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Interaction of monoamine oxidase inhibitors with dipalmitoyl phosphatidylcholine liposomes. A comparison between structure and calorimetric data

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Abstract

The effect exerted by some 1-alkoxybenzylidene-(4-toluyl-thiazol-2-yl)-hydrazines possessing monoamine oxidase inhibitory (MAOI) activity, on the thermotropic behaviour of model membranes constituted by dipalmitoylphosphatidylcholine (DPPC) vesicles, was studied by Differential Scanning Calorimetry (DSC). Attention was directed to evaluate eventual modifications in drug–lipid interaction induced by drug structural variations.

The examined drugs, as hydrobromide compounds and their free bases, were found to modify the gel-to-liquid crystal phase transition of DPPC liposomes, by causing a shift of the transition temperature (Tm) towards lower values and a negligible variation in the enthalpy changes (ΔH). These modifications were a function of the drug concentration.

These effects were differently modulated by the substituents present in the drug molecule backbone. Differences should be caused by the different polarity and pH influence of the salt or free base form.

The calorimetric results could be related to MAO inhibitory activity measured by fluorescence techniques. The hypotheses on a relation between drugs structure, inhibitory activity and membrane interaction are suggested. © 1997 Elsevier Science B.V.

Keywords: Differential Scanning Calorimetry; Membranes; Monoamine oxidase inhibitors; Phosphatidylcholine

1. Introduction

Monoamino oxidase (MAO, EC 1.4.3.4) is a class of flavin-dependent enzymes which catalyzes the oxidative deamination of biogenic, as well as exogenous amines. Located within the external mitochondrial membrane, they provide control of the intracellular concentration of most amine neurotransmitters, like dopamine, serotonin, noradrenaline and tryptamine [1].

Inhibitors of these enzymes (MAOI drugs) have always been considered as important agents in many therapeutical conditions. Among the number of structural classes of MAOIs, many heterocyclic hydrazines and hydrazides have been described, starting from the first reports on the in vitro effects on monoamine oxidase of Iproniazid [2]. Hydrazino compounds have been extensively evaluated as therapeutic agents for

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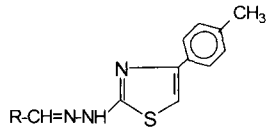
the treatment of hypertension and central nervous system depression [3,4]. It is known that the alkyl or phenyl residue present in such derivatives affects the MAOI activity by sustaining the nucleophilic nature of the linked nitrogen atom, which appears to be essential for the irreversible reaction with the specific amino acid residue in the enzyme [5]. Moreover, by replacing the alkyl or phenyl groups with some heterocyclic rings an increase in the potency of the inhibitors [7] could be achieved and compounds with a particular tissue specificity become available [6].

In a series of precedent papers, we extensively described the synthesis and in vitro MAOI activity of a large number of 2-thiazolyl-hydrazines [8,9]. Thereafter, some homogeneous series of them were studied to evaluate more deeply the structural requirements for optimal MAOI activity. In particular, lipophilicity of these compounds was measured both in the classical *n*-octanol/aqueous system and by DSC, using DPPC multilayered liposomes as a biomembrane model [9,10]. Relationships between lipophilicity and in vitro MAOI activity were thus obtained and reported. More recently, steric as well as electronic parameters were also taken into consideration for a further series of thiosemicarbazides structurally related to the initial MAOI thiazolyl-hydrazines [11].

For their synthesis, the first 2-thiazolyl-hydrazines [8] were obtained directly as hydrobromides and assayed, in the biochemical system, both in their original form and as their free bases. In the present work we planned to investigate the possible effects of such structural differences on the interaction with DPPC liposomes; we chose a unique series of derivatives, bearing a *p*-toluyl group as the 4-thiazolyl substituent and varying only for the alkoxy–phenyl radical in 1-position (compounds shown in Fig. 1).

To study this interaction, we have used L-α-dipalmitoylphosphatidylcholine liposomes as a synthetic simplified model membrane. They show a change of their thermotropic behaviour if other molecules are dissolved in their ordered structure (see Refs. [12–15]). Differential Scanning Calorimetry (DSC), being a non-perturbative technique, was employed to carry out the investigation.

