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Monoterpenes affect chlorodiazepoxide–micelle interaction through micellar dipole potential modifications

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Abstract

The ability of several natural terpenes to affect benzodiazepine (BZD)–micelle interaction through the membrane dipolar organization was investigated. The acid–base equilibrium of chlorodiazepoxide (CDX) and the spectroscopic behavior of the electrochromic dye merocyanine were tested in the presence and in the absence of Triton X-100 micelles (used to mimic a membrane environment) containing or not cineole, menthol, geraniol or camphor. CDX's apparent p*K* increased in the environment of terpene-containing micelles compared with pure Triton X-100 micelles. Decrements in electric potentials (between −111 and −128 mV with respect to pure detergent) were calculated from Boltzmann equation. This result suggested, that in the presence of terpenes, the tendency of $CDXH^+$ to remain in the membrane phase increased. The dielectric constant (*D*) of the microenvironment sensed by merocyanine within Triton X-100 micelles, determined from $\lambda_{max,2}$ of merocyanine monomer, was $D=9$ and increased in the presence of all the terpenes assayed ($D\cong 11$). The decrease in merocyanine partitioning (A_{peak1}/A_{peak2} increased) also reflected an increment in the negative dipole potential. The present results suggest that terpenes contributed to the whole dipolar arrangement of the micelle with a dipole moment vector which had an intense component oriented parallel to the intrinsic dipole of the Triton X-100 molecules in the micelles. This led to a more negative environment of the interface region where CDX was located, and increased the net polarity of the deepest micelle regions sensed by merocyanine.

Keywords: Chlorodiazepoxide; Monoterpene; Triton X-100; Acid–base equilibrium; Merocyanine; Dipolar potential

1. Introduction

Essential oils as well as many of their pure components are well known in popular medicine. Compounds like thymol, geraniol and cineole have sedative properties and are able to affect mouse motility after inhalation in controlled conditions [1–3]. Limonene [4] and the essential oil of *Tagetes minuta* L. have antidepressant and anxiogenic effects [5,6]. The essential oil from *T. minuta* (a complex mixture of terpenes) has also been used in popular medicine as diuretic, diaphoretic, antihelmintic, aphrodisiac and catartic [7]. The smoke produced during the burning of *T. minuta* leaves and those from mint are inhaled twice a day by some people in Perú, for the treatment of psychiatric diseases [8].

Upon analyzing the chemical structure of terpenes, it may be inferred that, from the biophysical point of view, these compounds should have either an amphypathic or a hydrophobic behavior. This fact should be taken into account to predict and to evaluate correctly their biological effects [9]. Terpenes have a tendency to partition from the aqueous phase toward membranes [10] and, as a consequence, they have the possibility of inducing changes in the membrane structural organization and surface electrostatics. For that reason, concomitant effects on membrane permeability and on the activity of intrinsic proteins should be expected [10,11]. Hence, geraniol inhibits the growth of *Candida albicans* through a mechanism involving changes in membrane dynamics [12]. The anxiogenic effects of *T. minuta* essential oil mentioned above were associated to its ability to affect gamma-aminobutyric acid (GABA) function [13].

Abbreviations: CDX, Chlorodiazepoxide; *D*, dielectric constant; $GABA_A$-R, GABA receptor; GABA, gamma-aminobutyric acid; BZD, benzodiazepine

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GABA is the major inhibitory neurotransmitter in central nervous system and its activation was associated with anxiolytic effects. The binding of GABA to $GABA_A$ receptor type A ($GABA_A$-R) enhances the membrane permeability to chloride ions inducing a hyperpolarization of neurons and blocking the transmission of the nerve impulse. Phenol derivatives bearing aliphatic substituents in *ortho* position to the phenolic hydroxyl, activated chloride-currents in the absence of GABA. Among this type of compounds, are some monoterpenes [14]. $GABA_A$-R is an intrinsic protein of postsynaptic brain membranes. It consists of five units containing five transmembrane domains each, that limits a chloride channel. In addition to the binding site for GABA, $GABA_A$-R contains binding sites for benzodiazepines (BZDs), barbiturates and neurosteroids that are allosterically coupled [15,16]. Tagetone, a major component of the essential oil from *T. minuta* has been shown to modulate the supramolecular coupling of the subunits of $GABA_A$ receptor ($GABA_A$-R) through a mechanism involving its incorporation into membranes [17]. Moreover, it was demonstrated that the essential oil from *T. minuta* decreased the affinity of the BZD flunitrazepam for its binding site at the $GABA_A$-R [13] and to increase the nonspecific binding of this drug to synaptic membranes from brain cortex. Previously, we have reported that membrane composition and organization modulates BZD partitioning and localization within the polar head groups of membrane lipids [18–22].

