LIPOSOMES - MODEL MEMBRANES TO STUDY THE BINDING OF TRICYCLIC ANTIDEPRESSANTS

Zdeněk Fišar, Radoslav Krulík and Dana Beitlová

Psychiatric Research Unit
Faculty of Medicine, Charles University
Ke Karlovu 11, 12821 Praha 2
Czechoslovakia

SUMMARY

Binding of four tricyclic antidepressants (TCAs) - imipramine, desipramine, didesmethylimipramine and amitriptyline - on the lipid part of biological membranes was studied. Heterogeneity in partitioning of these drugs in artificial lipid bilayers (liposomes) was quantified using a radioligand binding method. High-affinity binding sites on the liposomes were found and characterized by apparent dissociation constant (K_d) and by binding capacity (B_{max}). Change in the membrane lipid composition affected the binding parameters of the high-affinity binding, while the ligand modification affected non-specific binding (low-affinity adsorption) of TCAs. The possible role and methodological importance of high-affinity binding to the lipid part of biological membranes are both discussed. Determination of radioligand binding on model lipid membranes is recommended to avoid certain problems in interpretation of receptor binding studies.

KEY WORDS

tricyclic antidepressants, high-affinity binding, liposomes, lipid binding sites, fluorescence probes, membrane fluidity

Authenticated | xajibim@mail.ru
Download Date | 6/16/12 6:40 AM

INTRODUCTION

Identification and characterization of specific binding sites on biological membranes is a necessary step in the elucidation of molecular mechanisms of drug action. The study of effects of psychotropic drugs is one way of learning about biochemical processes in mental disorders. The binding sites for tricyclic antidepressants (TCAs) have not as yet been reliably characterized. The binding of TCAs to membranes both from blood platelets and from brain tissue has been studied. High-affinity binding sites, probably related to the uptake of serotonin (5-HT) or noradrenaline (NA), have been found for imipramine /1, 2/ and desipramine /3/. The specificity of the labeling by TCA of sites associated with transporters of biogenic amines has given rise to some controversy /4-6/. Further studies have suggested a heterogeneity of TCA-binding sites /7-9/. Treatment of brain membranes by protease has enabled the distinction of both protease-sensitive (truly specific) imipramine binding sites and those of a non-protein nature /10/. It was concluded that there are probably lipid low-affinity (K_d = 10^{-1} - 10^{0} μM) binding sites on membranes which do not correlate with regional distribution of 5-HT uptake.

Interactions of charged drugs with artificial phospholipid membranes have been studied and adsorption isotherms have been measured and analyzed with micromolar and millimolar concentrations of drugs /11-13/. Lipid binding sites for TCAs were found preferentially localized near the membrane surface at the phospholipids' polar groups /14/. Some new results indicate both surface and intramembrane localization of imipramine /15/.

It was therefore of interest to determine the binding properties of pure lipid bilayers for low concentrations of TCAs. The study was carried out on multilamellar liposomes using the radioligand binding method. The experimental data were analyzed by conventional methods for high-affinity binding studies. Four tritiated antidepressants - imipramine (IMI), desipramine (DMI), didesmethylimipramine (DDMI) and amitriptyline (AMI) - were studied. High-affinity binding sites for TCA were found and characterized. The parameters K_d and B_{max} were calculated from binding isotherms according to the equilibrium binding equation:

$$BS = \frac{B_{max} \cdot F}{K_d + F} \qquad [1]$$

Authenticated | xajibim@mail.ru
Download Date | 6/16/12 6:40 AM

where BS = BT-NSB, BT is total bound radioligand, NSB is the non-saturable part of BT (marked as "non-specific" binding) and F is free (unbound) ligand. The common form of Eq. (1) is identical with the Langmuir adsorption isotherm. In our paper we marked BS as "pseudospecific" to distinguish it from the classical specific binding to proteins. Some necessary criteria for characterization of binding sites /16/ of TCAs on artificial lipid membranes were verified including saturability and kinetics of association and dissociation.

MATERIALS AND METHODS

Liposomes

Liposomes were prepared either from phosphatidylcholine (PC) or from crude lipid extract (CE), both isolated from white tissue of bovine brain. Crude extract of lipids was prepared according to Folch *et al.* /17/. Phosphatidylcholine was purified from CE by a modified method of Singleton *et al.* /18/.

