Effects of Gabaergic Phenols on Phospholipid Bilayers as Evaluated by $^{1}$H-NMR

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Received April 22$^{nd}$, 2013; revised May 25$^{th}$, 2013; accepted June 5$^{th}$, 2013

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ABSTRACT

The phenols propofol and thymol, and lately carvacrol, eugenol and chlorothymol, have been shown to act as positive allosteric modulators on GABA$_{A}$ receptor, which is the main inhibitory receptor of the central nervous system. GABA$_{A}$ receptor is an intrinsic membrane protein which activity may be affected by surface-active compounds and by physical changes in the membrane. Recently, we demonstrated that these phenols interacted with the lipid membrane phase, suggesting their anesthetic activity could be the combined result of their specific (with receptor proteins) as well as nonspecific (with surrounding lipid molecules) interaction modulating the supramolecular organization of the receptor environment. In the current study, by using $^{1}$H-NMR spectroscopy, we have investigated the effects of the insertion and the possible preferential location of the five phenol derivatives with GABAergic activity on EPC membranes. The results indicate that all compounds are able to insert in EPC phospholipid vesicles and to locate in the region between the polar group (choline molecule), the glycerol and the first atoms of the acyl chains, being the more lipophilic compounds (propofol and chlorothymol) that seem to prefer a deeper bilayer insertion. The location of the phenol molecules would reduce the repulsive forces among phospholipids head groups allowing closer molecular packing and finally diminishing the mobility of the hydrocarbon chains, as revealed by $^{1}$H spin relaxation times.

Keywords: Phenols; Propofol; GABA-A Receptor; Membrane Interaction; $^{1}$H-RMN

1. Introduction

General anesthetics are substances that induce a reversible state of unconsciousness, characterized by amnesia and analgesia. Their exact action mechanism remains incompletely elucidated. They were originally believed to act via nonspecific interactions with the lipid bilayers, affecting membrane fluidity. More recently, general anesthetics have been shown to act by modulating ligand-gated ion channels such as the GABA$_{A}$ receptor (GABA-R) (see references in [1]).

The GABA-R, a ligand-gated ion channel, constitutes the main inhibitory receptor of the central nervous system. GABA-Rs, besides being activated by the GABA neurotransmitter, are modulated by numerous therapeutically important drugs, including barbiturates, anesthetics, benzodiazepines, neurosteroids and ethanol. These compounds are GABA-Rs allosteric modulators as they bind to distinct sites to potentiate GABA-evoked currents [1-5]. The phenols propofol and thymol, and lately carvacrol, eugenol and chlorothymol, have been shown to act as positive allosteric modulators on this receptor [6-8].

GABA-R is an intrinsic membrane protein which activity may be affected by surface-active compounds and by physical changes in the membrane [4,9-12]. Taking into account the lipophilicity of the above described phenols, their interaction with the lipid membrane phase, especially the lipids surrounding the receptor and a consequent non-specific receptor modulation cannot be discarded, justifying a detailed study of drug-membrane interaction.

Recently we determined several lipophilic parameters for these five gabaergic phenols. The results obtained, based on the octanol-water partition coefficient ($\log P$...
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2. Experimental

2.1. Materials

Propofol (2,6-bis(isopropyl)-phenol), thymol (5-methyl-2-isopropyl-phenol), carvacrol (2-methyl-5-isopropylphenol), eugenol (2-methoxy-4-prop-2-enyl-phenol) and chlorothymol (5-methyl-4-chloro-2-isopropyl-phenol) were obtained from Sigma Chemical Co. (St Louis, MO, USA), and used without further purification. Egg phosphatidylcholine (EPC) was from Avanti Polar Lipids (Alabaster, USA). Water was bidistilled in an all-glass apparatus (pH 6.5 ± 0.3). Other drugs and solvents used were of analytical grade.

2.2. Membrane Preparation

Liposomes were obtained by evaporating stock chloroform solutions of EPC under a stream of N2. The samples were left under vacuum for no less than 2 h to remove residual solvent. The lipids were then suspended in 0.05 M phosphate buffer solution, pH 7.4 and vortexed for 5 min to form large multilamellar vesicles (MLVs).

For NMR experiments, small unilamellar vesicles (SUV) were used. Briefly, MLVs, obtained as described above, but suspended in D2O, were sonicated until clear (ca. 15 min) in a Sonics and Materials equipment (Newtown, CT). During sonication the temperature was kept at 0°C - 4°C by intermittent (1 min) agitation cycles, in an ice-water bath.

