LIQUID MEMBRANE PHENOMENON IN THE ACTIONS OF GABAPENTIN

Anantha Naik Nagappa[1], Vivek D[2], Srinivas D[2] and Ullas D.P[2]

[1]Manipal College of Pharmaceutical Sciences, Manipal University, Manipal, India,576104
[2] Dept of Pharmacology, SCS College of Pharmacy, Harapanahalli, India,583131

Summary

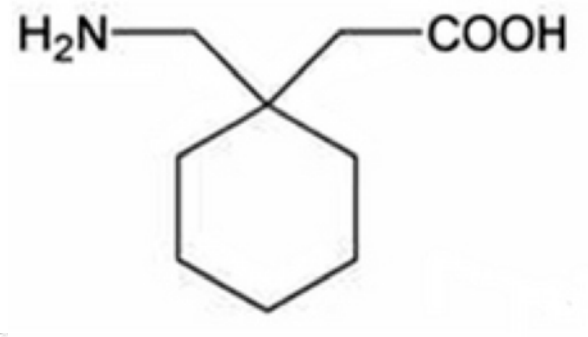
Role of surface activity in the mechanism of action of gabapentin (GBP) has been studied. GBP has been shown to generate liquid membrane it self and also in association with the relevant membrane lipids, sphingomyelin and cholesterol in series with a supporting membrane. Transport of relevant biogenic amines e.g. GABA, glycine, glutamic acid, aspartic acid, serotonin, nor-adrenaline, and dopamine and ions : sodium, potassium, calcium and chloride has been studied in the presence of liquid membranes generated by GBP and GBP in association with cholesterol – sphingomyelin. The data on modifications in the permeability of relevant biogenic amines and ions indicate that the liquid membranes generated by GBP may contribute to the mechanism of action of GBP.

Key words: Surface activity, Gabapentin, Liquid membrane hypothesis.

Introduction

Liquid membrane hypothesis was originally propounded to account for enhanced self rejection due to the addition of very small amounts; of the order of a few ppm, of surfactants like polyvinyl ether to the saline feed in reverse osmosis [1-3]. According to the hypothesis when a surfactant is added to an aqueous phase, the surfactant layer, which forms spontaneously at the interface, acts a liquid membrane and modifies transport across the phase boundary. The hypothesis further postulates that as the concentration of the surfactant is increased the interface gets progressively covered with the surfactant layer liquid membrane and at the critical micelle concentration (CMC) it is completely covered. Since molecules of surface active nature are crucial to living matter and its organization and biological implications of liquid membrane hypothesis have been investigated [4]. The investigators have given strong indications that liquid membrane bilayers generated from constituents of liquid membranes are capable of acting as mimetic systems of biological membranes [5].

Figure.1 Chemical structure of Gabapentin: 1-(aminoethyl) cyclohexane acetic acid, Neurontin®



Formation of cell membrane and location of receptor proteins in the lipid bilayers is a consequence of surface activity. It is therefore logical to expect that drugs by altering the permeability of cell membranes after interacting with them are likely to surface active. Wide variety of drugs infact, are known to be surface active [6-12]. This does not appear to be a fortuitous coincidence. In a number of cases excellent correlation between surface activity and biological effects have been demonstrated [13-18].

Since structural requirement for surface activity are often similar to those for interaction of drugs with receptor sites[19] , the correlation between surface activity and biological effects appear to indicate the possibility of a common mode of action for surface active drugs or at least some crucial step common to the mechanism of all surface active drugs. What can this common mode be? In view of the liquid membrane hypothesis, it was suspected that liquid membranes generated by surface active drugs either by themselves or in association with membrane lipids at the site of their action modifying the transport of relevant permeants to the action sites, might be an important step common to the mechanisms of all surface active drugs. This essentially is the core of liquid membrane hypothesis of drug action [20] . Investigations carried out on a wide variety of drugs have strongly substantiated this surmise [22-28].