The facility of the drug to interact and penetrate the lipid bilayer of bio-membranes, causing variations in their structure and fluidity, should be one of the major causes influencing the inhibitory activity exerted by



Compd.		R	m.p. (°C)[*1]	M.W.	% activity
IA	-	3,4,5-$(OCH_3)_3$-C_6H_2	137-138	383.5	72.7%
IB	.HBr	"	211-213	+81	-
IIA	-	3,4-(OCH_2O)-C_6H_3	197-198	337.4	18.0
IIB	.HBr	"	262-263	+81	-
IIIA	-	3,5-$(OCH_3)_2$-4-OC_2H_4-C_6H_2	193-194	397.5	5.3
IIIB	.HBr	"	199-200	+81	-

[*1] Salts were crystallized from methanol, free bases from ethanol

Fig. 1.

drugs on MAO. Though many factors, such as steric hindrance and electronic properties are known to modulate the interaction and/or penetration of foreign molecules into cell membranes, their lipophilicity should be more involved in such phenomena and their evaluation can support pharmacological evidences to draw optimal structure–activity relationships. The variations of the calorimetric parameters (transition temperature (Tm) and enthalpy changes (ΔH)), caused by structural features of a homogeneous series of drugs, can be compared with their MAOI activity and their apparent partition coefficient ($P_{o/w}$), that are known to be related to their structure [9,10,16,17]).

The results should give useful indications for understanding the role of substituents present in the drug molecule or the presence of HBr in the salt form in the interaction with the microenvironment of the lipid bilayer, also causing a variation in the activity and potency of enzymes present in natural membranes.

2. Materials and methods

2.1. Chemicals

Synthetic L-α-dipalmitoylphosphatidylcholine was obtained from Fluka Chemical Co. (Buchs, Switzerland). Solutions of the lipid were chromatographically pure as assessed by two-dimensional thin-layer chromatography (TLC). Lipid concentrations were determined by phosphorous analysis by the method of Bartlett [18].

The buffer consisting of Tris (50 mM) was adjusted to pH = 7.4 with hydrochloric acid.

1-(3,4,5-Trimethoxybenzylidene)-2-(4-toluyl-thiazol-2-yl)-hydrazine = IA

IA * HBr = IB

1-(3,4-methylenedioxybenzylidene)-2-(4-toluyl-thiazol-2-yl)-hydrazine = IIA

IIA * HBr = IIB

1-(3,5-dimethoxy-4-ethoxybenzylidene)-2-(4-toluyl-thiazol-2-yl)-hydrazine = IIIA

IIIA * HBr = IIIB

See Fig. 1 for structures and chemical data.

2.2. Synthesis and characterization of drugs

Thiazolyl-hydrazines were obtained as previously reported [8] by the cyclization between *p*-methyl-phenacylbromibe and 1-alkoxybenzylidene-thiosemicarbazones. The reaction, carried out in ethanol, gave the hydrobromides of thiazolyl-hydrazines directly ; the corresponding free bases were obtained by dissolving the salts in a small amount of water and adding a saturated sodium acetate aqueous solution. The solid was extracted with $CHCl_3$ (2 × 30 ml) and the organic layers were collected, dried (Na_2S0_4) and evaporated to dryness to give the hydrazines, which were recrystallized from ethanol.

IR, ^{1}H-NMR and MS spectroscopy, and CHNS elemental analysis confirmed the structure and molecular composition. In particular, mono-hydrobromide salts were confirmed as obtained.

