CHAPTER 4

MATERIALS AND METHODS

4.1 MATERIALS

Potassium ferrocyanide (K_4 [$Fe(CN)_6$]), Potassiumferricyanide ($K_3[Fe(CN)_6]$), Sodium bicarbonate ($NaHCO_3$) and Potassium Chloride (KCl) used were of analytical grade and were purchased from Merck, Germany.99 % of L-α-Phosphatidylcholine was purchased from Sigma Aldrich. Stock solution of L-α-Phosphatidylcholine (50 mg/mL) was prepared by using Analytical Grade chloroform. BLM forming dispersions were prepared in n-Decane (Merck, Germany). All the aqueous solutions used in our studies were prepared using Miili-Q water ($R>18M\Omega\ cm^{-1}$).

4.2 DRUG FOR THE STUDIES - LORAZEPAM

Lorazepam was extracted from commercially available calmese tablets, using 90% ethanol solution, in its hydrochloride form. It is precipitated by adding excess water to the 90% ethanolic solution. The precipitate was separated by filtration, dried and used for studies.

4.3 CONSTRUCTION OF BLM CHAMBERS

Interaction of Lorazepam with planar lipid membranes was studied using indigenously constructed BLM chambers. Two 24 mm diameter cavities, each of 5ml capacity were drilled in a polished PMMA block of dimension 85 x 60 x 22mm.The cavities are exactly aligned and almost touch

each other. The cavities were drilled in a computerized numerical control lathe. At the meeting point of the cavities the block was cut into two halves. A hole of diameter1.34 mm was drilled in a 1mm thick PMMA sheet and chamfered using a 1.25 mm drill bit from both sides to equal depths to form the membrane-supporting aperture. The aperture was examined through a travelling microscope with a magnification of 10 x and its diameter was measured. The septum having perfect circular aperture with minimum boundary ripples was used for the studies. This septum was inserted between the two halves of the chamber blocks such that the aperture is at one-third distance from the bottom of the cavity and glued with atmost care. The photograph of BLM chamber is shown in the following Figure.4.1.

Figure 4.1 The BLM chamber

4.3.1 Vibration Isolation Platform

The interferences from the floor vibrations are arrested by using a vibration isolated platform (MINUSK USA).

4.3.2 Fabrication of Electrodes

A two-electrode setup was used for studying the interaction of Lorazepam with planar or black lipid membrane. The Silver-Silver Chloride electrodes were fabricated by following standard procedures. Silver wire of 1mm diameter was anodized in 0.1M KCl solution to form the AgCl coating over the wire (Geddes, 1972) (Corvington, 1979). In AC measurements the two electrodes used are Ag/AgCl electrodes. The electrical connectivity between the electrolytic solution in the cavities and electrode immersed solutions was given by U shaped salt bridges made of glass filled with 1.5% agar-agar gel and saturated with KCl.

A three electrode assembly was used for studying interaction of Lorazepam with sb-BLM (salt bridge supported BLM) in KCl bath solutions where in a Pt foil served as the counter electrode, a Ag wire coated with AgCl served as the reference electrode, while the BLM formed on the agarose gel surface served as the working electrode. 0.3 g of agarose (Sigma Aldrich) was dissolved in 15 mL of boiling saturated KCl solution and the Teflon tubes were immersed so that the agar gel filled the tubes completely. Ag-AgCl reference electrode was prepared by anodizing Ag wire dipped in 0.1 M KCl solution. The anodized Ag - AgCl electrode was inserted into the agar gel

filled Teflon tube. The end of the Teflon tube was cut using a very sharp knife and 3 µL of lipid solution in n-decane was applied on the fresh surface of the agarose gel and allowed to dry under nitrogen atmosphere.

Studies on glassy carbon supported BLM, in the presence of marker ions were also made using a three electrode set up. The glassy carbon electrode of diameter 2 mm was used asa solid support for bilayer lipid membrane. The surface of glassy carbon electrode was polished with alumina slurry using polishing kit, washed with double distilled water and activated electrochemically by cyclic voltammetric method using the procedure described by Balamurugan et al (Balamurugan et al, 2013) and used for studies.All electrochemical experiments, using GCE-BLM, were performed with a potentiostat, GAMRY REFERENCE 3000.BLM covered GCE served as working electrode, Ag/AgCl electrode served as reference electrode and a Pt foil was used as the counter electrode.

4.3.3 Faraday Cage

The currents involved in the BLM experiments are extremely low, which are of the order 10^{-12} A. Any stray current pick-up will greatly affect the readings. Hence, the BLM set-up (the chamber electrode, stirring units etc) was placed in a metal cage called Faraday cage (Hanke and Schlue, 1993) (Sakmann and Neher, 1983) and the cage was grounded. Atmost care was taken to avoid stray-current leakage between the two aqueous chambers due to cracks in the septum in the case of black lipid membrane studies.