Large multilamellar vesicles (MLV) were prepared by modified Bangham's method /19/. Ten to 40 mg of phospholipid (PL) in chloroform solution was placed into a round-bottom vessel, chloroform was evaporated under a N_2 stream and the vessel was placed into vacuum for about 15 hours. A few ml of TRIS buffered salt solution (120 mM NaCl, 10 mM KCl, 1.5 mM $CaCl_2$, 1.5 mM $MgCl_2$, 30 mM TRIS, 0.02% NaN_3, pH = 7.3) was added and vortexed 5-10 min or briefly sonicated at 40°C to remove all lipids from the wall and to obtain a homogeneous suspension. This milky suspension was incubated at 50°C for 30 min, shaken thoroughly and sonicated in a Sonicator XL2020 (Heat Systems-Ultrasonics, Inc.) for 5 s. Buffer was added to obtain a final volume of 1 mg of PL per ml suspension of liposomes.

Isotopes

[^{3}H]-labeled imipramine (18.5 Ci/mmol), desipramine (55 Ci/mmol), didesmethylimipramine (58 Ci/mmol) and amitriptyline (118 Ci/mmol) (all from the Institute of Nuclear Biology and Radiochemistry, Czechoslovak Academy of Sciences, Prague, Czechoslovakia) were used in the binding study.

Authenticated | xajibim@mail.ru
Download Date | 6/16/12 6:40 AM

Binding assay

Modified method of Raisman *et al.* /20/ was used. Liposome suspension (50-100 μg of PL per sample) was incubated (30-40 min) at a temperature near to 0°C in a total volume of 250 μl containing the radioligand (in a final concentration of 0.5-20 nM). The samples were then diluted by 3 ml of ice-cold buffer, rapidly filtered through a Whatman GF/F glass fiber filter pretreated with 0.1% polyethylenimine and washed twice with 3 ml of the cold buffer solution. Concentration of PL was chosen to perform resulting binding with no more than 10% of initial free radioligand /21/. Pseudospecific binding was determined as that displaced by 50 μM chlorimipramine (for [^{3}H]IMI and [^{3}H]DMI), 100 μM didesmethylimipramine (for [^{3}H]DDMI) or 50 μM nortriptyline (for [^{3}H]AMI), respectively. Filters were counted using a Beckman LS6000IC liquid scintillation counter.

For association and dissociation kinetic studies liposomes were incubated with 5 nM of [^{3}H]imipramine under the same conditions as described above but the total sample volume was 4 ml. 200 μl of sample was filtered and washed at selected intervals. After equilibrium chlorimipramine was added (at 50 μM concentration) and 200 μl of sample was again filtered at selected intervals. Non-specific binding was measured and subtracted. Dissociation rate constant (k_{-1}) was determined from the slope of log (specific binding) versus time (40 sec - 20 min) and association rate constant (k_{+1}) was calculated from the k_{-1} value as described by Bennett and Yamamura /21/.

Fluorescence measurements

The method of fluorescence probes was used /22/ and changes in fluorescence anisotropy were measured before and after addition of TCA at concentrations of 1-50 μM. Three hydrophobic probes were used at a final concentration of 2 μM: N-phenyl-1-naphthylamine (NPN), 1,6-diphenyl-1,3,5-hexatriene (DPH) (both from ICN Pharmaceuticals) and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) (Molecular Probes). Samples were measured at 37°C on a SLM 4800 spectrofluorometer (SLM Instruments, Inc.).

Authenticated | xajibim@mail.ru
Download Date | 6/16/12 6:40 AM

RESULTS

The binding of [^{3}H]IMI, [^{3}H]DMI, [^{3}H]DDMI and [^{3}H]AMI (at final concentration of 5 nM) on PC- and CE-liposomes was measured. Both non-specific and displaceable pseudospecific binding was observed for these drugs (Fig. 1). Non-specific binding of DDMI on both PC- and CE-liposomes was found significantly higher ($p < 0.05$) in comparison with the other ligands. There were no significant differences between the values of pseudospecific binding of different TCAs. Pseudospecific binding to the PC-liposomes represented about 40% of the total binding at 5 nM radioligand concentrations except for [^{3}H]DDMI (18%). Mean percentages of pseudospecific binding to the CE-liposomes lay in the range 36-67% (the lowest value for [^{3}H]DDMI, the highest value for [^{3}H]AMI). These results show that the pseudospecific binding of TCA to artificial lipid membranes is very similar for all the tested ligands. Only the non-specific part of the total binding was significantly changed by ligand modification.

