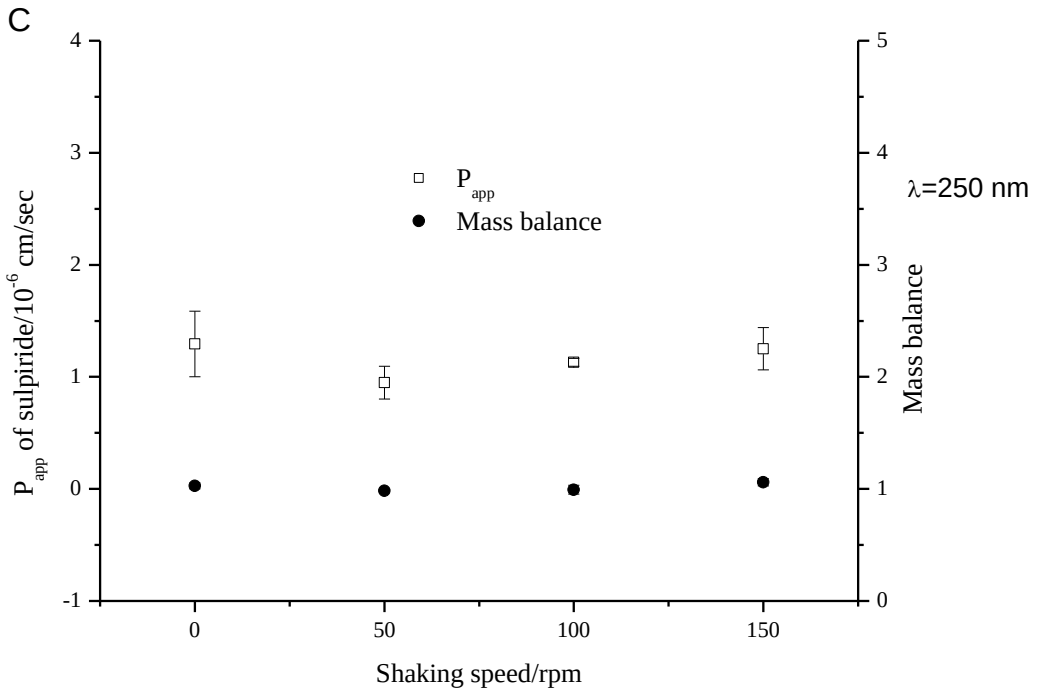


Figure 3. The permeability and mass balance values for testosterone (A), metoprolol (B) and sulpiride (C), respectively, plotted against shaking speed.

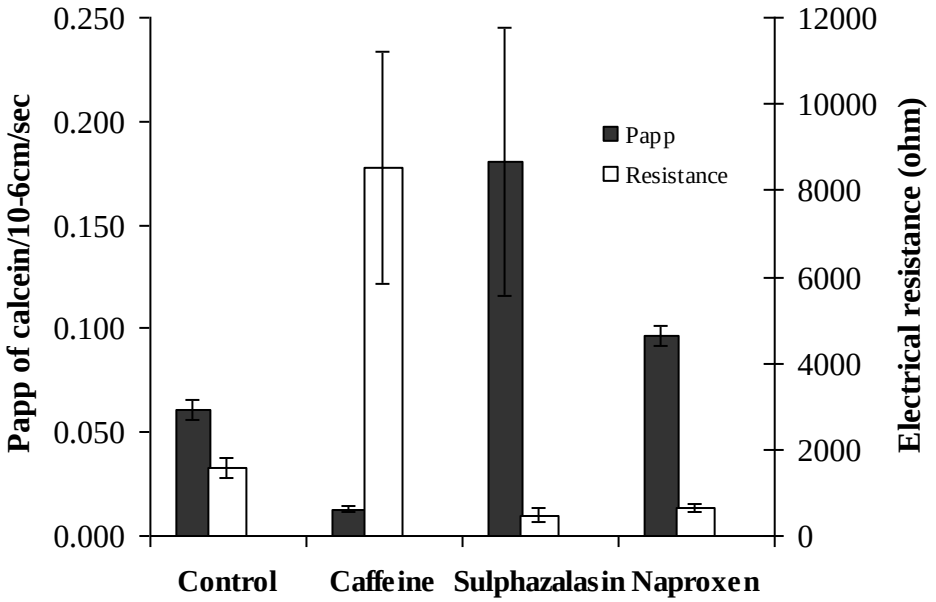


Figure 4. The electrical resistance and the permeability of calcein across the barrier in absence (control) and presence of caffeine, sulphasalazine and naproxen.