In the present work, we investigated the ability of several natural monoterpenes to affect the localization of a BZD within micelles of a neutral detergent to mimic the membrane environment. Micelles are frequently used to mimic biological membrane environments, and provide simple systems to study the physico-chemical properties of binding of amphipathic molecules to membranes [23,24]. Chlorodiazepoxide (CDX) was chosen due to its relatively high p*K* value. This property made it a more suitable probe for studies based on changes in an acid–base equilibrium, if compared with other BZDs available the p*K* of which are below 2 [20].

2. Materials and methods

2.1. Materials

7-Chloro-2-(methylamino)-5-phenyl-3*H*-1,4-benzodiazepine-4-oxide (CDX) was from Hoffman La Roche (Basel, Switzerland). Water was bidistilled in an all-glass apparatus. Other drugs and solvents used were of analytical grade. Terpenes used [geraniol (2,6-dimethyl-2,6-octadien-8-ol); (±)-camphor (1,7,7-trimethylbicyclo[2.2.1]-2-heptanone); menthol (5-methyl-2-(1-methylethyl)-cyclohexanol); and cineole (1,3,3-trimethyl-2-oxabicyclo[2.2.2.]octane)] were purchased from local drug stores, re-purified and the purity controlled by GC-MS [25]. The orthogonal views of the chemical structures in the minimal energy conformation as well as the absorbance spectra of the terpenes used are shown in Fig. 1.

2.2. CDX UV spectra

Aqueous solutions of 15 μM CDX were prepared. The pH was adjusted between 0 and 10 using a Cole Palmer 59003 pH-meter equipped with a glass electrode and a sensor for automated temperature compensation. The absorption spectra were measured against a blank prepared with the same solvent used for dissolving CDX, and at identical pH. Readings were recorded at 1-nm intervals within 200–400 nm wavelength range, using a Beckman DU 7500 spectrophotometer equipped with a diode array detector and 0.0001 AU sensitivity. The CDX spectrum was also determined in the presence of suspensions of Triton X-100 containing or not one of the terpenes indicated. Only the spectrum of camphor overlaps that of CDX at the wavelength used (264 nm) in the experiments of CDX's acid–base equilibrium (compare Figs. 1b and 2). In spite of that, the results with absorbance data obtained at 360 nm were

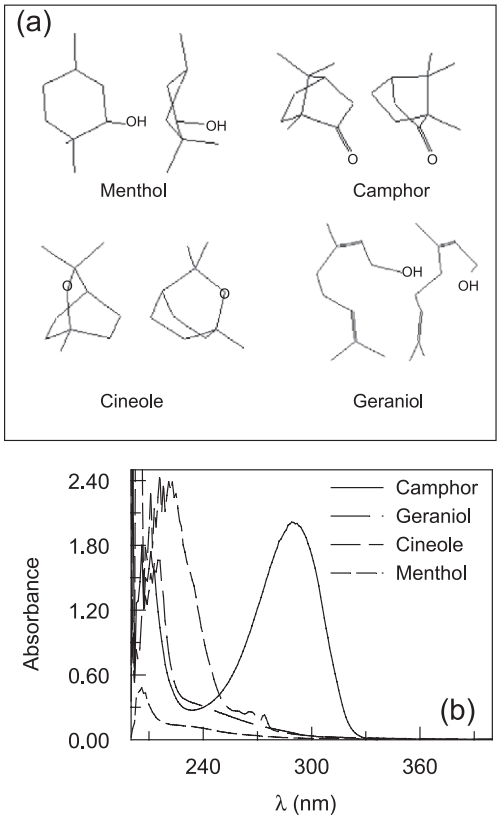

Fig. 1. Chemical structures and absorbance spectra of the terpenes used. (a) Orthogonal views of the chemical structures of terpenes in the conformations of minimal energy in the vacuum. The globular structure of menthol camphor and cineole is evident. The conformation of geraniol within the micelle environment is expected to be roughly linear. (b) Absorbance spectra of terpenes; the overlapping between camphor's and CDX spectra at the wavelength of 264 nm is evident from the comparison of b in this figure and Fig. 2a.

similar to those at 264 nm. So, the latter wavelength was preferred because, if compared with the former, it exhibited a higher sensitivity for CDX absorbance.