2.3. Partition Coefficient Determination

Phenols concentrations inside the membrane, expressed as molar ratios with respect to EPC, were calculated from the membrane—buffer partition coefficient, P, of each compound. In turn P was determined by phase-separation between MLVs and buffer at pH 7.4, according to the Equation (1) [16]:

\[
P = \frac{n_m/V_m}{n_w/V_w}
\]

where n denotes the number of phenol moles, V is the volume, and the subscripts m and w refer to the membrane and aqueous phase, respectively. The volume of the membrane phase, V_m, was calculated assuming a lipid density of 1 g/mL [16]. The amount of each phenolic compound bound to the lipid phase was optically determined at their corresponding wavelengths of maximal absorption between 270 and 282 nm [13] after ultra-centrifugation at 120,000 × g for 2 h, by subtracting the supernatant concentration from the total drug concentration measured before phase mixing.

2.4. Nuclear Magnetic Resonance (NMR) Experiments

Spectra were collected in a Varian Innova 600 MHz (LNBio, Campinas, Brazil) equipment. The samples were degassed to avoid the interference of dissolved O2 with longitudinal relaxation times (T1) measurements. For 1H-NMR, a 90˚ pulse was typically 10 - 15 μs and the recycling time was set to 5 times the largest T1 (those of the aromatic protons of phenols), typically 6 s. T1 were obtained by the conventional inversion-recovery technique, at 37°C. Using the determined partition coefficient values—see Section 2.3, all phenols were added to the sonicated vesicles up to 1:3 phenol:EPC molar ratio within the membrane.

3. Results and Discussion

Membrane-buffer partition coefficients were determined previously to the NMR experiments in order one could calculate the proper phenol amount to guarantee a 1:3 drug/lipid molar ratio in the membrane. P_EPC/w (between egg-phosphatidylcholine liposomes and phosphate buffer, pH 7.4) values, follow a similar behavior to the partition coefficient...
coefficient determined in other comparable systems reported before [13] with a hydrophobic profile of: chlorotymol \( \geq \) propofol \( > \) carvacrol \( > \) thymol \( > \) eugenol.

\(^{1}\)H-NMR spectra (600 MHz) of each compound, of EPC unilamellar vesicles and of samples containing vesicles in the presence of each compound, at a 1:3 (phenol:lipid) molar ratio, were collected. Typical spectra of phenol, EPC small vesicles and phenol in EPC vesicles (1:3 mole\%) are shown in Figure 1, for propofol.

The assignments of EPC and phenols hydrogen signals are indicated in Figure 2, where capital letters refer to the phospholipid, and lower-case letters identify the nonequivalent resonance peaks of each phenol compound. These assignments were in good agreement with those reported in the literature for EPC [15-17] and carvacrol, thymol and eugenol [18,19].

From the chemical shifts (C.S.) corresponding to the phenolic hydrogens in water or in EPC vesicles, changes in the chemical shifts between both systems (\( \Delta \)C.S.) were calculated. The same procedure was applied to determine the \( \Delta \)C.S. of EPC hydrogens in water or in the presence of each phenol. Table 1 shows the C.S. and \( \Delta \)C.S. for phenols and Figure 3 represent the \( \Delta \)C.S. values determined for EPC.

Changes in the chemical shifts of hydrogens (\( \Delta \)C.S. \( \neq \) 0) would indicate variations in the chemical environment of the nuclei, being considered more significant those changes higher than 0.05 ppm [15]. All phenolic compounds assayed showed significant \( \Delta \)C.S., especially in their aromatic hydrogens, indicating that, in the presence of EPC vesicles, they experience a different chemical environment, and confirming their interaction with the vesicles (Table 2).

Figure 3 shows the effect (\( \Delta \)C.S.) of the five phenol compounds on chemical shift of EPC hydrogens. Eugenol, the less hydrophobic analog essentially affected hydrogen I (choline group nearby the phosphate atom of EPC), changing it downfield. All the other phenol compounds induced upfield shifts in EPC hydrogens around the glycerol backbone region. In the presence of thymol, the main \( \Delta \)C.S. found in the EPC molecule was observed in hydrogens H, E and C. In the presence of propofol, a significant alteration was observed in hydrogen E and,

![Figure 1](image1.png)

**Figure 1.** \(^{1}\)H-NMR spectra (600 MHz) of propofol in \( \mathrm{D}_2\mathrm{O} \), EPC small unilamellar vesicles and propofol in EPC vesicles (pD 7.4; 25°C).