4.4 PREPARATION OF BLM FORMING DISPERSION

100μL of chloroform stock solution of the L-α-Phosphatidylcholine (50 mg/mL) was taken in a screw - cap bottle and nitrogen gas was purged forcibly into the tube in order to form a dry thin film of phospholipid and to evaporate chloroform. The phospholipid film was then immediately dispersed in 200μl of n-decane and used as the BLM forming dispersion.

4.5 FORMATION OF MODEL BLM SYSTEMS

4.5.1 Formation of planar or black lipid membrane

About 1μL of the lipid dispersion in n-decane was applied on the septum aperture as a preconditioning step (Tien, 1974) and dried. Then required bathing solutionswere taken in both compartments of the BLM chamber. Salt bridges were placed on both the sides. After placing the block on a stand electrical connections were made. Approximately 5μL of the BLM forming lipid dispersion was taken in a microlitre syringe. The tip of the syringe was immersed in the bath solution and held touching the aperture. The lipid dispersion was then delivered directly over the aperture. The dispersion spreads and sticks to the aperture as a lens and then slowly thins down. The solvent molecules were slowly excluded and the phospholipid molecules self assemble into a bilayer to form planar or black lipid membrane.

Brushes were used by workers previously to apply lipid dispersions over the aperture and this technique was found to associate with lot of uncertainties. To avoid the troubles caused by such uncertainties, H.Ti.Tien suggesteda more

elegant technique (Tien, 1974) where syringe is used to deliver the lipid dispersion at the aperture which is called syringe technique. Besides this a number of techniques are available for the formation of BLMs (Hanke and Schlue, 1993) (Tien, 1974). Being simple and suitable to form BLM at the aperture between two cavities; the syringe technique was used throughout the work.

4.5.2 Formation of sb-BLM

The end of the Teflon tube filled with agar gel saturated with KCl was cut by a very sharp knife and 3 µL of the lipid dispersion in n-decane was applied on the fresh surface and allowed to dry under nitrogen atmosphere. Again 3 µL of the lipid solution was applied and immersed in 3.5 mL NaCl bath solution for 30 minutes, where the lipid (L-α-Phosphatidylcholine) molecules self assemble into a lipid bilayer.

4.5.3 Formation of solid supported BLM (GCE-BLM)

Using cylic voltammetric technique the surface of GCE was activated in 0.1 M $NaHCO_3$ solution.Placing Ag/AgCl electrode as reference electrode, a Pt foil as counter electrode, the surface of glassy carbon electrode was activated keeping it as working electrodein 0.1 M $NaHCO_3$ solution by cyclic voltammetry. The glassy carbon electrode was mechanically polished well before electrochemical activation using alumina slurry. The cyclic voltammograms were recorded in the potential range -1.0 to 2.0 V at the scan rate of 100 mV/s. The cyclic voltammograms recorded during electrochemical activation of GCE is shown in Figure 4.2.

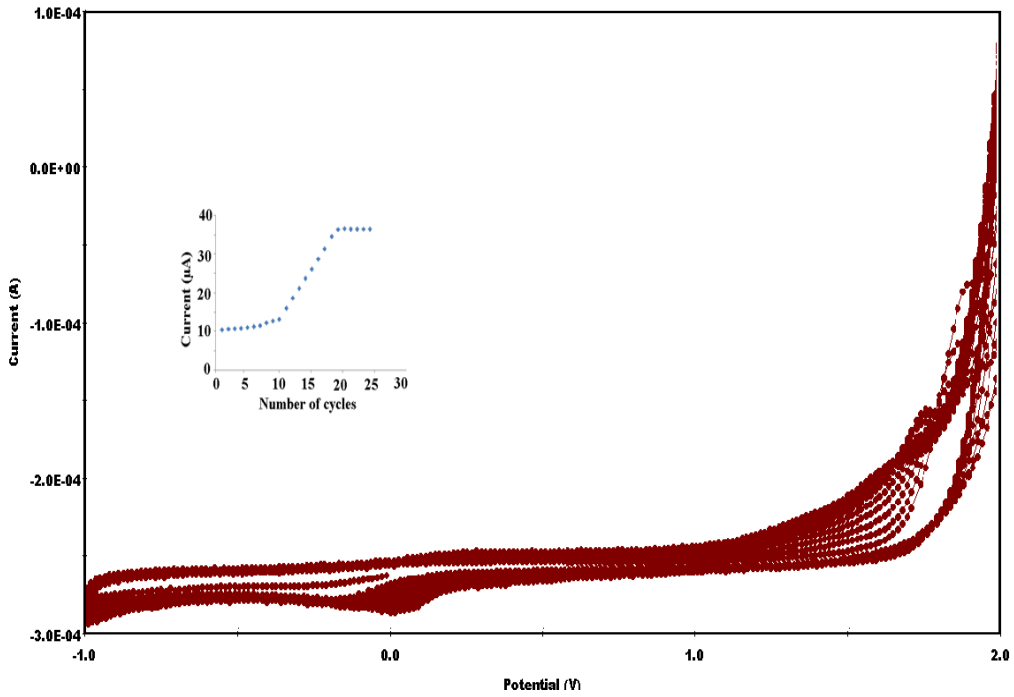

Figure 4.2 Cyclic voltammograms obtained during electrochemical activation of GCE

The formation of highly porous, highly reactive oxygen containing functional groups and oxidized carbon on the GCE surface is related to the anodic peak observed around 1.8 V (Ilangovan and Chandrasekar pillai, 1999). This anodic peak current increased significantly after 10 cycles and reached a maximum at 21st cycle (Inset of Figure 4.2). Hence, before forming BLM on GCE, it was electrochemically activated atleastby 20 voltammetric cycles in the above mentioned potential range.