2.3. Determination of in vitro MAOI activity

MAO activity was determined by a method reported in the literature [19]. Aliquots of rat brain mitochondrial suspension (90 μg of protein) were preincubated at 37°C for 5 min adjusting the volume to 970 μl with 10 mM KH_2PO_4, pH = 7.2. The reaction was started by the addition of 30 l 3.07 mM kynuramine (equivalent to a final concentration in the assay of 92 μM), and was shaken for 15 min before it was terminated by the addition of 300 μl 0.4 M perchloric acid. The tubes were then plugged, mixed briefly, and centrifuged at 12 000 rpm for 20 s in a microcentrifuge HERMLEZ 230M, to remove precipitated proteins. A 1 ml aliquot of the supernatant was transferred to test tubes that contained 2 ml 1 M sodium hydroxide. After mixing, the fluorescence intensity due to the formation of 4-hydroxyquinoline was measured at an excitation wavelength of 315 nm and an emission wavelength of 380 nm in a Perkin–Elmer mod. LS-5 fluorescence spectrophotometer. The concentration of the product was calculated from a standard curve of 4-hydroxyquinoline (0–10 nmol) carried through the assay procedure. Protein was measured by the method of Lowry [20], with bovine serum albumin used as the standard. Enzyme activity was expressed as nanomoles of 4-hydroxyquinoline formed/milligram protein/h.

2.4. Preparation of liposomes

Multilamellar liposomes were prepared in the presence and absence of drugs following this procedure. Chloroform–methanol (1 : 1, v : v) stock solutions of lipid and drugs were mixed to obtain the chosen mole fraction of drugs. The solvents were removed under a nitrogen flow and the resulting film was freeze-dried to remove the residual solvents.

Liposomes were prepared by adding to the film 50 mM Tris buffer (pH = 7.4), then heating at 55°C, a temperature above that of the gel–liquid crystalline phase transition, and vortexing three times for 1 min.

The samples were shaken for 1 h in a water bath at 55°C to homogenize the liposomes. Afterwards, aliquots of 120 μl (5 mg of lipid) were transferred in a 150 μl DSC aluminium pan and submitted to DSC analysis.

2.5. Differential Scanning Calorimetry

DSC was performed by using a Mettler TA 3000 system equipped with a DSC-30 cell and a TC-10 processor. The scan rate employed was 2°C/min in the temperature range 10–55°C. The sensitivity was 1.72 mW, and the reference pan was filled with the Tris buffer solution. Palmitic acid was employed to calibrate the temperature scale and transition enthalpies. Enthalpies were evaluated from the peak areas using the integration program of the TA processor, permitting the choice of different baselines and ranges of integration. For curves showing an ill-defined baseline a fixed arm planimeter was employed. The areas calculated with these different methods lie within the experimental error (±5%).

The samples were cooled and heated four times to achieve reproducible results. All samples, after calorimetric scans, were extracted from the pan and aliquots were used to determine the amount of phospholipid by the phosphorous assay.

3. Results and discussions

Multilayered DPPC vesicles were chosen as an experimental model to assess the lipophilicity of our compounds (Fig. 1), as a function of the substituents *n* position-1 and of the presence of Hbz. It is noteworthy that this 'three-dimensional' model can more widely resemble the micro-environment of a cell membrane, and it has already shown to give better correlations with biological data, than the classical two-solvent system [10].

In Fig. 2(a)–(c) we report the calorimetric heating curves of DPPC liposomes in the presence of different mole fractions of the compounds IA, IIA and IIIA, respectively. All drugs interact with DPPC bilayers causing different shifts of the transitional temperature (Tm) of the calorimetric peak, associated with the well-known gel to liquid–crystal phase transition typical for DPPC multilayers (Fig. 3, curves a–c and Table 1). The enthalpy changes (ΔH), related to the peak area, remained nearly constant (Table 2).

Looking at the curves in Fig. 3, the effects caused by different drugs are of the same order of magnitude (effects are considered on the basis of Tm depression) only for low molar fractions: in fact, by increasing the X_{drug}, the compound IA continues to perturb the ordered lipid structure, while IIA and IIIA have no further effects on the lipid transition. This behaviour should find justifications by considering the formulas

Table 1
Main transition peak temperature (Tm, °C) of DPPC dispersions at different molar fractions of drugs and their hydrobromide salts

Molar fraction	IA	IIA	IIIA	IB	IIB	IIIB
0.00	42.1	42.1	42.1	42.1	42.1	42.1
0.03	41.2	41.3	41.1	41.4	41.1	41.4
0.06	40.4	41.3	41.1	40.8	41.1	41.4
0.09	39.8	41.6	41.5	40.3	41.9	42.2
0.12	39.3	41.8	41.6	39.9	42.8	43.2