For better characterization of the discovered pseudospecific binding, its saturability, kinetics and binding parameters were measured. An example of saturability of pseudospecific binding of [^{3}H]IMI to PC-liposomes is shown in Fig. 2. Similar saturation curves were obtained for all ligands. Calculation of binding parameters (apparent dissociation constant, K_d, and binding capacity, B_{max}) was made by nonlinear regression analysis using the Accufit Saturation-Two Site program (Beckman) and results are summarized in Table 1. Significantly higher values of K_d were found for binding on CE-liposomes. Significant differences between binding capacities of single drugs were not found but B_{max} values of CE-liposomes were increased in comparison with PC-liposomes. It must be taken into account that B_{max} values, as well as the values of binding in Figs. 1-3, are underestimated due to the multilamellar structure of the liposomes (see Discussion). The positively charged form of the TCAs both in solution and in bilayer is most probable because the pK_a values of these drugs are 9.4 or higher. Neither surface charge effects nor surface area effects /11-13/ were included in the binding isotherms because the radioligand concentrations were too small (0.5-20 nM).

The binding of imipramine to PC-liposomes is reversible and occurs at a reasonable speed as shown in Fig. 3. The dissociation rate constant was determined ($k_{-1} = 0.26 . 10^6 s^{-1} M^{-1}$) and the association

Authenticated | xajibim@mail.ru
Download Date | 6/16/12 6:40 AM

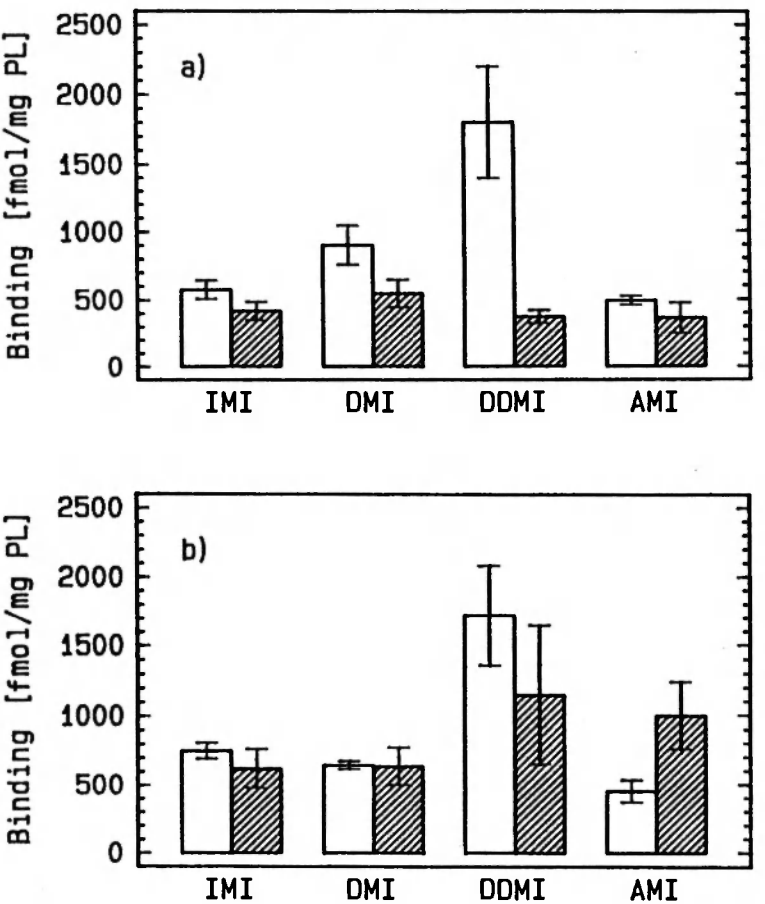

Fig. 1: Binding of tricyclic antidepressants on liposomes prepared a) from phosphatidylcholine, b) from a mixture of brain lipids. Both non-specific (☐) and pseudospecific (▨) binding is displayed as mean ±SEM values from at least 3 experiments. Liposomes were incubated with radiolabeled TCAs at a final concentration of 5 nM.