The electrochemical activation of GCE provided highly porous, rough and highly electrochemically active surface compared to that of bare GCE. 3 µL of lipid (L-α-Phosphatidylcholine) dispersion in n-decane was applied on the dry electrochemically activated GCE surface using a micro syringe and

immediately immersed in the KCl bath solution, where the phospholipid molecules undergo self assembly spontaneously into a bilayer lipid membrane. The electrical properties of s-BLMsattained stable values after 30 minutes. This indicates the formation of a stable membrane on the electrochemically activated GCE surface.

The interaction between Lorazepam and s-BLM in the presence of marker (ferri-ferro cyanide) ions was studied in KCl bath solutions at pH 7, using BR buffer. pH 7 BR buffer was prepared by adding 57.2 mL of 0.2 N NaOH to 100 mL of pH 1.86 BR buffer. BR buffer solution of pH 1.86 was prepared by adding 2.4761 g of boric acid to a 1.0 L solution containing 2.3 mL of 17.4 N acetic acid and 2.7 mL of 14.7 N phosphoric acid.

REFERENCES

1. Issaku Ueda, "***Molecular Mechanism of Anesthesia***", Keio. J. Med. 50, (2001), 20.
2. Asydney, E, Salmon and Alan C, Sartorelli in "***Basic and clinical pharmacology***", 5th edition, Ed. Bertram G Katzung, Prentice-Hall International, (1992).
3. Sternbach, L.H, Randall L.O, Banziger R and Lehr H in "***Drug Affecting the Central Nervous System***", Burger A; Editor, Marcel Dekker: New York, 2, (1968).
4. Sternbach, L.H, "***The Benzodiazepine Story***", J. Med. Chem, 22, (1978), 1.
5. Martin, I.L, "***The Benzodiazepines and their Receptor-25: Years of Progress***", Neuropharmacology, 26, (1987), 957.
6. Schwartz, M.A, in "***The Benzodiazepines***", Garattini S, Mussini L, Randall L.O, Editors, Raven Press: New York, (1973), 53.
7. Möhler, H, and Okada, T, "***Benzodiazepine Receptor: Demonstration in the Central Nervous System***", Science 198, (1977), 849.
8. Squires, R.F, and Braestrup C, "***Benzodiazepine Receptors in Rat Brain***", Nature 266, (1977), 732.
9. Haefely, W, Kyburz, E, Gerecke, M and Möhler, H in "***Advances in Drug Researches: Recent Advances in the Molecular Pharmacology of Benzodiazepine Receptors and in the Structure-Activity Relationships of their Agonists and Antagonists***", Testa B; Editor, Academic Press, 14, (1985), 156.

10. Fryer R.I in "***Comprehensive Medicinal Chemistry: Ligand Interactions at the Benzodiazepine Receptor***", Hansch C, Sammes P.G, Taylor J.B, Editors, C.A. Ramsden Pergamon Press: New York, 3, (1990), 539.
11. Fryer R.I in "***The Benzodiazepines: From Molecular Biology to Clinical Practice***", Costa E; Editor, Raven Press: New York, (1983), 7.
12. Roche Laboratories, "***Verse (Midazolam hydrochloride) prescribing information***", Nutley, NJ, (1997).
13. Tomas, W.G in "***Martindale-The Extra Pharmacopeias***", Reynolds E.F, Editor, The pharmaceutical Press: London, U.K., (1993), 605.
14. Reves, J.G, Fragen R.J, Vinik H.R and Greenblatt, "***Midazolam: pharmacology and uses***", Anesthesiology, 62, (1985), 310.
15. Dundee, J.W, Hallida, N.J, Harper K.W et al. "***Midazolam: a review of its pharmacological properties and therapeutic uses***", Drugs, 28, (1984), 519.
16. Khanderia, U and Pandit, S.K, "***Use of midazolam hydrochloride in anesthesia***", 6, (1987), 533.
17. Kanto, J.H, "***Midazolam: the first water soluble benzodiazepine: pharmacology, pharmacokinetics and efficacy in insomnia and anesthesia***", Pharmacotherapy, 5, (1985), 138.
18. Albert Lehninger, Davis L. Nelson and Michael M. Cox in "***Principles of Biochemistry***" 2nd Edition, CBS Publishers and Distributors, (1993).