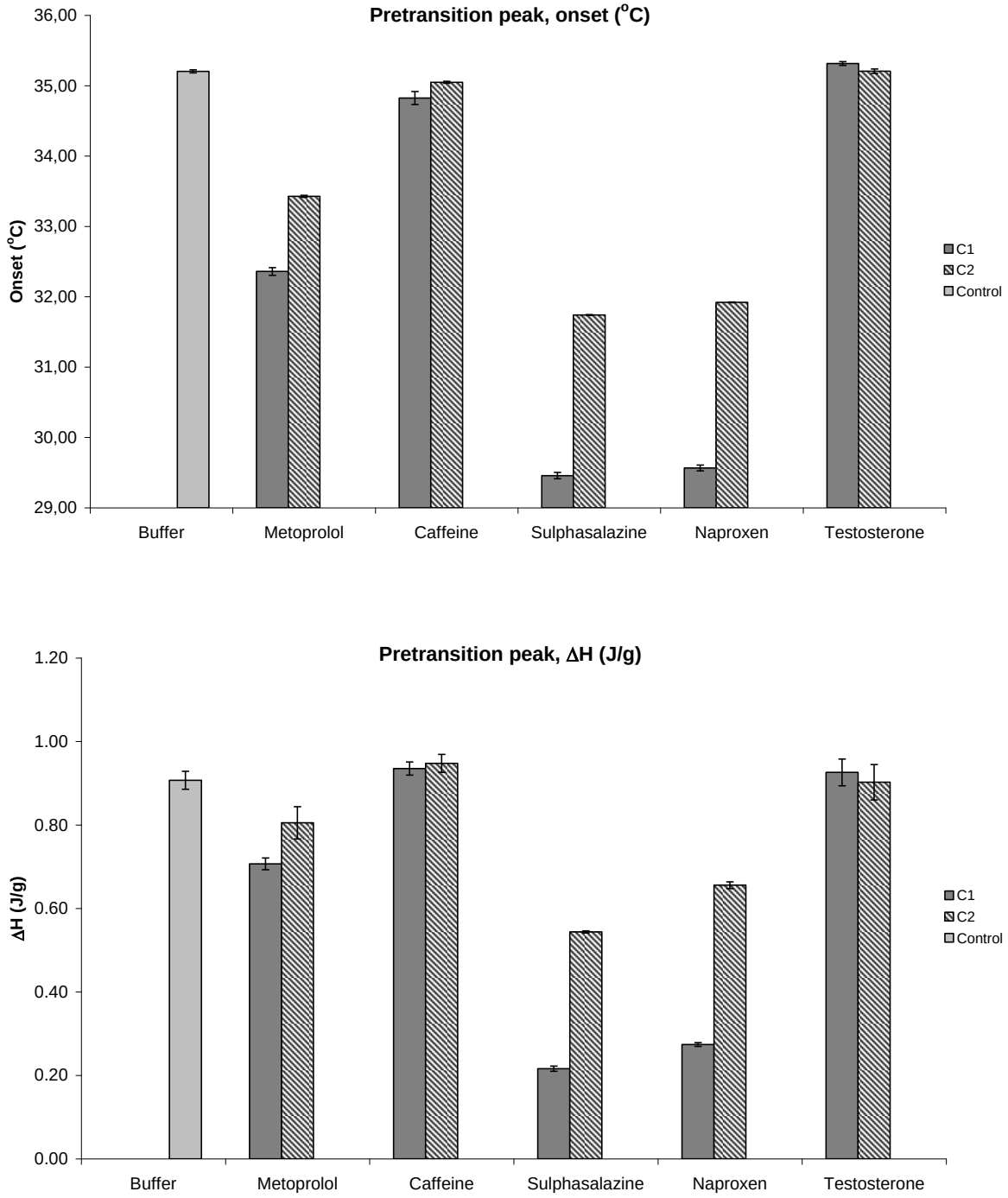
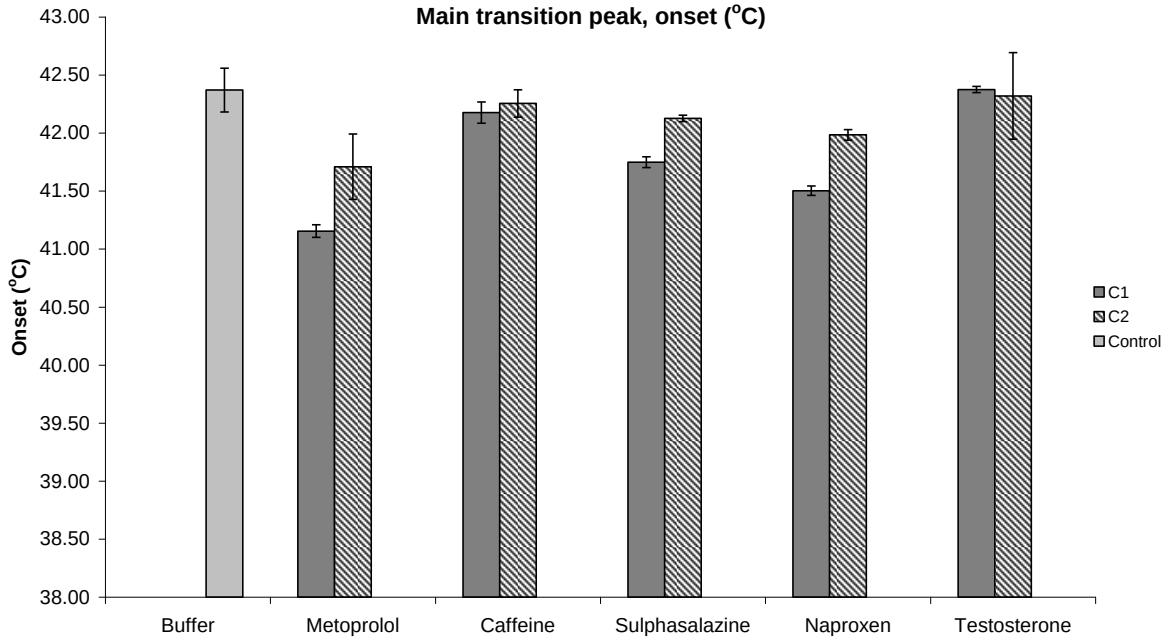