Table 2
Main transition enthalpy changes (ΔH, Kcal/mol) of DPPC dispersions at different molar fractions of drugs and their hydrobromide salts

Molar fraction	IA	IIA	IIIA	IB	IIB	IIIB
0.00	8.2	8.2	8.2	8.2	8.2	8.2
0.03	7.9	7.7	6.7	8.4	7.7	8.1
0.06	7.7	8.0	7.6	7.3	7.3	8.6
0.09	7.3	8.7	6.0	6.6	7.9	6.8
0.12	7.3	7.4	6.7	7.3	7.7	8.5

of the compounds. All of them show the same thiazolic ring with a *p*-toluyl group in 4-position, changing only the substituent **R** in position **1**. The presence of different moieties linked to hydrazine chain seems to modify the lipophilicity of the drugs, allowing them to interact to a different extent with the DPPC liposomes.

In Fig. 4(a)–(c), the heating curves of HBr salts (IB, IIB and IIIB) are reported. They would increase the water solubility of the drugs, and we suppose it would be worthwhile to study them, for the different lipophilicity that they could show with respect to the free base forms. These investigations gave results close to those discussed above, showing for all the drugs a similar behaviour (decrease in the Tm and a nearly constant ΔH at low molar fraction (< 0.03)); only the compound IB continued to cause a Tm decrease also at higher mole fractions, whereas IIB and IIIB were found to increase the Tm (Fig. 5); this last effect should be due to the HBr concentration that causes a pH decrease, which overlaps the buffer capacity of Tris solution, and the subsequent different lipid packing within the bilayer [21].

The calorimetric results obtained from the free bases as well as from the hydrobromides, suggest that at low-drug molar fraction, all the compounds are able to penetrate the lipid bilayer perturbing the order of the structure, while at higher molar fraction only the drug I (A or B) continues to interact with the lipids, whereas II and III remain unadsorbed on the membrane surface, probably preferring to aggregate each other out or over the membrane surface. Differences in the thermotropics behaviour between the base and the salt form have also been observed: in fact, the effect of the free base of the drugs is greater than the hydro-