19. Arthur, C. Giese, "***Cell Physiology***", 4th Edition, W. B. Saunders Company, (1973), 265.
20. Loewy, Siekevitz, Menninger and Gallant in "***Cell Structure and Function: An integrated approach***", 3rd Edition. Saunders Publications, (1991), 2.
21. Alessandra, G, and Mauro, R, "***Model Systems: A strategy for Studying biological membranes***", Colloids & Surfaces, 35, (1989), 135.
22. David, E. Sadava in "***Cell Biology: Organelle Structure and Function***", Jone and Bartlett Publishers, (1993), 2.
23. Israelachvili, "***Intermolecular and surface forces***", Academic Press, (1992).
24. Heller, H, Schaefer, M and Schulten K, "***Molecular dynamics simulation of a bilayer of 200 lipids in the gel and in the liquid crystal phases***", J Phys Chem, 97, (1993), 8343.
25. Uma Maheswari K, "***Studies on the Interaction of certain Anticancerous Drugs with Planar Lipid Bilayers to assess their Non-specific action***", Bharathiar University, Coimbatore, India, (1999).
26. Uma Maheswari. K, Ramachandran. T, and Rajaji D. "***Interaction of cisplatin with planar model membranes – dose dependent change in electrical characteristics***", Biochimica et Biophysica Acta, 1463, (2000), 230.

27. Tien H.Ti, in "***Bilayer Lipid Membranes (BLM) – Theory and Practice***", Marcel Dekker Inc., (1974).
28. Mahendra, K, Jain in "**The *Bimolecular Lipid Membrane: A System***", Van Nostrand reinhold Company, (1972).
29. Hanke, W and Schlue, W.R, in "***Planar Lipid Bilayers-Methods and Applications***" Academic Press, (1993), 78.
30. Dilger, J.P, McLauglin, S.G, McIntosh, T.J and Simon SA, "***The dielectric constant of phospholipid bilayers and the permeability of membranes to ions***", Science, 206, (1979), 1196.
31. Rubin, E, Miller, K.W and Roth, S.H in "***Molecular and Cellular Mechanisms of Alcohols and Anesthetics***", Annals of the New YorkAcademy of Science, 625, (1991).
32. Ueda, I and Suzuki, A, "***Irreversible phase transition of firefly luciferace; contrasting effect of volatile anesthetics and myristic acid***", Biochimica et Biophysica Acta, 1380, (1998), 313-319.
33. Ueda. I and Suzuki, A, "***Is there a specific receptor for anesthetics Contrary effects of alcohols and fatty acids on phase transition temperature and bioluminescence of firefly luciferase***", Biophysical Journal, 75, (1998),1052.
34. Doris, P Flugmacher and Heinrich Sandermann Jr, "***The lipid/protein interface as a target site for general anesthetics: a multiple-site kinetic***

analysis of synaptosomal Ca^{2+}-ATPase", Biochimica et Biophysica Acta 1415, (1998), 174.

35. Theresa, M.A, Newman, M.S, Woodle, M.C, Mayhew, E and Uster, P.S, "***Pharmacokinetics and anti tumer activity of vincristine encapsulated in sterically stabilized liposomes***", International Journal of Cancer, 62, (1995), 199.
36. George, Z, Edvin, O, Jan, V, Yolanda, Q, Mary, N, Charles, E and Uster. P, "***The effect of vincristine-polyanion complexes in stealth liposomes on pharmacokinetics, toxicity and anti tumor activity***", Cancer Chemotherapy and Pharmacology, 39, (1996), 138.
37. Tien, H.T, Barish R.H, Gu, L.Q. and Ottova, A.L, "***Supported bilayer lipid membranes as ion and molecular probes***", Analytical Science, 14, (1998), 3.
38. Darold, W, "***A capacitance meter for bilayer membrane studies***", The Review of Scientific Instruments, 43, (1972), 1723.
39. Stephen, H, White and Thompson T.E, "***Capacitance, area and thickness variations in thin lipid films***", Biochimica et Biophysica Acta, 323, (1973), 7.
40. StanisIawa, K, SIawomir, K and Krzysztof, B, "***Changes of structural and dynamic properties of model lipid membranes induced by tocopherol: implication to the membrane stabilization under external electric field***", Biochimica et Biophysica Acta, 1510, (2001), 300.