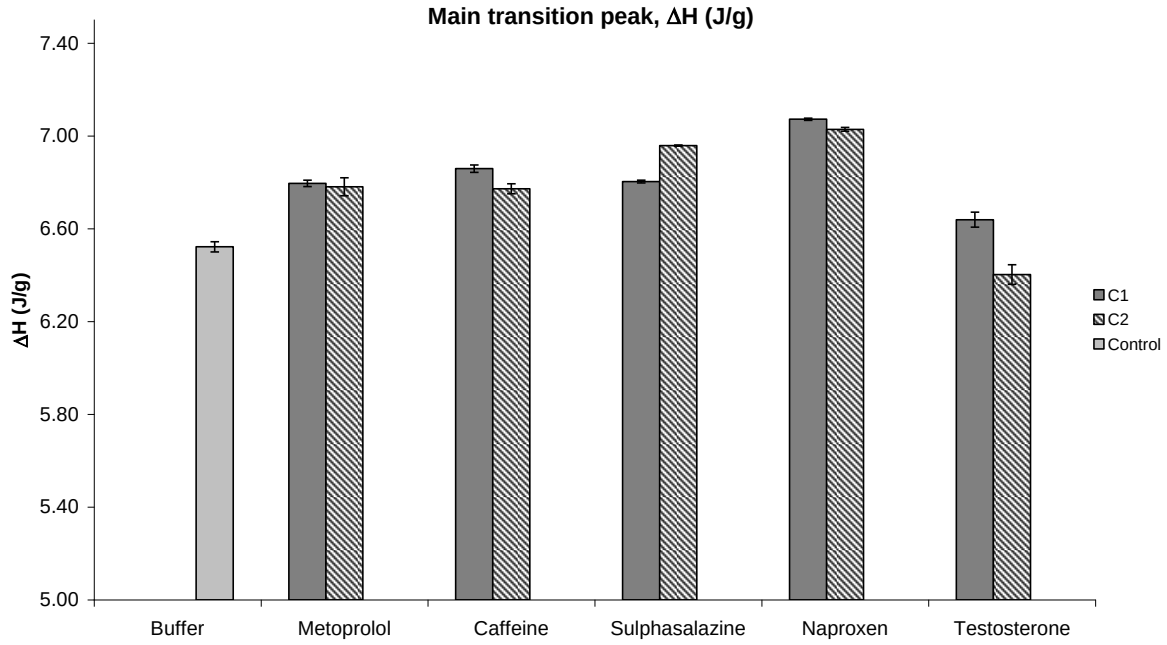