Authenticated | xajibim@mail.ru
Download Date | 6/16/12 6:40 AM

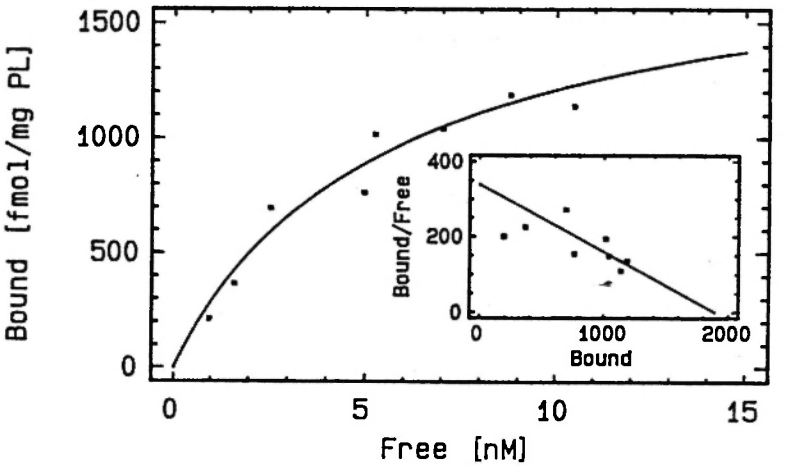

Fig. 2: Saturation isotherm of pseudospecific [^{3}H]imipramine binding to PC-liposomes. The data were analyzed by nonlinear regression analysis. Inset: Scatchard plot of the pseudospecific [^{3}H]imipramine binding to PC-liposomes.

TABLE 1
Effect of ligand modification and lipid composition on binding parameters of TCAs on liposomes: a) PC-MLV, b) CE-MLV

	IMI	DMI	DDMI	AMI
a)				
K_d[nM]	6.7±1.3	3.3±0.3	8.6±1.9	8.6±3.0
B_{max} [fmol/mg PL]	1163±299	728±184	923±147	1014±402
N	6	2	3	3
b)				
K_d [nM]	20.0±7.1	28.9±2.7	19.3±7.3	26.6±2.0
B_{max} [fmol/mg PL]	3266±1473	3952±683	3401±1771	5087±1517
N	3	3	3	3

The values are means±SEM of N separate experiments.

Authenticated | xajibim@mail.ru
Download Date | 6/16/12 6:40 AM

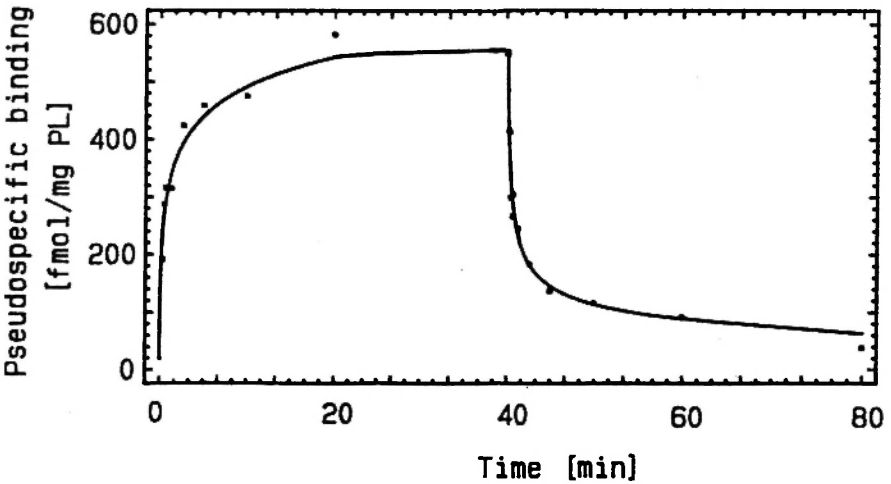

Fig. 3: Time course of pseudospecific [^{3}H]imipramine binding to PC-liposomes. [^{3}H]imipramine (5 nM) was incubated with liposomes as described in the text and filtered at various time intervals. At 40 min, chlorimipramine (50 μM) was added to displace [^{3}H]imipramine binding.