2.3. Preparation of model membranes

Triton X-100 was dispersed in water at room temperature at a final concentration of 0.86 mM (over the critical micellar concentration, c.m.c. = 0.24 mM) [26]. In some cases, the detergent was dispersed in the presence of one of the terpenes (cineole, menthol, camphor and geraniol), which were tested at a final concentration of 0.65 mM.

2.4. Corrections of pH meter readings

Dioxane–water solutions with known stoichiometric proton concentrations were prepared by the addition of known amounts of HCl or NaOH from standardized solutions, and their pH values were calculated as $pH_{calc} = -\log[HCl]$ or $pH_{calc} = 14 + \log[NaOH]$ and measured as indicated above to obtain pH_{obs} values. The plots of pH_{corr} vs. pH_{obs} were fitted to second-degree equations on pH_{obs}, by non-linear regression using a computer-aided least squares method. The resulting function was used to correct pH_{obs} values in other experiments [18,20]. This calibration allows elimination of the combined and indistinguishable contributions of 'primary medium effect' and the 'liquid junction potential' from the pH readings [27].

2.5. Calculation of CDX equilibrium dissociation constant

CDX can be protonated at the methylamine group at C2 (see Ref. [20]). The method used for calculation of the constant for the acid–base equilibrium was described previously [18,20]. Briefly:

For the dissociation reaction (see Ref. [20] for details):

$$CDXH^+ \rightleftarrows CDX + H^+ \quad (1)$$

the equilibrium constant results:

$$K = \frac{[CDX][H^+]}{[CDXH^+]} \quad (2)$$

In homogeneous systems, K can be calculated from absorbance data of CDX as a function of pH adjusted to the following equation:

$$A_{obs} = \frac{[H^+]A_{obs1} + KA_{obs2}}{[H^+] + K} \quad (3)$$

where the absorbance of the protonated and dissociated species are A_{obs1} and A_{obs2}, respectively.

In heterogeneous systems such as dispersions of detergents

$$A_{obs} = A_m + A_w \quad (4)$$

where A_m and A_w are the absorbance values of the drug partitioned into the membrane and dissolved in water, respectively. Then Eq. (4) results from the sums of two terms representing the contribution of the drug present in each phase (water and micelle).

$$A_{obs} = \frac{[H^+]A_{obs1m} + K_{app}A_{obs2m}}{[H^+] + K_{app}} + \frac{[H^+]A_{obs1w} + K_wA_{obs2w}}{[H^+] + K_w} \quad (5)$$

where $A_{obs1m}=[A_{obs1} - A_{obs1w}]$ and $A_{obs2m}=[A_{obs2} - A_{obs2w}]$, K_{app} is the apparent equilibrium dissociation constant in heterogeneous media, and K_w is the equilibrium dissociation constant in water; A_{obs1} and A_{obs2} are the experimental absorbance values measured in heterogeneous systems at both extremes of the titration curve. The subindex w is refers to the absorbance of species $CDXH^+$ and CDX in water, measured in a separate sample.

2.6. Analysis of the number of chemical species presented in CDX solutions

The CDX absorption spectra were analysed according to Nowika-Jankowsika [28]. The analysis consisted of a linear combination of any two absorbances of solution **j** found at λ_k and λ_p that gives a new value denoted $\zeta_{k,p}$ calculated as: $\zeta_{k,p} = \alpha A_{j,k} + \beta A_{j,p}$.

A constraint is imposed to the linear combination:

$$(\lambda_k + \lambda_p) = \text{constant or } (\lambda_k - \lambda_p) = \text{constant}$$

Then, the A values for each **j** solution are plotted against the two wavelength scales. If a set of plots intersects at one point, then a genuine isosbestic point is present in the original spectra implying that there is a reasonable evidence that only two absorbing species are present in the system studied. The conditions of the transformation used were: $(\lambda_k - \lambda_p) = 5$ nm.