![Figure 2](image2.png)

**Figure 2.** Hydrogen peaks assignments for each compound (lower-case letters) and EPC molecule (capital letters) in NMR spectra.

![Figure 3](image3.png)

**Figure 3.** shows the effect (\( \Delta \)C.S.) of the five phenol compounds on chemical shift of EPC hydrogens. Eugenol, the less hydrophobic analog essentially affected hydrogen I (choline group nearby the phosphate atom of EPC), changing it downfield. All the other phenol compounds induced upfield shifts in EPC hydrogens around the glycerol backbone region. In the presence of thymol, the main \( \Delta \)C.S. found in the EPC molecule was observed in hydrogens H, E and C. In the presence of propofol, a significant alteration was observed in hydrogen E and,
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Figure 3. Changes in the chemical shifts of EPC hydrogen peaks in NMR spectra induced by the presence of phenolic compounds. Assignments are given in Figure 2. [EPC] = 65 mM, pH 7.4, 25°C, 600 MHz. Just ΔC.S. higher than 0.05 ppm is represented.

Table 2. Changes in the chemical shifts of $^1$H-NMR signals of phenolic compounds in the aqueous phase (D$_2$O) and with EPC vesicles, at pH 7.4 and 25°C.

| Compound | Hydrogen | D$_2$O (ppm) | EPC (ppm) | ΔC.S. (ppm) |
|----------|----------|--------------|-----------|-------------|
| Eugenol  | a -CH$_2$- | 2.432 | 2.461 | 0.029 |
|          | b CH$_3$-O | 2.940 | 2.938 | -0.002 |
|          | c = CH2(9) | 4.172 | 4.139 | -0.033 |
|          | d = CH-     | 4.673 | *     | *          |
|          | e = CH$_2$(9') | 5.112 | 5.050 | -0.062 |
|          | f = CH-     | 5.864 | 5.702 | -0.162 |
|          | g -CH = C-OH | 5.963 | 5.809 | -0.154 |
|          | h -CH = C-O | 6.026 | 5.943 | -0.083 |
| Thymol   | a (CH$_3$)$_2$ | 0.291 | 0.270 | -0.021 |
|          | b -CH$_3$ | 1.360 | 1.313 | -0.047 |
|          | c -CH(CH$_3$)$_2$ | 2.276 | *     | *          |
|          | d -CH = C-OH | 5.868 | 5.619 | -0.249 |
|          | e = CH-     | 5.941 | 5.837 | -0.104 |
|          | f -CH=     | 6.329 | 6.011 | -0.318 |
| Propofol | a -(CH$_3$)$_4$ | 0.309 | *     | *          |
|          | b ((CH$_3$)$_2$-CH)$_2$ | 2.380 | *     | *          |
|          | c = CH-     | 6.155 | 5.846 | -0.309 |
|          | d -CH=     | 6.310 | 6.035 | -0.275 |
| Carvacrol | a -(CH$_3$)$_2$ | 0.305 | 0.292 | -0.013 |
|          | b -CH$_3$ | 1.282 | 1.242 | -0.040 |
|          | c -CH(CH$_3$)$_2$ | 1.956 | 1.867 | -0.099 |
|          | d -CH = C-OH | 5.928 | 5.624 | -0.304 |
|          | e = CH-     | 5.957 | 5.884 | -0.073 |
|          | f -CH=     | 6.265 | 5.985 | -0.280 |
| Chlorothymol | a -(CH$_3$)$_2$ | 0.292 | *     | *          |
|          | b -CH$_3$ | 1.382 | 1.359 | -0.023 |
|          | c -CH(CH$_3$)$_2$ | 2.267 | *     | *          |
|          | d -CH = C-OH | 5.944 | 5.779 | -0.165 |
|          | e = CH-C-Cl | 6.412 | 6.091 | -0.321 |

*not determined

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Figure 4 shows the $T_1$ profile of EPC hydrogens with and without phenolic compounds. In this work we presented the $T_1$ data as described by Fraceto et al. [15, 16], with an EPC molecule shown in its extended form, from hydrogens of the polar head-group (left) to those of the acyl chain region (right).

The presence of phenolic compounds within the EPC molecules induced also changes in the molecular dynamics, as revealed by relaxation studies. A decrease in $T_1$ values was detected for all several hydrogens belonging to the phenols (data not shown), indicating an important mobility restriction and suggesting a strong phenol-EPC interaction.