TABLE 2

Effect of imipramine on fluorescence anisotropy of PC- and CE-liposomes treated with different hydrophobic fluorescence probes

fluorescence probe	NPN	TMA-DPH	DPH
PC-MLV - control	0.080±0.004	0.204±0.005	0.097±0.003
+10 μM IMI	0.076±0.005	0.206+0.007	0.101±0.004
CE-MLV - control	0.104±0.001	0.285±0.001	0.257±0.001
+10 μM IMI	0.105±0.001	0.287±0.001	0.255±0.001

The values are means±SEM of six separate experiments.

Authenticated | xajibim@mail.ru
Download Date | 6/16/12 6:40 AM

rate constant was calculated ($k_{+1} = 0.96 . 10^{-3} s^{-1}$). The equilibrium dissociation constant $K_D = 3.7$ nM was calculated as the k_{-1}/k_{+1} ratio. These values are similar to the values obtained in receptor binding studies.

The values of fluorescence steady-state anisotropy before and after addition of imipramine (10 μM) to liposomes treated with fluorescence probes are shown in Table 2. No effect on membrane fluidity was observed for all tested ligands in the concentration interval 1-50 μM. An effect on the temperature phase transition cannot be observed because the lipids isolated from bovine brain do not show evident phase transition. The differences in fluorescence anisotropy of individual fluorescence probes are caused by their different localization in the lipid bilayer and by their different molecular shape. The differences between PC- and CE-liposomes are due to their lipid composition. Accordingly, the binding of TCAs did not influence the structure and fluidity of the lipid bilayers.

DISCUSSION

An important role for 5-HT and NA has been proved in depressive disorders. The high-affinity binding of TCAs to biological membranes and its relation to the uptake of these neuromediators was therefore studied. It was concluded that only high-affinity binding is related to a proteinaceous carrier for 5-HT or NA. Lipid binding sites, whose role is not known, are supposed to be of only low affinity.

In our experiments, we found both low- and high-affinity binding of different TCAs to artificial lipid membranes. It is obvious that different lipid binding sites arise due to the specificity in partitioning of drugs into the lipid bilayer. This specificity is determined both by the molecular shape of the ligand and by membrane heterogeneity, both in the horizontal and in the vertical plane /23/. There are two explanations of the role of the binding of drugs and other biologically active small molecules to the lipid bilayer: 1. effect on the physical properties of the membrane and resulting changes in the accessibility of embedded receptors due to their vertical and lateral displacement /24/; 2. lateral diffusion of drugs and binding to a hydrophobic intrabilayer protein site /15, 25, 26/. A combination of both mechanisms is possible, and a requirement for a certain accumulation of the drugs into the bilayer is probable. The second hypothesis appears

Authenticated | xajibim@mail.ru
Download Date | 6/16/12 6:40 AM

more likely in the case of TCAs, because we did not observe any general changes in membrane structure after addition of these drugs (in micromolar concentrations) to the liposomes (Table 2). The possibility exists that the binding of TCAs into lipid bilayers is either without any biological role or that these interactions are related only to the side-effects of these drugs. This is, however, not probable because evidence exists about lipid regulation both of the high-affinity binding of TCAs to biological membranes and of the uptake of 5-HT and NA into platelets and lymphocytes /27/. Stockert *et al.* /28/ observed that the effect of antidepressants was potentiated when the compounds were used with phosphatidylserine liposomes. Many other results support the idea that the lipid environment has an important role in the regulation of high-affinity TCA binding; this effect cannot be explained entirely by changes of electrostatic interactions.