2.7. Effect of terpenes on the absorbance spectra of merocyanine

Merocyanine solutions (15 μM final concentration) were prepared in dioxane–water mixtures containing increasing dioxane proportions (0%, 20%, 45%, 70% and 82%) to obtain media with different polarities and of known dielectric constant. The absorbance spectra of merocyanine were recorded as indicated in Section 2.2 for CDX. In separate experiments, the spectra of merocyanine were recorded in samples consisting of aqueous dispersions of Triton X-100 or terpene–Triton X-100 mixed micelles.

2.8. Calculation of electrical potentials from CDX acid–base equilibrium data

A variation in the apparent value of $[H^+]$ at the surface as a function of the surface potential can be approximated by using a Boltzmann relation

$$[H^+] = [H^+]\exp(-ze_o\varphi_o/kT)$$

This shift in the "local pH" will cause a shift in pK from pK_w in water (Eq. (7)) that will be added to the shift caused by the partitioning of CDX and $CDXH^+$ toward the interface (Eq. (6)).

$$\Delta pK_i = pK_i - pK_w \quad (6)$$

then,

$$\Delta pK_{app} = [\Delta pK_i - (e\varphi_o/2.3kT)] \quad (7)$$

where pK_i is the interfacial pK and pK_{app} is the apparent pK, measured in the presence of terpenes. The latter may include the electrostatic effects caused by a charged membrane–water interface. In Eq. (7), φ_o represents the surface potential; it depends on the net surface charge and is usually calculated according to Gouy-Chapman equation using surface charge density values [29]. Although neither Triton X-100 nor the terpenes used bears any ionizable group, they posses strong dipolar moments which might exert its effect according to

$$\varphi_d = \frac{\mu\cos\theta}{\varepsilon_0\pi r^2} \quad (8)$$

where μ: dipolar moment of the terpene molecule; ε_o: permitivity of free space (8.85×10^{-12} CV^{-1} m^{-1}); r: distance from the dipole to the probe-charge (here represented by $CDXH^+$). The distance x between the pair of charges $\pm q$ was assumed to be $x \ll r$ [30]. In addition, the electrostatic effect of the dipole sensed is in the same orientation as its vector, then $\theta = 0$. Hence, the changes in pK_{app} induced by terpenes, with respect to Triton X-100, were assumed to be due to a dipolar potential added by the former compounds to the detergent micelles. So, φ_o in Eq. (7) was taken as a dipolar potential φ_d. Moreover, from Eq. (8), using the values of φ_d obtained from Eq. (7), theoretical distances between $CDXH^+$ and the terpene were calculated.