41. Kalinowski, S, Zenon, L and Jacek W. M, "***The influence of bolaamphiphilic steroid dimer, on the formation and structure of bilayer lipid membranes***", Cellular & Molecular Biology Letters, 5, (2000), 107.
42. Karin, B, Dieter, H, Martin K, Llaus, M and Bernd, M, "***The ability of cationic amphiphilic compounds to depress the transition temperature of dipalmitoylphosphatidic acid liposomes depends on the spatial arrangements of the liphophilic moiety***", Biochemical Pharmacology, 42, (1991), 61.
43. Lee, A. G, "***The interactions between phospatidylcholines and anesthetic steroids***", Biochemical Pharmacology, 28, (1979), 91.
44. Hiroshi, K, Issaku, U, Patrick, S, Moore and Henry, E, "***Antagonism between high pressure and anesthetics in the thermal phase transition of dipalmitoylphospatidylcholine bilayer***", Biochimica et Biophysica Acta, 550, (1979), 131.
45. Cooper, C.E, Wrigglesworth, J.M and Nicholls, P, "***The mechanism of potassium movement across the liposomal membrane***", Biochemical and Biophysical Research Communications, 173, (1990), 1008.
46. Sharpe M.A, Cooper C.E and Wrigglesworth J.M, "***Transport of K^+ and other cations across phospholipid membranes by non-esterified fatty acids***", Journal of Membrane Biology, 141, (1994), 21.

47. Sabra, M.C, Jorgensen, K and Mouritsen, O.G, "***Lindane suppresses the lipid-bilayer permeability in the main transition region***", Biochimica et Biophysica Acta, (1995), 1.

48. Mauro, D and Gianfranco, M, "***A fast-kinetic approach to the fluorimetric determination of the effects of membrane perturbing agents on lipid bilayers***", Biophysics, 12, (1993), 109.

49. Paul, E, Fraser, R, Peter, R and Charles M. Deber, "***Bilayer-stabilizing properties of myelin basic protein in dioleoylphospatidylethanolamine systems***", Biochimica et Biophysica Acta, 983, (1989), 23.

50. Bauer, M, Megret, C, Lamure, A, Lacavanne, C and Fauran, M Clavel, Journal of Pharmaceutical sciences, 79, (1990), 897.

51. Arnhold, J, Mops, A, Krumbeigel, M and Arnold K, "***A comparative study of the main phase transition in phospolipid liposomes by fluorescence quenching and differential scanning calorimetry***", Studia biophysica, 133, (1989), 101.

52. Paz Sanchez, M. M, Francisco J, Juan C, Gomez, F, "***Interaction between alpha tocopherol and heteroacid phospatidylcholine with different amounts of unsaturation***", Biochimica et Biophysica Acta, 1279, (1996), 251.

53. Christensen, B, Gutweiler M, Grell E, Wagner N, Pabst R, Dose K and Bamderg E, "***Pump and displacement currents of reconstituted ATP synthase on black lipid membranes***", Journal of Membrane Biology, 104, (1988), 179.

54. Ramon, L and James E.H, "***Dipole potential measurements in asymmetric membranes***", Nature, 264, (1976), 36.

55. Dietmar, P and Ernst, G, "***Electric Parameters of Na^+/K^+- ATPase by measurements of fluorescence-detected electric dichroism***", Biochimica et Biophysica Acta, 1231, (1995), 181.

56. Tadayoshi, Y, Hiroshi, K and Issaku, U***, "Gaint Planar Lipid Bilayer***", Journal of Colloid and Interface Science, 105, (1985), 129.

57. Schwan, H.P, Takashima, S., Miyamoto, Y.K. and Stoeckenius W, "***Electrical properties of phospholipid vesicles***", Biophysica Journal, 10, (1970), 1102.

58. James, P, Digler, Stuart G.A. McLaughlin, Thomas J. McIntosh and Sidney A. Ssimon, "***The dielectric constant of phospholipid bilayers and the permeability of membranes to ions***", Science, 206, (1979), 1196.

59. Arnold, D.P and William D. A, "***Alternating current studies of charge carrier transport in lipid bilayers–Pentachlorophenol in lecithin-chloestral membranes***", Biochimica et Biophysica Acta, 455, (1976), 36.

60. Colome, C, Alsina, M.A, Busquets, A.M, Haro, I and Reig F, "***Interaction of amino glycosides and colistin with model membranes: Liposomes and monolayers***", International Journal of Pharmaceutics, 90, (1993), 59.

61. Ludwig, L and Gregor, C, "***Effects of short chain alcohols on the phase behavior and interdigitation of phosphatidylcholine bilayer membranes***", Biochimica et Biophysica Acta, 1237, (1995), 56.

62. Issaku, U in "***Drug and anesthetic effects on membrane structure and function***", Wiley-Liss Inc., (1991), 15.
63. Sterk, G. J, Thijsse P.A and Epand, R.F, "***New fluorescent probes for polarity estimations at different distances from the membrane interface***", Journal of fluorescence, (1996).
64. Diaz, R.S and Monreal, J, "***Unusual low proton permeability of liposomes prepared from the endogenous myelin lipids***", Journal of neurochemistry, 62, (1994), 2022.
65. Seelig, A, "***Local anesthetics and pressure; a comparison of dibucaine binding to lipid monolayers and bilayers***", Biochimica et Biophysica Acta, 899, (1987), 196.
66. Pope, J.M, Littlemore, L. A and Westerman P.W, "***Chain length dependency of n-alkane solubility in phosphatidylcholine bilayers: a ^{2}H-NMR study***", Biochimica et Biophysica Acta, 980, (1989), 69.
67. Gail L. Barchfeld and David W. Deamer, "***The effect of general anesthetics on the proton and potassium permeabilities of liposomes***", Biochimica et Biophysica Acta, 819, (1985), 161.
68. Barchfeld G.L and Deamer D.W, "***Alcohol effects on lipid bilayer permeability to proton and potassium: relation to the action of general anesthetics***", Biochimica et Biophysica Acta, 944, (1988), 40.