Regardless of the biological effect of the pseudospecific binding of TCAs into lipid bilayers there is the methodological importance of the characterization of the high-affinity binding sites in the lipid part of membranes. This lipid binding can influence the measured high-affinity binding parameters of different radioligands to membrane proteinaceous receptors. As the lipid bilayer is an essential part of all biological membranes, it is obvious that if this binding is not taken into account, incorrect interpretation of binding studies may arise with respect to the nature of the binding tissue (i.e., lipid composition of membranes) and to the character of the radioligand molecule. As it is difficult to distinguish between two or more binding sites with similar dissociation constants the binding of radioligands to liposomes may be a good test to select the best ligand from a family of ligands related to the same biological effect or to the same receptor complex in receptor binding studies.

The binding affinity rather than the number of binding sites was affected by the lipid composition of the liposomes (Table 1), while imipramine demethylation affected the non-specific part of the total binding (Fig. 1). This points to the importance of the aromatic moiety of TCAs in the pseudospecific binding. The nature of the binding medium and similarity of binding parameters of different TCAs indicates a similarity or identity of binding sites. The binding affinity constants of unlabeled ligands determined from competitive inhibition studies were found to be in the micromolar range for PC-

Authenticated | xajibim@mail.ru
Download Date | 6/16/12 6:40 AM

liposomes (2.8-8.9 μM, single-site model). This can be explained by the existence of both low- and high-affinity lipid binding sites.

Neither the partition coefficients nor the binding capacity can be calculated properly for multilamellar vesicles because the inner bilayers are less accessible to drugs (radioligands) than the outer bilayer. This problem can be resolved by using very large unilamellar vesicles or spherical supported vesicles (SSVs; i.e., single bilayers adsorbed on spherical glass micro beads) /29/. Difficulties with unilamellar liposome size distribution and with their filtration do not arise in binding studies with SSVs (unpublished results).

CONCLUSIONS

There is a saturable, displaceable binding of TCAs to artificial lipid bilayers. The kinetics and binding parameters of TCAs on liposomes are similar to the values of high-affinity receptor binding. Lipid composition but not ligand modification can significantly influence binding parameters.

A possible existence of both high- and low-affinity binding to the lipid part of membranes must be taken into account when interpreting the results of receptor binding studies employing mainly lipophilic ligands.

ACKNOWLEDGMENTS

The authors would like to thank Eva Richtrová and Eliška Kašparová for skillful technical assistance.

REFERENCES

1. Tavenheimo J, Nelson PJ, Rudnick G. Mechanism of imipramine inhibition of platelet 5-hydroxytryptamine transport. J Biol Chem 1979; 254: 4631-4635.
2. Langer SZ, Moret C, Raisman R, Dubocovich ML, Briley M. High-affinity [^{3}H]imipramine binding in rat hypothalamus: Association with uptake of serotonin but not of norepinephrine. Science 1980; 210: 1133-1135.
3. Raisman R, Sette M, Pimoule C, Briley M, Langer SZ. High-affinity [^{3}H] desipramine binding in the peripheral and central nervous system: A specific site associated with the neuronal uptake of noradrenaline. Eur J Pharmacol 1982; 78: 345-351.