In the presence of eugenol, hydrogens A, B and D of EPC showed large decrements in $T_1$ values, indicating a decrease in mobility in the hydrocarbon chains region. In the presence of chlorothymol, the hydrogens more affected were I, K and A, indicating the insertion of its aromatic (voluminous) group in that zone of the bilayer (principally hydrogens I and K), also explaining the changes found in chemical shifts (Figure 3) which could be due to the ring current effect inducing changes in the chemical environment of the hydrogens below the glycerol zone (E and C). Similar results have been described for local anesthetics [25]. Propofol induced $T_1$ reduction mainly in the hydrocarbon chains (hydrogens D, F, B and A) but changed C.S. at the glycerol level and the beginning of the acyl chains. Thymol also caused comparable

with minor intensity, in hydrogens I and K. Carvacrol and chlorothymol induced important alterations in hydrogens E and C.

The NMR literature reports that aromatic or electronegative molecules in the bilayer can shift resonances by the short-range ring current effect [15,20]. Chemical shifts to minor frequencies (upfield) reveal that the group is inducing the shield of the spins of the observed nuclei, indicating the proximity of the aromatic ring and probably the localization of the compound in that spot at the bilayer [15].

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The $T_1$ values measured for EPC hydrogens on unilamellar vesicles (Figures 4(a)-(e), full symbol) are in good agreement with those reported before [15]. The $T_1$ are small for the polar headgroup hydrogens (peaks G, H), reflecting the restrictions caused by the electrostatic interaction between the amine group of one phospholipid and the phosphate groups of the next phospholipid [21, 22]. In the glycerol backbone region (peaks I, J, K) hydrogens have intermediate, smoothly higher mobility than hydrogen at carbons $\alpha$ and $\beta$ of the acyl chain (peaks E, C) due to the high molecular packing of that region. The profile of acyl chain EPC hydrogens (peaks B to A) dynamics is in agreement with that described by other authors and obtained through $^{13}$C, $^2$H and $^1$H-NMR or EPR [23,24] showing increased mobility towards the terminal methyl group—peak A.

In the presence of eugenol, hydrogens A, B and D of EPC showed large decrements in $T_1$ values, indicating a decrease in mobility in the hydrocarbon chains region. In the presence of chlorothymol, the hydrogens more affected were I, K and A, indicating the insertion of its aromatic (voluminous) group in that zone of the bilayer (principally hydrogens I and K), also explaining the changes found in chemical shifts (Figure 3) which could be due to the ring current effect inducing changes in the chemical environment of the hydrogens below the glycerol zone (E and C). Similar results have been described for local anesthetics [25]. Propofol induced $T_1$ reduction mainly in the hydrocarbon chains (hydrogens D, F, B and A) but changed C.S. at the glycerol level and the beginning of the acyl chains. Thymol also caused comparable
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Figure 4. Changes in the longitudinal relaxation times (T\textsubscript{1}, s) of EPC hydrogens at SUV in the presence of propofol, thymol, carvacrol, eugenol and chlorothymol. [EPC] = 65 mM, pH 7.4, 25°C, 600 MHz. The letters without values were not determined.

4. Final Remarks

These results indicate that the phenols studied are clearly able to interact with membranes. Considering that many drugs have intracellular targets, which require their transport across phospholipid bilayers, and drug-lipid interactions are unavoidable. Furthermore, although specific pharmacological regulation of membrane protein functions, such as GABA\textsubscript{A} receptor, can be analyzed using well-described theories of ligand-receptor interactions, it...
Figure 5. Schematic representation of the proposed insertion of the five phenol groups in EPC liposomes, as revealed by different NMR approaches (see text). More significant changes in C.S. (ΔC.S.) and T_1 values are represented by triangles (dark gray: >0.05 ppm; light gray: 0.03 - 0.05 ppm) or circles (>0.06 s), respectively.

should be considered that many compounds that regulate the receptor protein function are noticeably lipophilic, which may change the physical properties of the lipid bilayer. Thus, it is possible that any anesthetic activity of lipophilic phenols could be the combined result of the interaction of the phenol molecules with specific receptor proteins (GABA<sub>A</sub> receptor) and with the surrounding lipid molecules modulating the supramolecular organization of the receptor environment.

5. Acknowledgements

This work was partially financed with grants from a bilateral CAPES (Brasil)/MINCyT (Argentina) project, SECyT—Universidad Nacional de Córdoba, MINCyT (Córdoba) and CONICET (Argentina). DAG and MAP are career investigators from CONICET.

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