In the present communication role of liquid membranes in the actions of Gabapentin (GBP) has been studied. GBP, structure shown in figure 1 has both hydrophilic and lipophilic domains with structure. GBP is therefore, likely to be surface active in nature and hence capable of formation of liquid membrane. In the present study formation of liquid membrane by GBP and GBP in association with sphingomyelin and cholesterol in series with supporting membrane has been demonstrated.

GBP is an anti-convulsant drug approved by USFDA in early 1990s. The molecular bases of the anti-convulsant and anti-epileptic activities of GBP are unknown. GBP has been approved for the treatment of neuropathic pain in six European countries, New Zealand and Australia, and numerous countries in Latin America [29].

GBP is a gamma amino butyric acid (GABA) analog, but is not a GABA mimetic, although some neurons that respond to GBP are GABAergic. Recently it is reported that GBP at relevant concentrations, binds to an auxiliary protein of voltage-gated calcium channels (α2δ) [30] and as a result, modulates the action of calcium channels and neurotransmitter release [31-32]. This may account for many of its pharmacological actions. GBP is also a substrate for the large neutral amino acid transporter, and this may be the major route allowing GBP access to the CNS [33].

Modulation of synaptic transmission between primary afferents and *substantia gelatinosa* neurons, and blockade of signal transduction, are two potential mechanisms of action, in addition to' inhibition of glutamate release by voltage-sensitive calcium channels [34]. GBP also exerts an indirect effect on the voltage dependent sodium channels, slightly inhibits the release of certain neurotransmitters [35]. GBP appears to be potentially useful in the adjunctive treatment of drug-resistant bipolar mixed states, and that it was particularly effective in relation to depressive symptomatology [36].

Materials and Methods

Materials

GBP was gifted by Parke-Davis Hyderabad, potassium chloride, calcium chloride, - sodium chloride, disodium hydrogen phosphate, potassium dihydrogen phosphate, serotonin, GABA, glutamic acid, aspartic acid, glycine (Analytical Grade) were purchased from Genuine chemicals, Mumbai, India. Dopamine hydrochloride (Biological Grade) from Ranbaxy Laboratories Ltd., Nagar, nor-adrenaline (Biological Grade) from Intas Laboratories, Ahmedabad, cholesterol (Biological Grade) from Rolex Laboratories, Mumbai, sphingomyelin from Sigma Chemicals, USA and deionized water were used in the study.

Methods

Determination of critical micelle concentration (CMC)

CMC of aqueous GBP solutions was determined from variation of surface tension with concentration of GBP prepared. The surface tensions of various concentrations of GBP were measured by using Du-Nouy Tensiomat Model 144. pH of the various concentrations of the GBP solutions remained more or less constant at constant temperature. All the measurements were carried out at constant temperature 37 ± 0.1 ^{0}C. The CMC of GBP was found to be 2×10^{-3} *M.*

Determination of hydraulic permeability data (L_p)

Hydraulic permeability data is used to demonstrate the formation of liquid membrane in series with the supporting membrane. All glass transport cell as shown in figure 2 [27], was used for the transport studies. A sartorious cellulose acetate micro-filtration membrane (Cat. no. 11107, pore size 0.2 μm and thickness of 1×10^{-4} m and area 2.55×10^{-5} m^2) was used as support for liquid membrane and separated the transport cell in

to two compartments, C and D. Measurements of hydraulic permeability were carried out at various concentration of GBP. The aqueous solution of GBP of various concentration ranges, were placed in compartment C of the transport cell, where as compartment D was filled with deionised water. The concentration range selected for this study was chosen so as to obtain the data from both lower and the higher sides of the CMC of GBP. The known pressures were applied in the compartment C by adjusting the pressure head and the consequent volume flux was measured by noting the rate of advancement of liquid meniscus in the capillary L_1L_2 using a cathetometer of least count 0.001 cm and stop watch reading up to 0.1 seconds. The magnitude of the applied pressure difference was also measured by noting the position of the pressure head with the cathetometer. An important precaution in the measurement of volume flux was taken by allowing a sufficient time after the application of pressure on compartment C before measurement of liquid meniscus in the capillary L_1L_2. This was done to ensure that flow in the capillary was steady flow. The distance traveled by the liquid meniscus was plotted against time. If such plot were found to be straight line passing through origin, the flow was taken as steady. During the volume flux measurement, the solution in the compartment C was well stirred and the electrode E_1 and E_2 were electrically short-circuited (figure 2).