Authenticated | xajibim@mail.ru
Download Date | 6/16/12 6:46 AM

4. Laduron PM, Robbyns M, Schotte A. [^{3}H]Desipramine and [^{3}H]imipramine binding are not associated with noradrenaline and serotonin uptake in the brain. Eur J Pharmacol 1982; 78: 491-493.
5. Langer SL. [^{3}H]Imipramine and [^{3}H]desipramine binding: non-specific displaceable sites or physiologically relevant sites associated with the uptake of serotonin and noradrenaline? Trends Pharmacol Sci 1984; 5: 51-52.
6. Laduron PM. Pierre M. Laduron replies. Trends Pharmacol Sci 1984; 5: 52-53.
7. Rehavi M, Skolnick P, Paul SM. Subcellular distribution of high affinity [^{3}H]imipramine binding and [^{3}H]serotonin uptake in rat brain. Eur J Pharmacol 1983; 87: 335-339.
8. Reith MEA, Sershen H, Allen D, Lajtha A. High- and low-affinity binding of [^{3}H]imipramine in mouse cerebral cortex. J Neurochem 1983; 40: 389-395.
9. Bäckström IT, Marcusson JO. High- and low-affinity [^{3}H]desipramine-binding sites in human postmortem brain tissue. Neuropsychobiology 1990; 23: 68-73.
10. Marcusson J, Fowler CJ, Hall H, Ross SB, Winblad B. "Specific" binding of [^{3}H]imipramine to protease-sensitive and protease-resistant sites. J Neurochem 1985; 44: 705-711.
11. Lee AG. Effects of charged drugs on the phase transition temperatures of phospholipid bilayers. Biochim Biophys Acta 1978; 514: 95-104.
12. Seelig A, Allegrini PR, Seelig J. Partitioning of local anesthetics into membranes: surface charge effects monitored by the phospholipid head-group. Biochim Biophys Acta 1988; 939: 267-276.
13. Barthel D, Zschoernig O, Lange K, Lenk R, Arnold K. Interaction of electrically charged drug molecules with phospholipid membranes. Biochim Biophys Acta 1988; 945: 361-366.
14. Zimmer G, Schulze P. Membrane action of tricyclic drugs: Spectroscopic studies of a series of phenothiazines compared with tricyclic antidepressive substances in red cell membrane, using the spin labelling technique. Arzneim-Forsch 1981; 31: 1389-1392.
15. Bauer M, Megret C, Lamure A, Lacabanne C, Fauran-Clavel M-J. Differential scanning calorimetry study of the interaction of antidepressant drugs, noradrenaline and 5-hydroxytryptamine with a membrane model. J Pharmaceut Sci 1990; 79: 897-901.
16. Burt DR. Criteria for receptor identification. In: Yamamura HI et al., eds, Neurotransmitter Receptor Binding, 2nd ed. New York: Raven Press, 1985; 41-60.
17. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 1957; 226: 497-509.
18. Singleton WS, Gray HS, Brown ML, White JL. Chromatographically homogeneous lecithin from egg phospholipids. J Am Oil Chem Soc 1965; 42: 53-56.
19. Bangham AD, Standish MM, Watkins JC. Diffusion of univalent ions across the lamellae of swollen phospholipids. J Mol Biol 1965; 13: 238-252.
20. Raisman R, Briley M, Langer SZ. High-affinity [^{3}H]-imipramine binding in rat cerebral cortex. Eur J Pharmacol 1979; 54: 307-308.

Authenticated | xajibim@mail.ru
Download Date | 6/16/12 6:40 AM

21. Bennet JP Jr, Yamamura HI. Neurotransmitter, hormone, or drug receptor binding methods. In: Yamamura HI et al., eds, Neurotransmitter Receptor Binding, 2nd ed. New York: Raven Press, 1985; 61-89.
22. Brand L, Gohlke JR. Fluorescence probes for structure. Ann Rev Biochem 1972; 41: 843-868.
23. Jain MK, Wu NM. Effect of small molecules on the dipalmitoyl lecithin liposomal bilayer: III. Phase transition in lipid bilayer. J Membrane Biol 1977; 34: 157-201.
24. Shinitzky M. Membrane fluidity and receptor function. In: Kates M, Manson LA, eds, Membrane Fluidity. New York: Plenum Publ Corp 1984; 585-601.
25. Herbette LG, Chester DW, Rhodes DG. Structural analysis of drug molecules in biological membranes. Biophys J 1986; 49: 91-94.
26. Mason RP, Rhodes DG, Herbette LG. Reevaluating equilibrium and kinetic binding parameters for lipophilic drugs based on a structural model for drug interaction with biological membranes. J Med Chem 1991; 34: 869-877.
27. Krulík R, Sikora J, Bures P, Fuchsová K. Methylated and demethylated tricyclic antidepressants and their binding to cell membranes. Drug Metabol Drug Interact 1991; 9: 283-291.
28. Stockert M, Buscaglia V, De Robertis E. In vivo action of phosphatidylserine, amitryptiline and stress on the binding of [^{3}H]imipramine to membranes of the rat cerebral cortex. Eur J Pharmacol 1989; 160: 11-16.
29. Bayerl TM, Bloom M. Physical properties of single phospholipid bilayers adsorbed to micro glass beads. A new vesicular model system studied by ^{2}H-nuclear magnetic resonance. Biophys J 1990; 58: 357-362.

Authenticated | xajibim@mail.ru
Download Date | 6/16/12 6:40 AM

Authenticated | xajibim@mail.ru
Download Date | 6/16/12 6:40 AM