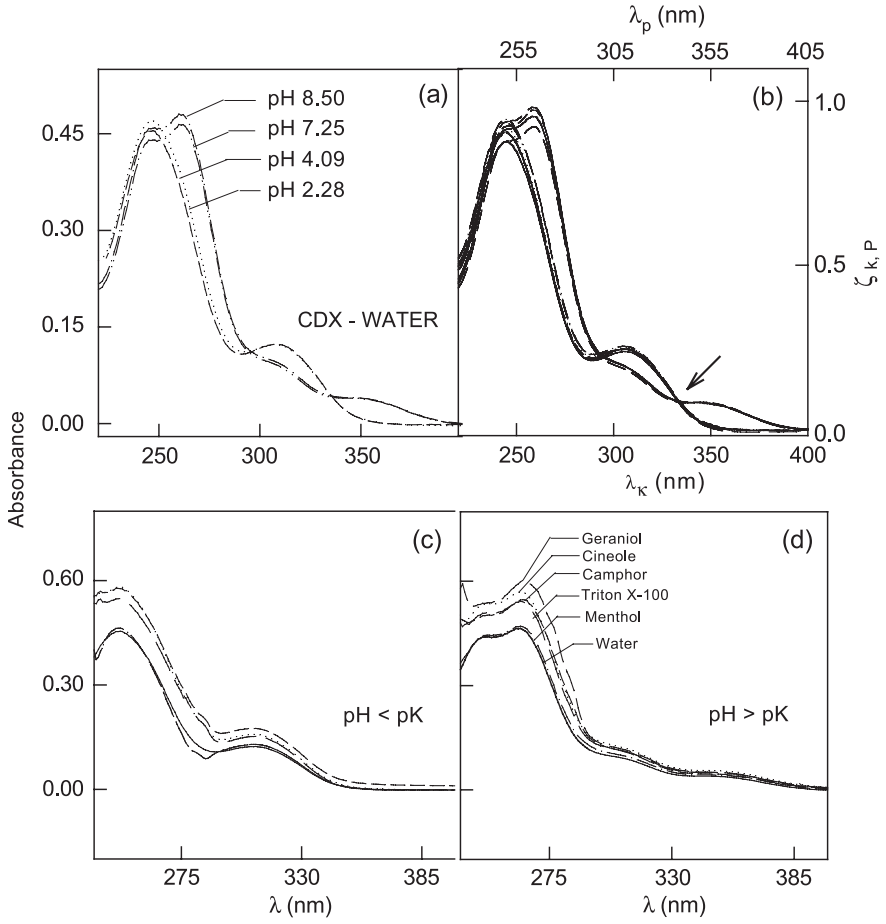

Fig. 2. Spectroscopic behavior of CDX as a function of pH. (a) Absorbance spectra; (b) Nowika-Jankowsika analysis to confirm the existence of an isosbestic point; (c) absorbance spectra of the charged form of $CDXH^+$; (d) absorbance spectra of the non-charged form of CDX.

3. Results and discussion

3.1. Terpenes affect the acid–base equilibrium of CDX in a micellar environment

The effect of pH and the presence of pure Triton X-100 or mixed terpene–Triton X-100 micelles on the UV absorption spectrum of CDX is shown in Fig. 2. The presence of an isosbestic point at the wavelength value where spectra crossed up (335 nm) (Fig. 2a) was confirmed by a linear combination analysis as proposed by Nowika-Jankowsika [28] (see Fig. 2b). This indicates the presence of only two absorbing chemical species and supports the use of the present absorbance data to the analysis of CDX acid–base equilibrium. The presence of Triton X-100 micelles exerted an hyper-chromic effect on both the protonated (Fig. 2c) and on the neutral (Fig. 2d) form of CDX. The spectra of these two chemical species were obtained at pH values several units below and above the pK of CDX, respectively. Subtle variations were induced by the presence of the different terpenes in the mixed micelles.

Fig. 3 shows the titration curves of CDX in water and in the presence of Triton X-100 or mixed terpene–Triton X-100 micelles; points correspond to the experimental values of observed absorbance (A_{obs}) at 264 nm vs. pH_{corr} (pH values corrected as explained in the Materials and methods section). The theoretical fitting of the experimental data A_{obs} to Eq. (3) (for homogeneous systems) or to Eq. (5) (for micellar dispersions) was good and allowed the calculation of CDX's pK values in each condition (Table 1).

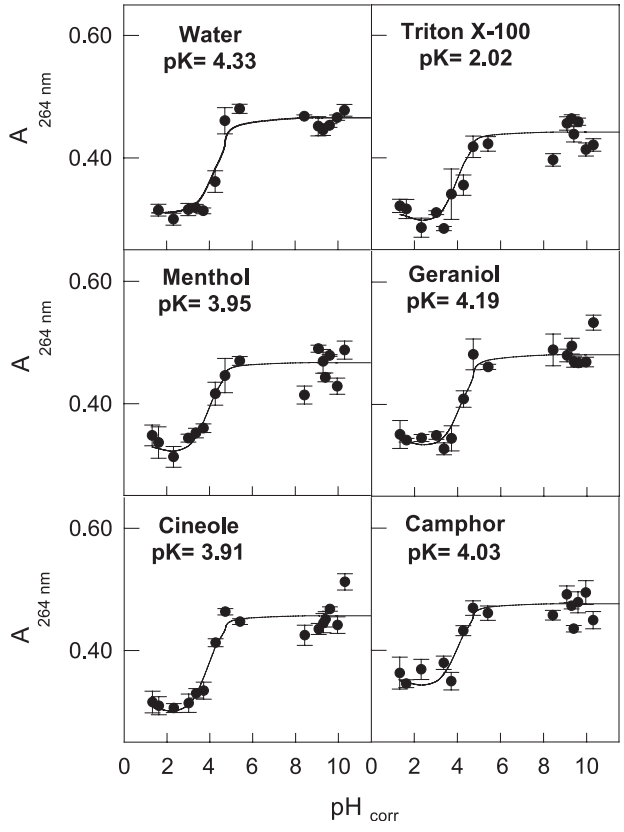

Fig. 3. Titration curves of CDX in heterogeneous systems. See experimental procedures for details.