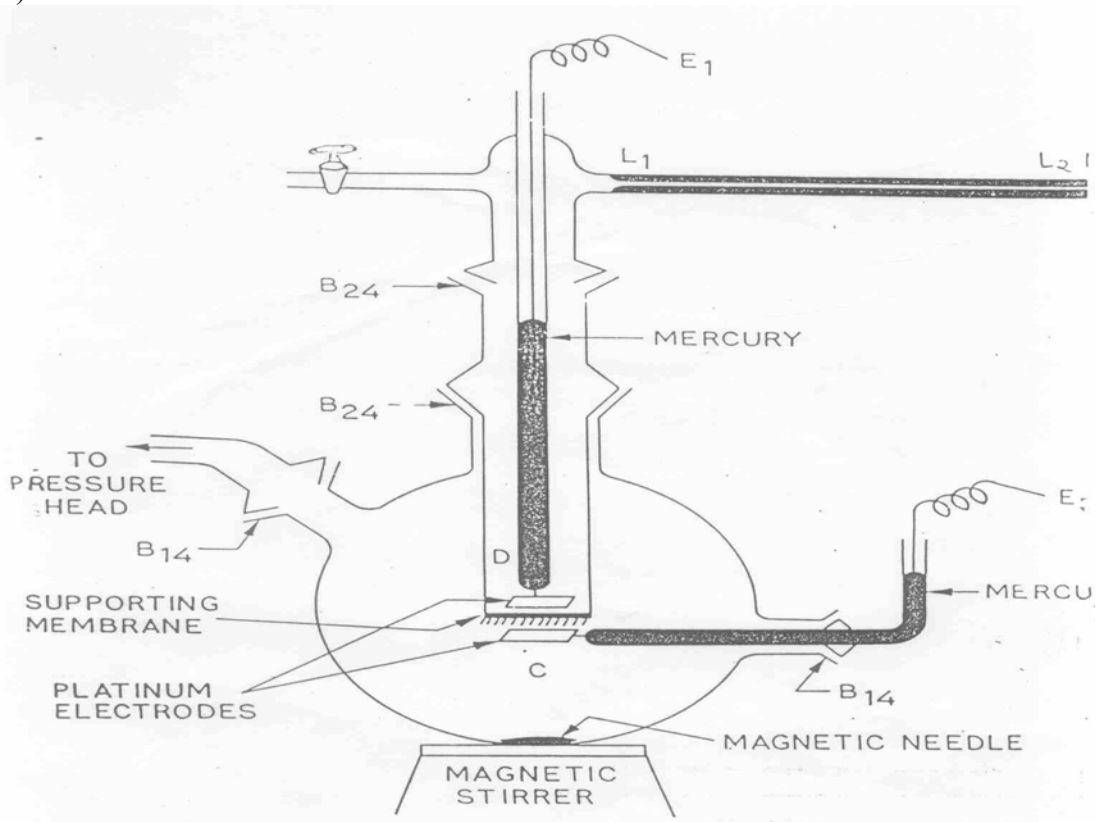


Figure 2.All Glass Transport Cell : E_1 ,E_2 - Electrode terminals, L_1, L_2 - capillary, B14, B24 are ground glass joints, Supporting membrane- Cellulose Acetate micro filtration membrane (Sartorius Cat. No 11107)

The volume fluxes J_V at various values of applied pressure difference (ΔP) were calculated by using relation;

$J_V = \pi r^2 l / \pi R^2 t = l/t$ -------- [1]

Where r and R are radii of the capillary L_1L_2 , l is the distance traveled by liquid meniscus at time t. Total of ten replicates were carried out and values are presented as mean ± S.D. of 10 replicates (Figure 2).