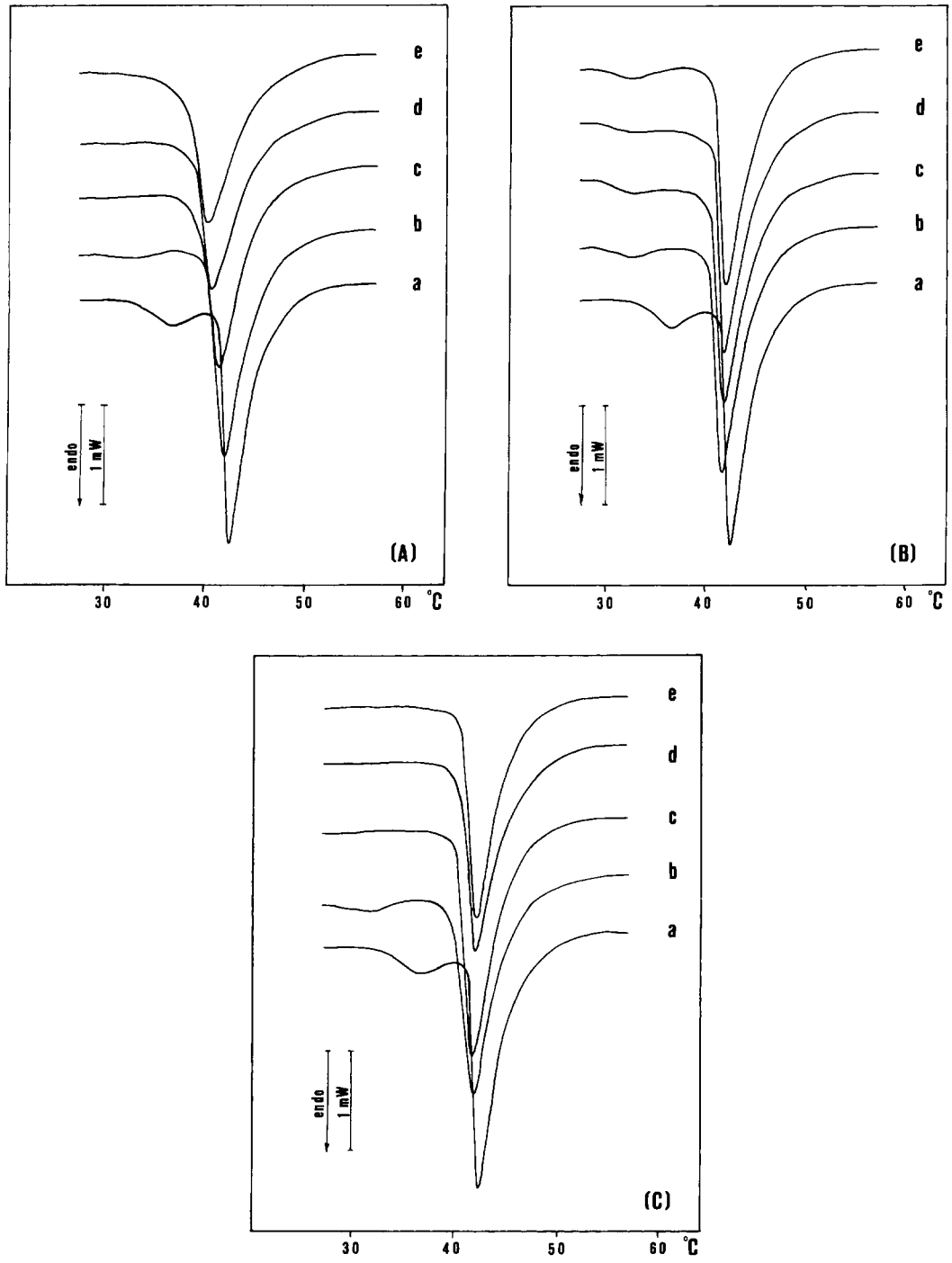


Fig. 2. Differential scanning calorimetry heating curves of hydrated DPPC containing (a) IA, (b) IIA and (c) IIIA at a drug mole fraction: $a = 0$; $b = 0.03$; $c = 0.06$; $d = 0.09$; $e = 0.12$.

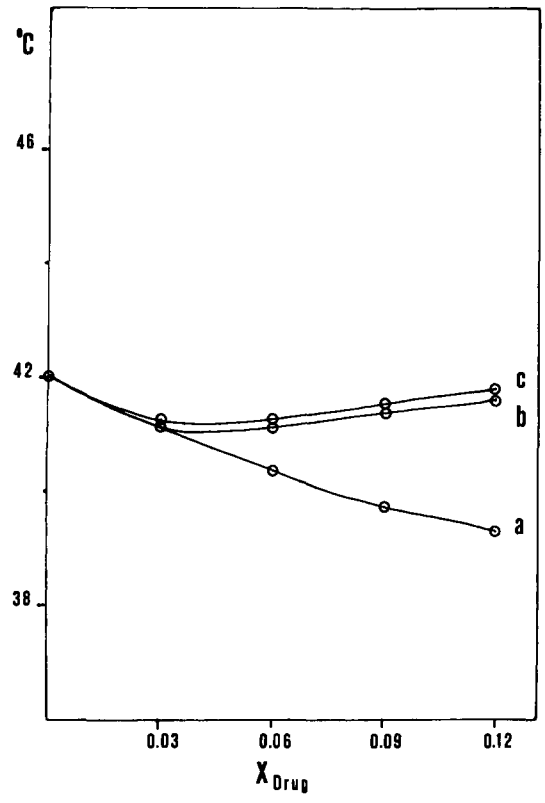


Fig. 3. Transition temperature (Tm, °C) values (average of at least 4 runs), in heating mode, as a function of mole fraction of (a) IA, (b) IIA, (c) IIIA.

bromide salts. Such differences should be due to the greater lipophilic character of the base drug (IA, IIA, IIIA).

The different modification of the 'fluidity' of the membrane could be a critical factor in determining many of its biological functions and the activity of the enzymes embedded.