69. Rajaji, D and Jayaraman, J, "***Interaction of barbiturates with bimolecular lipid membranes***", Indian journal of biochemistry and biophysics, 21, (1984), 365.

70. Farideh, B, Qing, Y and Per, L, "***Immobilized liposome chromatographic analysis of drug partitioning into lipid bilayers***", Journal of chromatography A, 704, (1995), 315.

71. Qiang H, Li, y, Hong, y, Yan, Z, Gui, G, Qizhi, Y and Qingshan, L "***Aggregation of phospholipid vesicles induced by the ribosome inactivating protein Saporein, Biochemistry and molecular biology international***", 38, (1996), 701.

72. Brian, D. W and Scott C.H, "***Osmotic stress sensitizers sterol free phospholipid bilayer to the action of amphotercin-B"***, Biochimica et Biophysica acta, 1238, (1995), 156.

73. Lundbaek, J.A, Birn, P, Grishman, J and Hansen A. J and Andersen O.S, "***Membrane stiffness and channel function***", Biochemistry, 35, (1996), 3825.

74. Gianfranco, M, Claudio, M, Shahaireen, P and Rodney, W, "***Pore formation by Eschershei Coli Hemolysin (HlyA) and other members of the RTX toxins family***", Toxicology 87, (1994), 249.

75. Agawa, Y, Lee, S, Aoyagi, H, Ohno M, Taniguchi, T, anzai, K and kirino, Y,"***Interaction with phospholipid bilayer, ion-channel formation, anti-microbial activity of basic amphipathic alpha- helical model***

peptides of various chain lengths", The Journal of biological Chemistry, 266, (1991), 20218.

76. Antunes-Madeira, L. M.C, M. Almeida and Madeira V.M.C, "***Depth-dependent effects of DDT and lindane on the fluidity of native membranes and extracted lipids. Implications for mechanisms of toxicity***", Bulletin of Environmental Contamination and Toxicology, 51, (1993), 787.
77. Autunes –Madeira M. C, Videira R. A and.Madeira V.M.C, "***Effects of parathion on membrane organization and its implications for the mechanisms of toxicity***", Biochimica et Biophysica Acta, 1190, (1994), 149.
78. Romen, A.Videira, Luis P.S.Peca, Mario do Carmo Antunes-Madeira and Vitro M.C. Madeira, "***Effectsof malathion on membrane fluidity and its implications for the mechanisms of toxicity***", Medical Science Research, 22, (1994), 551.
79. Maria Rosa Moya-Quiles, Encarncion Munoz- Delgado and Cecilio l.Vidal, "***Interactions of the pyrethroid insecticide allethrin with liposomes***", Archives of Biochemistry and Biophysics, 312, (1994), 95.
80. Romen, A.Videria, Mario do Carmo Antunes-Madeira, Maria Lucia W.Kluppel and Vitor M.C.Madeira, "***Perhexiline effects on membrane organization detected by fluorescence polarization of DPH and DPH-PA***", Medical Science Research, 22, (1994), 391.

81. Mario do Carmo Antunes-Madeira, Romen A. Vieira, Maria Lucia W. Kluppel and Vitor M. C. Madeira, "***Amiodarone effects on membrane organisaton evaluated by fluorescence polarization***", International Journal of Cardiology, 48, (1995), 211.

82. Andrew Pohorille in "***Phenomena at aqueous interfaces,The Encyclopedia of Computational Chemistry****", Schleyer P. V. R, Allinger, N. L, Clark, T, Gasteiger* J, Kollman P. A, Schaefer, Schreiner P. R (Eds.), John Wiley & Sons: Chichester, (1998), 30.

83. Andrew Pohorille, Michael H. New, Karl Schweighofer and Michael A, "***Computer simulations of small molecules in membranes: Insights from computer simulations into the interactions of small molecules with lipid bilayers***", Wilson Exobiology Branch, NASA Ames Research Center Moffett Field, CA 94035.

84. North, C and Cafiso D.S, "***Contrasting membrane localization behaviors of halogenated cyclobutanes that follow or violate the Mayor - Overtonn hypothesis of general anesthesia potency***", Biophysica Journal 72, (1997), 1754.

85. Eger, E.I, Koblin, D. D, Harris, R. A, Kendig, J. J, Pohorille, A, Halsey, M. J and Trudell, J. R, "***Hypothesis: inhaled anesthetics produce immobility and amnesia by different mechanisms at different sites***", Anesth. Analg, 84, (1997), 915.