Solute and Hydraulic permeability studies using sphingomyelin – cholesterol mixture

Solute permeability data is an important determinant in the view of transport of the ions and the biogenic amines across the liquid membranes generated by the GBP. The selection of the permeants as ions and biogenic amines was done on the basis of present mechanism of actions of the GBP. The findings of the solute permeabilities will be a useful outcome to support the present views of the mechanism of actions of the GBP. In the present study, various ion permeants evaluated are, calcium chloride for calcium transport, potassium chloride for potassium transport, sodium chloride for sodium transport and potassium chloride for chloride transport. Endogenous amines like GABA, glycine, glutamic acid, aspartic acid, serotonin, nor-adrenaline, and dopamine were evaluated so as to study the effect of the liquid membranes generated by GBP on the transport of these amines.

Hydraulic and Solute permeability studies using sphingomyelin – cholesterol mixture were carried out so as to impose the in vivo conditions. It has been established that sphingomyelin – cholesterol aqueous mixture at cholesterol concentration 1.919×10^{-5} *M* and sphingomyelin concentration of 1.175×10^{-6} *M* generates liquid membranes at the interface in such a way that the interface is completely saturated with cholesterol [37]. For these permeability studies, buffer solutions (Phosphate buffer pH 7.4) of the sphingomyelin – cholesterol mixture were prepared by adding necessary volume of ethanolic stock solutions of known concentrations of the respective components to the buffer phase with constant stirring. All the solutions thus prepared, the final concentration of ethanol never exceeded 0.4 % by volume as it was shown by control experiments that a 0.4 % solution of ethanol in water did not affect the surface tension of water to any measurable extent. In these studies sartorious cellulose micro filtration membrane (Cat. no. 11107, pore size 0.2 μm, thickness of 1×10^{-4} m and area 2.55×10^{-5} m^2) was chosen as supporting membrane to highlight passive transport through the liquid membrane generated by GBP.

The solute permeability (ω) of the relevant permeants in presence of liquid membranes generated by GBP was determined by equation,

$$(J_s / \Delta\pi)_{Jv=0} = \omega \text{ ------- (1)}$$

where J_s and J_v are the solute flux and volume flux per unit area of the membrane, respectively and $\Delta\pi$ is the osmotic pressure difference.

The condition Jv = 0 was imposed on the system and the amount of permeant transported to the compartment filled with deionised water in a known period of time was estimated. All measurements were performed at constant temperature, using thermostat setting of 37 ± 0.1 ^{0}C. After a period of two hours, concentration in the compartment D was measured. For solute permeability measurements, concentration of GBP chosen was the one at which the liquid membrane generated by GBP, completely covered the supporting membrane and was saturated with GBP. This concentration was derived from the present data on hydraulic permeability in presence of various concentrations of GBP.

For measuring solute permeability study compartment C was filled with the liquid membrane generating drug solution along with permeants of known concentrations. Compartment D was filled with the buffer. The solute permeability study was done above CMC to make sure that supporting membrane would be fully covered by the liquid membrane generated by GBP.

Hydraulic permeability study using sphingomyelin – cholesterol mixture was also undertaken so as to have an additional check on the effect of GBP liquid membranes in simulated conditions on the transport of the water across the membranes. For the measurement of the hydraulic permeability study, Compartment C of the transport cell was filled with solutions of various concentrations of the GBP prepared in the buffer solutions of the sphingomyelin – cholesterol. All measurements were performed at constant temperature, using thermostat setting of 37 ± 0.1 ^{0}C. Total of ten replicates were carried out for solute permeability and hydraulic permeability with sphingomyelin – cholesterol mixture and values are presented as mean ± S.D. of 10 replicates (Table 1)

As GBP have both hydrophobic and hydrophilic domains in its structure, it is supposed that they are involved in the formation of the liquid membrane. The hydrophobic tails of GBP will preferentially orient towards the hydrophobic part of the supporting membranes and hydrophilic part of these, would be drawn outwards i.e. away from it. So in these experiments, the permeants would face the hydrophilic surface of the liquid membranes generated by GBP.

Estimation of ions and biogenic amines

Amount of various permeants transported across the compartment D were estimated as follows;

Ions

The amount of sodium, potassium and calcium ions was determined by using atomic absorption Spectrophotometer (Perkins – Elmer model 306). The estimation of chlorides was conducted using Mohr's method [38].