Figure 5. The onset temperatures and transition enthalpies (ΔH-values) at pretransition of DPPC both in absence and presence of drug compounds at two different concentrations.


Main transition peak, onset (°C)
Onset (°C)
43.00
42.50
42.00
41.50
41.00
40.50
40.00
39.50
39.00
38.50
38.00
Buffer
Metoprolol
Caffeine
Sulphasalazine
Naproxen
Testosterone
C1
C2
Control


Main transition peak, ΔH (J/g)
ΔH (J/g)
7.40
7.00
6.60
6.20
5.80
5.40
5.00
Buffer
Metoprolol
Caffeine
Sulphasalazine
Naproxen
Testosterone
C1
C2
Control

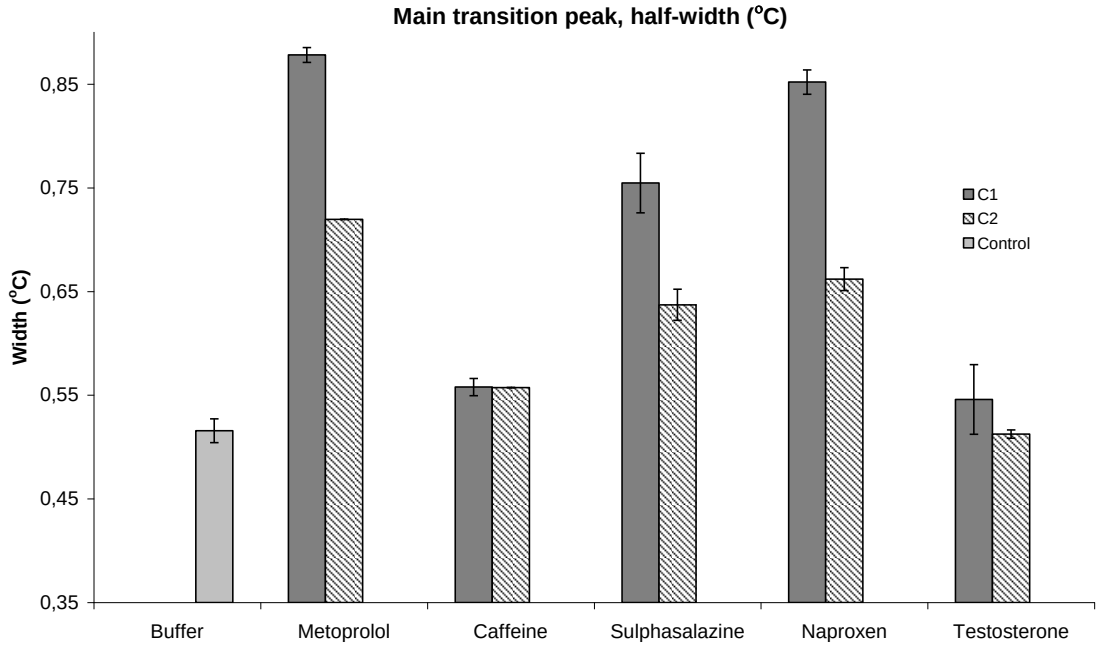


Figure 6. The onset temperatures, transition enthalpies (ΔH-values) and the half-width values ($\Delta T_{m1/2}$) at main transition of DPPC both in absence and presence of drug compounds at two different concentrations.