Table 1
Effect of terpenes in the acid–base equilibrium of CDX at the membrane–water interface

Sample	pK_w (intrinsic)	pK_i (interfacial)	pK_{app}	φ_d (mV)	μ (D)	r (nm)
Water	4.33	–	–			
Triton X-100	–	2.02	–	0		
Triton X-100 + menthol	–	–	3.95	– 114	1.531	1.27
Triton X-100 + cineole	–	–	3.91	– 111	1.454	1.25
Triton X-100 + geraniol	–	–	4.19	– 128	1.726	1.27
Triton X-100 + camphor	–	–	4.03	– 118	2.523	1.6

φ_d values are the differences induced by the terpene with respect to Triton X-100 and were calculated from Eq. (7); μ: resultant dipole moment of the terpene in debyes; r: theoretical distance between CDX and the electrostatic attracting point, calculated from Eq. (8).

The theory applied to analyse the results was the one developed by Fernández and Fromherz [31]. According to it, the drug titration in a media of known polarity let determine the intrinsic K (such as pK_w in water). This pK_w reflects the tendency of the drug to dissociate in that medium and can be calculated from the true concentrations of all the chemical species taking part in the equilibrium (CDX, $CDXH^+$ and H^+) in that media. The values of K_{app} determined in heterogeneous systems, as is described by Eq. (5), is not an intrinsic pK but an "interfacial pK" because the concentration of protons used in its calculation is the one present in water and not in the membrane [18,20,31].

Similarly to what we described previously [20], in the presence of Triton X-100, CDX's pK_{app} was significantly lower than pK_w, indicating an enhanced tendency of BZDs to dissociate in low-polarity media compared with a polar isotropic solvent like water (see Refs. [18,20] and present work). All the terpenes assayed induced an increment on the value of CDX pK with respect to that obtained in the presence of pure Triton X-100 micelles. This result evidenced that terpenes displaced the dissociation reaction toward the reactants, favoring the existence of the positively charged form of CDX ($CDZH^+$). These increments in pK were too big to be ascribed to a mere dielectric change in the microenvironment. This suggested an effect due to an electrostatic binding (Table 1). However, Triton X-100 do not have ionizable groups, so the surface of the micelles resulting form the self-aggregation of this detergent remained neutral within the whole pH range tested. The same can be said about all the terpenes used. As a consequence, an electrostatic potential other than a typical Gouy-Chapman potential might be involved.

3.2. Changes in membrane electrostatics induced by terpenes?

Several electrical potentials can be measured in a biomembrane: (a) a trans-membrane potential, coming from the charge separation across the membrane; (b) surface potentials, coming from the charge bound to the membrane–water interface; and (c) the dipole component of the membrane boundary potential, which is an integral parameter that may report on the conformational state of the lipid head-group and their orientation dipole potentials, as well as the effect of the water molecules structured at the membrane–water interface. Micelles do not have an internal hydrophilic compartment, so a trans-membrane potential cannot be established. As explained above, in our system, the micelle–water interface is not expected to exhibit a formal net charge so, the surface potential will also be zero. Only a dipole potential could be non-zero in our system. It can be estimated by measurements of permeability and binding of hydrophobic ions (Refs. [32–34] and references therein). It is interesting to mention that a dependence on the dipole potential of the partitioning of positive ions into membranes with zero net surface charge was demonstrated [32,33].

Negative potentials were obtained when Eq. (7) was applied to ΔpK_{app} values (Table 1). Those potentials represented the difference, respect to pure Triton X-100, introduced by the terpenes. They were the resultant (projected on the micelle water interface) of the vectorial sum of: (i) terpene dipole moments, plus the changes they exerted on (ii) Triton X-100 polar head group dipoles orientation and (iii) structured-water dipoles organization. To determine whether the modulus of the intrinsic dipole moments of the terpenes assayed were consistent with the changes these drugs produced on the membrane dipole potential, their energy-minimized structures and dipole moments were calculated. Terpenes showed a potency to shift CDX's pK in the environment of Triton X-100 micelles, which was independent from the modulus of their dipole moments (Table 1). This suggested that terpenes differed in either the distance from the CDX molecule or the orientation of their dipole moment vectors, with respect to the normal to the surface. It is important to note that the conformation of minimal energy of the cyclic molecules of cineole, menthol and camphor (calculated in the vacuum) (Fig. 1a) are roughly globular and are expected to be conserved in the micellar environment. On the contrary, up on incorporating in the micelle, a non-cyclic molecule such us geraniol, might be forced to acquire a roughly linear conformation different from the minimal energy conformation in vacuum. As a consequence, the net dipole moment of the molecule is expected to be somewhat different form the value calculated in the present work. To clarify this point, surface potential measurements should be done.