Biogenic amines

The amount of glycine, aspartic acid, GABA and glutamic acid were estimated form the amount of their reaction products with ninhydrin, and the colored product measured at 570 nm [39] using spectrophotometer. (Model SL –159, Elico India). The amounts of

serotonin and nor-adrenaline were estimated by measurement of absorbance at 281 nm in 0.1 N HCl and dopamine was estimated at absorbance at 280nm using 0.1 N Hcl [40-41].

Result and discussion

Amphiphilic drugs interact with membranes and biological systems, causing a variety of effects. Mouritsen et al. has discussed the theoretical analysis of the effects of drugs on lipid bilayer [42-43]. There are number of examples where drugs have been inserted into membranes as interstitial components and are known to alter the organization and properties of the membranes and proteins. Computer simulations indicated that partitioning of the drugs, accumulate heterogeneously in the membrane, higher concentrations being attained at the interface between gel phase and liquid crystal domains of membranes [42-43]. The interest in understanding the capacity of drugs, for instance for manipulate the local lipid – bilayer structure is put in a particular perspective when considering that this capacity is may be related to potency of drug and therefore its molecular mechanism of action [44]. The aggregation of many of the surface active drugs follows the same pathways as that of the classical surfactants, while some drugs shows the ability to self associate forming closed micelle like structures, others aggregates by continuous stacking [12] .

In present study, hydraulic permeability data at varying concentrations of GBP was found to be linear with,

$$J_v = L_p \Delta p \qquad \ldots\ldots\ldots\ldots\ldots [2]$$

Where, J_v is the volume flux per unit area of the membrane, Δp is the applied pressure difference, and L_p is the hydraulic conductivity co-efficient. The values of L_p recorded at varying concentrations of the drug, estimated from the slopes of J_v versus Δp plots are given in Figure 3. It has been shown that a progressive decrease of L_p values are observed with increase in the concentration of the GBP up to its CMC value, beyond which it becomes more or less constant.

This trend indicates the progressive coverage of the supporting membrane with the liquid membrane generated by GBP in accordance with the Kesting's hypothesis. At CMC, coverage of the supporting membrane with the GBP liquid membrane is complete. The marginal increase beyond the CMC is most probably due to an increase in density of the liquid membrane as postulated by Kesting's et al. [1-2]. Analysis of the values of L_p in the light of the mosaic model [45-46] also furnishes further evidence in favor of the formation of liquid membrane in series with the supporting membrane. When concentration of the surfactant is 'n' times its CMC ≤ 1, the value of L_p should be $[(1-n) L^s_p + n L^c_p]$. Where L^s_p and L^c_p respectively represent the values of hydraulic conductivity co-efficient for the supporting membrane and surfactant layer liquid membrane. Functionally L^s_p and L^c_p would be the values of L_p at 0 and 1 CMC of the surfactant. The values of L_p thus calculated compare favorably with the experimentally determined values (Figure 3).