86. Pohorille, A, Wilson, M. A, Chipot, C, New M. H. and Schweighofer K, in "***Computational Molecular Biology, Interactions of Small Molecules and Peptides in Membranes***",J. Leszczynski; Editor, Elsevier, Amsterdam, (1999), 485.

87. Wilson, M. A and Pohorille, A, "***Mechanism of unassisted ion transport across membrane bilayers***", J. Am. Chem. Soc, 118, (1996), 6580.

88. Pohorille, A and Wilson, M. A, "***Molecular dynamics studies of simple membrane-water interfaces: structure and functions in the beginnings of cellular life***", Origins of Life and Evolution of Biosphere 25, (1995) 21.

89. Wilson, M. A and Pohorille, A, "***Molecular dynamics of a water - lipid bilayer interface***", J. Am. Chem. Soc. 116, (1994), 1490.

90. Ashok, K. Mehta, Maharaj, K. Ticku, "***An update on $GABA_A$ receptors***", Brain Research Review, 29, (1999), 196.

91. Braestrup, C and Nielsen M, "***Benzodiazepine receptors***", Clin. Neuropharmocol, 8, (1985), 52.

92. Siegel, E and Buhr, A, "***The benzodiazepine binding site of $GABA_A$ receptors***", TIPS, 18, (1997), 425.

93. Wang, Q, Han, Y and Xue, H, "***Ligands of the $GABA_A$ Receptor Benzodiazepine Binding Site***", CNS Drug Rewiews, 5, (1999), 125.

94. Sieghart, W, "***Structure and pharmacology of aminobutyric acid A receptor subtypes***", Pharmacol Rev, 47, (1995), 181.

95. Hafley W.E, Martin J.R, Ricgards J.G and. Schoch P, "***The multiplicity of actions of Benzodiazepine Receptor Ligands***", Canadian Journal of Psychiatry, 38, (1993), 5102.

96. Gardner, C.R, Tully, W.R and Hedgecock, C.J.R, "***The rapidly expanding range of neuronal benzodiazepine receptor ligands***", Prog Neurobiol, 40, (1993), 1.

97. Fryer, R.I, Leimgruber, W and Trybulski, K, "***Quinazolines and 1,4- benzodiazepines, structure – activity relationship between substituted 2-amino-N-(2-benzoyl-4-chlorophenyl) acetamides and 1,4-benzodiazepines***", J. Med. Chem, 25, (1982), 1050.

98. Borca, P.A and Bonora, A, "***Brain receptor binding and lipophilic character of benzodiazepines***", Biochem. Pharmocol, 32, (1983), 603.

99. Gupta, S.P, Saha, R.N and Mulchandani, V, "***Quantitative structure-activity relationship studies on benzodiazepine receptor binding: Recognition of active sites in receptor and modeling of interaction***", J. Mol. Recognit, 5, (1992), 75.

100. Maddelena, D.J and Johnson G.A.R, "***Prediction of receptor properties and biding affinity of ligands to benzodiazepine/$GABA_A$ receptors using artificial neural networks***", J. Med. Chem, 38, (1995), 715.

101. So, S.S, Karplus, M, "***Genetic neural networks for quantitative structure- activity relationships: Improvements and applications ofbenzodiazepine/ $GABA_A$ receptors***", J. Med. Chem, 39, (1996), 5246.

102. Hasegawa, K, Funatsu, K, "***GA Strategy for variable selection in QSAR studies; GAPLS and D- optimal desugns for predictive QSAR model***", J. Mol. Struct (Theochem), 425, (1998), 255.

103. Doble, A, Martin, I.L and Richards D.A, "***GABA modulation predicts biological activity of ligands for the benzodiazepine receptor***", Br. J. Pharmocol, 76, (1982), 238.

104. Menard, J and Treit, D, "***Effects of centrally administered anxiolytics compounds in animal models of anxiety***", Neuroscience and Behavioral Reviews, 23, (1999), 591.

105. Smith, M.T, Eadie, M.J and Brophy T.O***, "The pharmacokinetics of midazolam in man***", Eur J Clin Pharmacol, 19, (1981), 271.

106. Allonen, H, Ziegler, G and Klotz, U, "***Midazolam kinetics***", Clin pharmacol Ther, 30, (1981), 653.

107. Garzone, P.D and Krobath, P.D, ***"Pharmacokinetics of the newer benzodiazepines***", Reviews of the adsorption and fate of Midazolam, Clin pharmaco kinet, 16, (1989), 337.

108. Smith, M.T, Eadie, M. J and Brophy T.O***, "The phamacokinetics of midazolam in man***", Eur.J Clin. Pharmacol, 19, (1981), 271.

109. Allonen, H, Ziegler, G and Klotz, U, "***Midazolam kinetics***", Clin. Pharmacol. Ther.30, (1981), 653.

110. Greenbalt, D.J, Arendt, R.M, Abernethy, D.R, Giles, H.G, Sellers, E.M and Shader, R.I, "***In vitro quantitation of benzodiazepine lipophilicity: relation to in vivo distribution***" Br J Anaesth, 55, (1983), 985.