Assuming a constant orientation of the dipole vectors, from Eq. (8), a distance between the terpenes and $CDXH^+$ between 1.25 and 1.6 nm was calculated (Table 1). This result was consistent with the micellar model proposed by Robson and Denis [35]. Moreover, it also indicated a localization of $CDXH^+$ within the outer polar head group region of the micelle, according to previous evidence of BZD localization [19,21].

3.3. Changes in membrane electrostatics sensed by merocyanine

Changes in surface potentials induced by the presence of terpenes were also evaluated by the analysis of merocyanine absorbance spectra. Merocyanine had already been used as an electrochromic dye [36]. The peak positions of membrane-associated merocyanine reflect the microenvironment of dye molecules, specially the polarity if the contribution of the refractive index term is small [37]. Fig. 4a shows that merocyanine exhibit two absorbing peaks in water, one of them at 500 nm and the other one at 540 nm representing the dimer in water (peak1) and monomer in the membrane (peak2), respectively [38]. As demonstrated by other authors [36], the $\lambda_{max,2}$ of the latter peak suffered hypsochromic and hipochromic shifts as the polarity of the media increased (here represented by the decrement in dioxane concentration) (Fig. 4a and b). The local dielectric constants were calibrated in terms of the peak positions of absorption spectra of merocyanine dissolved in various dioxane–water solutions (Fig. 4a). The value of $\lambda_{max,2}$ obtained in the presence of Triton X-100 with or without terpenes was interpolated in the plot of $\lambda_{max,2}$ vs. D in Fig. 4b and the values of dielectric constants of the environment sensed by merocyanine were obtained (Table 2). The probable polarity of the microenvironment, estimated from the positions of the absorption peaks, increased in the presence of terpenes (Table 2). The increment in the value of D, with respect to the terpene free micelles, indicated that terpenes induced an increase in the polarity of the Triton X-100–water interface. This result was consistent with the inhibition of $CDXH^+$ dissociation (pK of CDX increases).

Increasing merocyanine/Triton molar ratio (Fig. 4c) as well as the presence of terpenes in addition to Triton X-100 (Fig. 4d) induced changes in the intensity and also in the shape of the spectra. This effect can be more clearly described by the ratio between the absorbance of both peaks. This ratio increased with the polarity of the media as well as with the merocyanine–Triton X-100 molar ratio. A linear relationship with positive slope was found between the A_{peak1}/A_{peak2} ratio and the dielectric constant of the media (Fig. 4b). The A_{peak1}/A_{peak2} increased in the presence of terpenes compared with pure Triton X-100 micelles (Table 2). The D values obtained up on interpolation of those A_{peak1}/A_{peak2} values in the corresponding plot of Fig. 4b gave results qualitatively similar to those obtained from $\lambda_{max,2}$ values (Table 2). However, the latter ratio should better be interpreted in terms of a decreased partitioning of