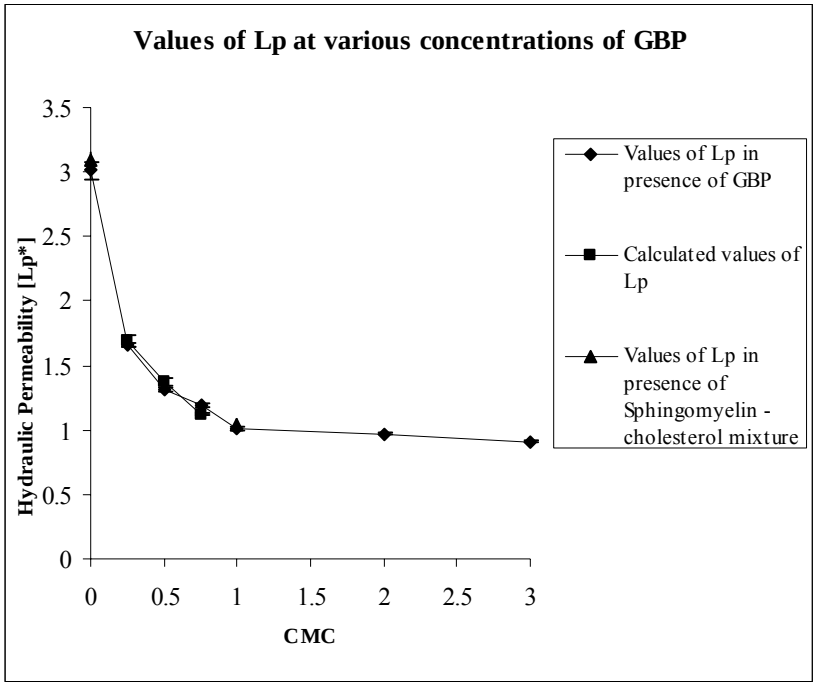


Figure 3. Plot of hydraulic permeability (Lp) vs. critical micellar concentration of GBP. The values are presented as arithmetic mean ± standard deviation of ten determinations.
* Lp X 10^9 (m3 S^{-1} N^{-1})

Although cellular mechanisms of pharmacological actions of GBP remain incompletely described, several hypotheses have been proposed. Wide range of therapeutic indications for GBP and its remarkable safety profile has stimulated a large amount of effort in pursuit of its mechanism of action, which continues to be the subject of interest and much speculation. GBP was originally designed as a gabamimetic drug, capable of crossing the blood brain barrier and is going to accumulate in CNS in therapeutic concentration.

Data on transport studies of ions and neurotransmitters across liquid membrane generated by GBP in association of sphingomyelin and cholesterol can be correlated with the mechanism of action of GBP. The studies on passive diffusion of cations were carried out as they play an important role in the electrophysiological activities associated with the neuronal membranes. Values of the solute permeability for ions (sodium, calcium, potassium and chloride) amino acids (GABA, glycine, glutamate, aspartic acid) and biogenic amines (serotonin, nor-adrenalin and dopamine) in presence of sphingomyelin – cholesterol and GBP mixture are presented in Table 1.

Table 1: Solute permeability of various permeants in the presence of liquid membrane generated by GBP in presence of sphingomyelin – cholesterol mixture

Permeant	Initial concentration	$\omega_0 \times 10^6$ (Mole $s^{-1}N^{-1}$)	$\omega_1 \times 10^6$ (Mole $s^{-1}N^{-1}$)
GABA	1.940 (mole/L)	0.783 ± 0.06	0.753 ± 0.02
Glycine	1.333 (mole/L)	2.074 ± 0.03	2.482 ± 0.03
Glutamate	1.204 (mole/L)	0.659 ± 0.04	0.364 ± 0.01
Aspartic acid	1.127 (mole/L)	0.360 ± 0.04	0.130 ± 0.02
Serotonin	0.0247 (mole/L)	0.253 ± 0.01	0.307 ± 0.04
Nor-adrenaline	0.059 (mole/L)	0.322 ± 0.02	0.141 ± 0.03
Dopamine	0.0527 (mole/L)	0.453 ± 0.06	N.D.
Potassium (KCl)	10.43 (mg/ml)	344 ± 0.52	586 ± 0.37
Calcium (CaCl2)	10 (mg/ml)	530 ± 0.71	356 ± 0.47
Sodium (NaCl)	5.382 (mg/ml)	647 ± 0.42	535 ± 0.22
Chloride (KCl)	10.43 (mg/ml)	121 ± 0.21	182 ± 0.16

All the values of ω are reported as arithmetic mean of 10 repeats ± S.D.

ω_0: Values of ω when no drug is used in presence of sphingomyelin – cholesterol mixture
ω_1: Values of ω in presence of GBP and sphingomyelin – cholesterol mixture.
N D.: Not detectable

GBP has been shown to be effective and having a broad spectrum of anti-seizure activity in a number of animal seizures models elicited by both physical (Electroshock or audiogenic) and chemical (Pentelene tetrazole, thiosemicarbazide, isoniazid, bicuculine, picrotoxin, 3-mercaptopropianate) induced models [47-49] indicating its broad spectrum of activity. Three basic mechanisms of anti-epileptic actions of drugs are attributed to

inactivation of sodium channels, opening of chloride channels, and diminution of calcium currents, which exclusively highlights the role of ions, amino acids and biogenic amines.