111. Short, T.G, Galletly, D.C and Plummer, J.L "***Hypnotic and anaesthetic action of thiopentone and midazolam alone and in combination***", British Journal of Anesthesia, 66, (1991), 13.

112. Daniel, A. Garcia and Perillo M.A, "***Localisation of flunitrazepam in artificial membranes. A spectrophotometric study about the effect the polarity of the medium exerts on flunitrazepam acid-base equilibrium***", Biochimica et Biophysica Acta, 1324, (1997), 76.

113. Daniel, A. Garcia and Perillo, M.A, "***Benzodiazepine localization at the lipid-water interface: effect of membrane composition and drug chemical struc*ture**", Biochimica et Biophysica Acta, 1418, (1999), 221.

114. Kurishingal, H, Barain, P and Restall, C, "***Benzodiazepine-induced changes in biomembrane fluidity***", Biochem. Soc. Trans, 20, (1992) 1575.

115. Li, D.F, Bahar, M, Cole, G and Rosen, M "***Neurological toxicity of the subarachnoid infusion of bupivacaine, lignocaine or 2-chloroprocaine in the rat***", British Journal of Anesthesia, 57, (1985), 424.

116. Rawal, N, "***Spinal antinociception: clinical aspects***", Ann. Med, 27, (1995). 263.

117. Malinovsky, J M, Pinaud, M, "***Neurotoxicity of intrathecally administrated agents***", Ann Fr Anesth Reanim, 15, (1996), 647.

118. Peter, S, Hodgson, Joseph, M, Neal, Julia, E, Pollock, and Spencer, S.Liu,"***The Neurotoxicity of Drugs Given Intrathecally (Spinal)***", Anesth Analg, 88, (1999), 797.

119. Tamic Takenamic, Saburo Yagishita, Tumino Asato, Sumio Haka, "***Neurotoxity of intrathetically administered tetracaine commences at the posterior roots near entry into spinal cord***", Reg. Anesth Pain Med 25, (2000), 372.

120. Erdine, S, Yucel, A, Ozyalcin, S, Ozyuvaci, E, Talu, G.K, Ahiskali B, Apak, H and Savci N. "***Neurotoxicity of midazolam in the rabbit***", Pain, 80, (1999), 419.

121. Svensson, B. A, Welin, M, Gordh, T. Jr and Westman, J, "***Chronic subarachnoid midazolam (Dormicum) in the rat. Morphologic evidence of spinal cord neurotoxicity***", Reg. Anesth, 20, (1995), 426.

122. Malinovsky, J.M, Cozian, A, Lepage, J.Y, Mussini, J.M, Pinaud, M and Souron R, "***Ketamine and midazolam neurotoxicity in the rabbit***", Anesthesiology, 75, (1991), 91.

123. Bozkurt, P, Tunali, Y, Kaya and Gokar I, "***Histological changes following epidural injection of midazolam in the neonatal rabbit***", Paediatr Anaesth, 7, (1997), 385.

124. Schwieger, I. M, Jorge-Costa, M, Pizzolato, G.P, Forster A and Morel D.R, "***Intrathecal midazolam reduces isoflurane MAC and increases the apnoeic threshold in rats***", Can J Anaesth, 41, (1994), 144.

125. Carassiti, M, Ysasi, A and Gonzalo, L.M, "***Effects of the chronic administration of midazolam on the rat hippocampus***", Rev Med. Univ Navarra, 42, (1998), 18.

126. Bahar, M, Cohen, M.L, Grinshpoon, Y, Kopolovic, U, Herbert, M, Nass, D and Chanimov, M, "***An investigation of the possible neurotoxic effects of intrathecal midazolam combined with fentanyl in the rat***", Eur J Anaesthesiol, 15, (1998), 695.

127. Leah, J.D, Malik, R and Curtis, D.R, "***Actions of midazolam in the spinal cord of the cat***", Neuropharmacology, 22, (1983), 1349.

128. Nishiyama, T, Matsukawa, T and Hanaoka, K, ***"Acute phase histopathological study of spinally administered midazolam in cats***", Anesth Analg, 89, (1999), 717.

129. Nishiyama, T, Sugai, N and Hanaoka, K, "***In vitro changes in the transparency and pH of cerebrospinal fluid caused by adding midazolam***", Eur J Anaesthesiol, 15, (1998), 27.

130. Sinz, E.H, Kofke, W.A and Garman, R.H, "***Phenytoin, midazolam, and naloxone protect against fentanyl-induced brain damage in rats***", Anesth Analg, 91, (2000), 1443.

131. Reiter, P.D and Stiles, A.D, "***Lorazepam toxicity in a premature infant***", Ann. Pharmacother, 27, (1993), 727.

132. Cronin, C, "***Neurotoxicity of lorazepam in a premature infant***", Pediatrics, 89, (1992), 1129.