The interaction between drugs and DPPC liposomes was largely explained by us as well as in the literature [12,22–29] in terms of a 'fluidifying' effect due to the introduction of lipophilic drug molecules into the ordered structure of the lipidic bilayer. Drug molecules act as spacers in such a structure, causing a destabilization of the lipid mosaic with a decrease in the Tm of the gel-to-liquid crystal phase transition. The negligible variation in the ΔH is explained as a superficial interaction between amphipatic molecules and DPPC polar heads, which occurs only at the surface of lipid layers without deeply interesting the acyl chains. The presence of different substituents in the backbone structure should influence the incorporation into, or at least the weak interaction of drugs with lipids in the model membrane.

For instance, the influence of drugs like morphine on the shift of the lipid phase transition increases as the aliphatic side chain is decreased. Shortening the aliphatic side chain causes an increase in the hydrophilic nature of the alcohol residue on C-7 and this perhaps could potentiate its interaction with the polar headgroup of the lipid [12]. Furthermore, when a substituent on the alcohol function of the aromatic ring in the benzomorphane structure is present on the codeine and ethylmorphine molecules, a relationship between the Tm shift towards lower values and increasing the substituents length at this position was observed [22].

It is then possible to presume the existence of a similar structure-dependent relation between MAOI drugs and perturbing effects on the thermotropic behaviour of lipid membranes (changes in the fluidity), but since these drugs possess a MAO inhibitory activity, it is interesting to examine the relation, if existing, between increasing in the membrane fluidity and biological activity.

By comparing the previous calorimetric results with the data on MAOI activity, obtained by fluorimetric studies and reported in Refs. [8–11], we found some interesting relationships. Compound IA, which possesses a MAOI activity greater than both IIA and IIIA, also shows a higher fluidifying effect on the model membrane (Fig. 1). (For the corresponding hydrobromide compounds the measured enzymatic activity was not reliable.)

Therefore, such high inhibitory activity should be due to the capacity to better penetrate the lipid bilayer of a membrane. Instead, compounds II and III, having a comparable or lower effect on the membrane fluidity, show a low inhibitory effect.

The presence of a different substituent in position 1 seems to be the reason for differences in the potency of the drug, by affecting the lipophilicity of the drugs. We should then conclude that a more lipophilic group present in this position allows the drugs to penetrate deeply the lipid bilayer, thus interfering with the action of MAO.