Transport data in Table 1 indicates that transport of potassium, chloride ions and glycine was enhanced where as transport of sodium, calcium ions and aspartic acid, glutamic acid are reduced. The trends observed indicate an overall tendency of increased hyperpolarization and decrease in depolarization of the neuronal membranes. The influence on passive transport observed in our studies may also be contributing to the anti-epileptic action of drug. The solute permeability data on calcium passive diffusion was reduced in presence of GBP sphingomyelin-cholesterol liquid membranes. Recent studies using electrophysiological and calcium imaging techniques has proven that GBP interferes with the transport of the calcium in the calcium channels [32,50-51]. These studies have shown that sensitivity of the calcium currents by GBP is greatly influenced by the physiological state of cell and the calcium channel sub units.
The auto radiographic studies have also indicated the localization of [^{3}H] GBP accumulation that occurs in the specific layers of frontal, parietal, enterolinal, and occipital cortex, where as the localization in the white matter was almost non existent [52]. In the subsequent autoradiographical studies with [^{3}H] GBP demonstrated that these binding sites are probably located on neurons rather than glia. The presence of GBP in higher concentration in specific neurons in environment of sphingomyelin and cholesterol is likely to form a liquid membrane in neurons, which by its virtue may be altering the calcium diffusion leading to the anti-epileptic action.

The hyperpolarization of the cell is also achieved by loss of potassium ions from the cell or gain / entry of chloride ions in the cell. This is explained by opening of the specific channels and subsequent diffusion, which is mainly driven by electrochemical gradients. As the potassium ions concentration is being higher in intra cellular fluids and chloride ion concentration is higher in extracellular fluids, the opening of these channels lead to hyperpolarization due to movement of the ions in the opposite directions. In our study, the potassium and chloride ion transport are enhanced in the presence of liquid membrane of GBP (Table 1) the observed effect on transport trends of these ions may be contributing to the hyperpolarization of the neurons observed after administration of GBP. It is interesting to know that GBP does not binds to GABA A or GABA B receptors [53] and it was also not converted to GABA in vivo. NMR studies in vivo, have shown that GABA concentration are elevated in human patients taking GBP and that this elevation of GABA is related to seizure control [54-55]. The transport of GABA is reduced in presence of sphingomyelin – cholesterol and GBP liquid membrane**.** It is possible that the reduction in diffusion of GABA in presence of GBP is may be because of entrapment of GABA in the sphingomyelin – cholesterol mixture membranes by reducing the rate of diffusion.

Several independent studies have now shown that GBP can produce reduction in calcium influx in pre-synaptic nerve terminals and inhibit the release of excitatory amino acids in regionally selective manner [56-57]. Recent data also described inhibitory effect of GBP

on potassium stimulated [^{3}H] nor-adrenalin release from human neocortex. Other early reports mentioning an inhibitory effect of GBP and dopamine release in the rabbit stratium [58] and 5-HT levels in whole blood of young man [59] remain anecdotal and have been substantiated by present investigations. In general GBP effects mentioned in this study are consistent with our observation on diffusion trends of GABA, glutamic acid, aspartic acids, nor adrenalin and dopamine that, are inhibited and where as glycine and serotonin are enhanced. Observed effect on passive diffusion may also be contributing to the diffusion characteristics of neurotransmitters after administration of GBP in the humans.

Conclusion

The liquid membrane phenomenon in the mechanism of action of GBP has been studied. It has been observed that, liquid membranes generated by GBP interfere with the transport of the important ions and biogenic amines. The present study in no way refutes the already established the mechanism of action of GBP, but provides a more rational and dynamic approach to their mechanism by highlighting the role played by the liquid membranes generated by this drug. Further, in-vivo studies are required to be designed and done for further confirmation of the hypothesis

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