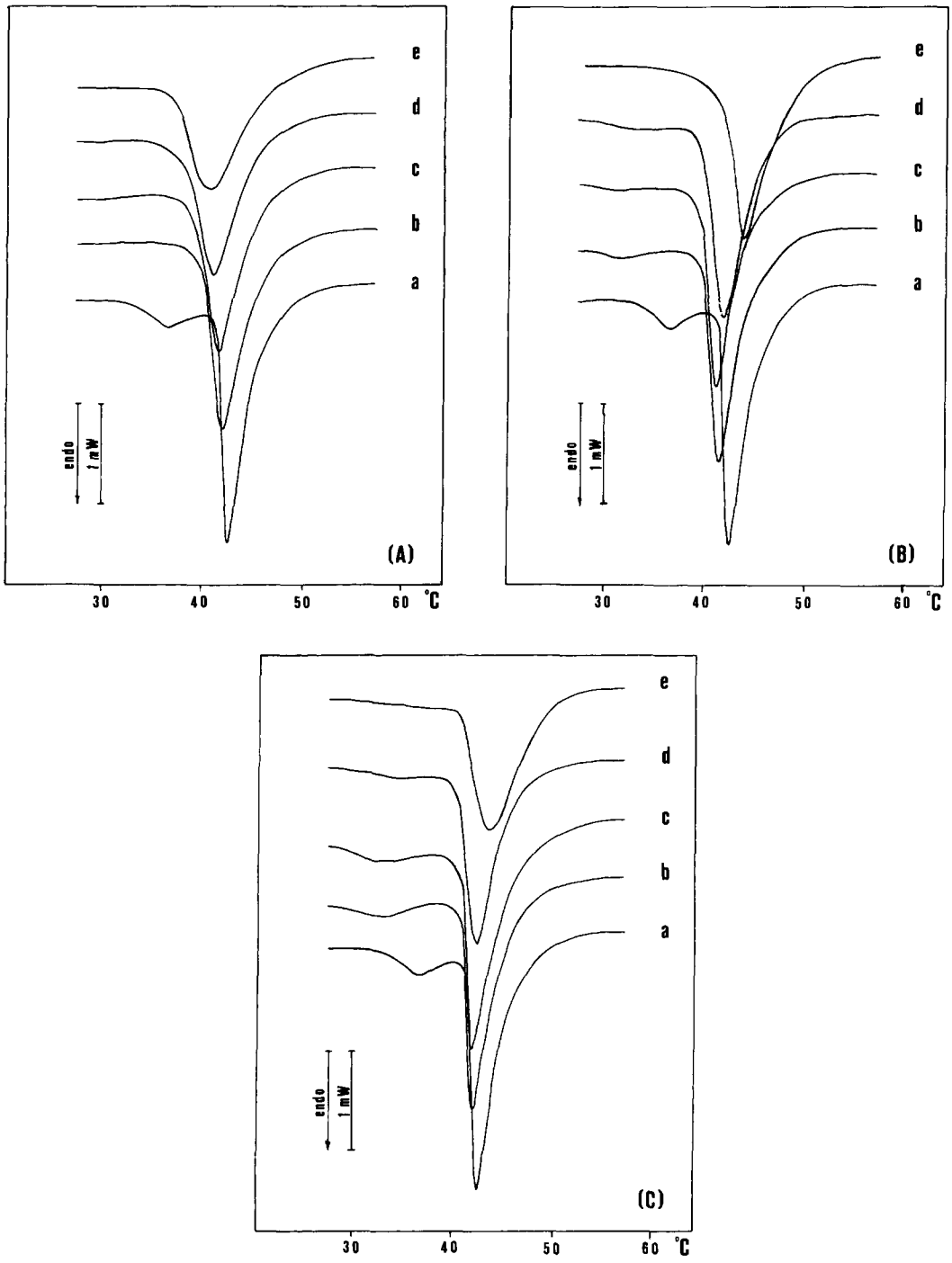


Fig. 4. Differential scanning calorimetry heating curves of hydrated DPPC containing (a) IB, (b) IIB and (c) IIIB at a drug mole fraction: $a = 0$; $b = 0.03$; $c = 0.06$; $d = 0.09$; $e = 0.12$.

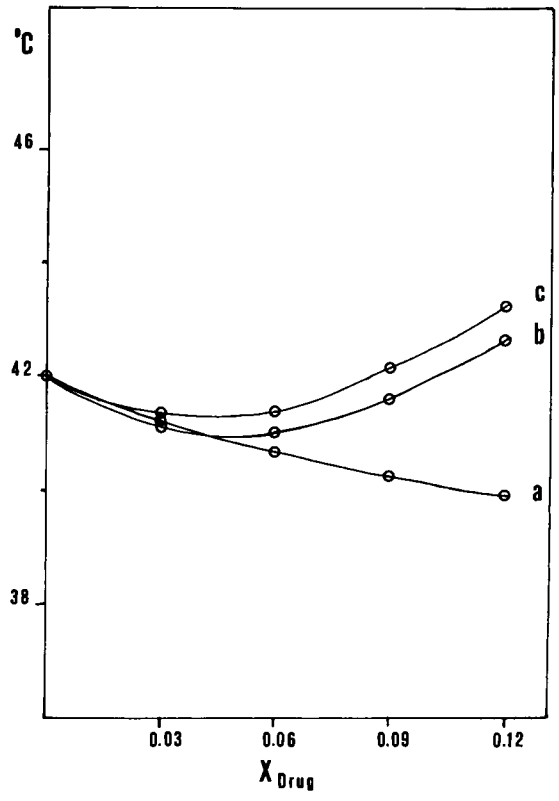


Fig. 5. Transition temperature (Tm, °C) values (average of at least 4 runs), in heating mode, as a function of mole fraction of (a) IB, (b) IIB, (c) IIB.

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