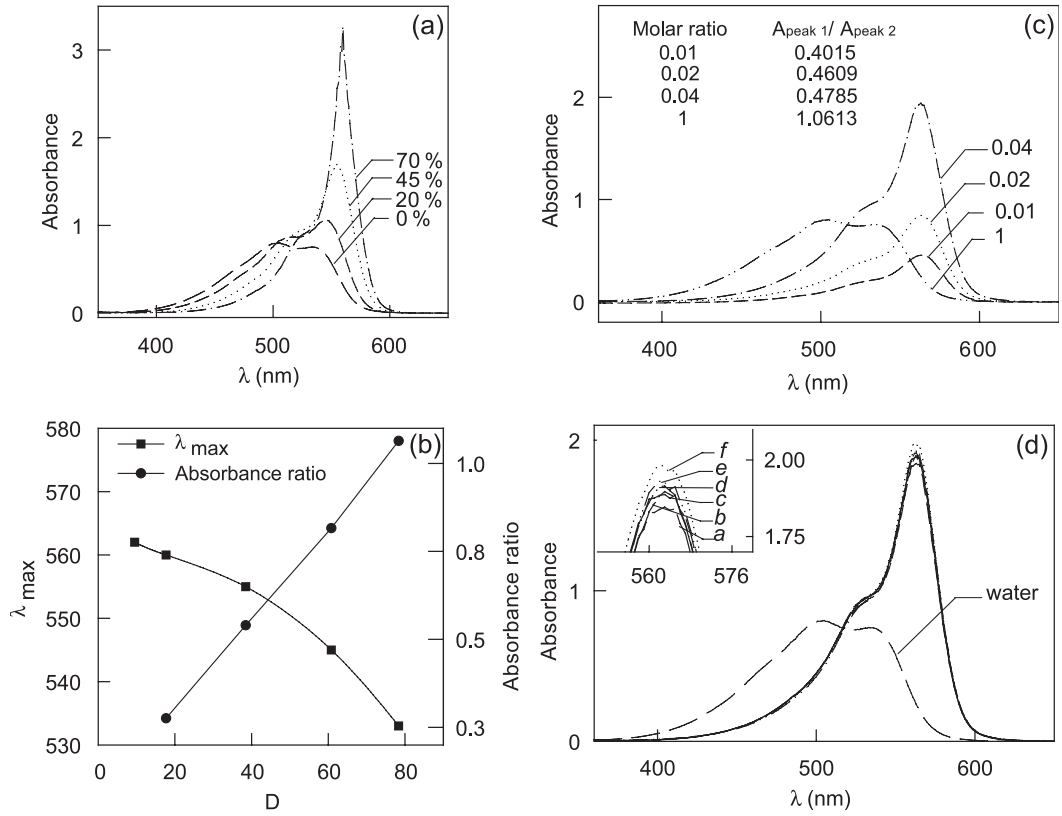

Fig. 4. Effect of medium polarity on the absorbance spectra of merocyanine. (a) Merocyanine in dioxane–water solutions; (b) variation of $\lambda_{max,2}$ and A_{peak1}/A_{peak2} absorbance ratio of merocyanine as a function of dielectric constants (peak1 and peak2 were those observed at the lower and the higher wavelength, respectively); (c) the numbers indicate the values of merocyanine/Triton X-100 molar ratio; (d) effects of terpenes on merocyanine spectroscopic behavior; the references from a to f correspond to Triton X-100, cineole, thymol, camphor, geraniol and menthol, respectively.

the monomeric form of merocyanine into membrane but not a direct reflection of the polarity of the microenvironment (the latter should be applicable only to homogeneous systems like the dioxane solutions). So, from A_{peak1}/A_{peak2} data, it was inferred that in the presence of terpenes, the partitioning of merocyanine decreased as an expression of the appearance of a negative potential.

Merocyanine is known to be located in very low polar regions of membranes ($D \cong 4-9$) [36]. Consistently with this, the present results (Fig. 4, Table 2) showed that this dye evidenced the existence of low polar microenvironments, in the deepest regions of Triton X-100 micelles (see below) whose polarity increased in the presence of terpenes.

Table 2
Effect of terpenes in the absorbance spectrum of merocyanine

Sample	A_{dim}/A_{mon}	λ_{max}	D
Water	1.1018	533	78
Triton X-100	0.4724	565	9
Triton X-100 + menthol	0.4797	563	11
Triton X-100 + cineole	0.4961	563	11
Triton X-100 + geraniol	0.4856	563	11
Triton X-100 + camphor	0.5019	563	11

D values were calculated by interpolating λ_{max} values of merocyanine monomer in the graph of Fig. 4b; using $\Delta A_{570-610}$.

3.4. The dipole potentials as the source of drug activity

The increments in CDX pK as well as the changes in the spectroscopic behavior of the electrochromic dye merocyanine induced by terpenes in the present system, supported the hypothesis that terpenes affect the membrane dipolar organization. Assuming an orientation of the hydroxyl ends of all the terpenes toward the membrane–water interface, the dipole moments for those molecules should produce a component parallel to the intrinsic dipole of the membrane (Fig. 5). This analysis is qualitatively consistent with the results obtained; however, as was mentioned above, reorientation of Triton X-100 polar head group and water dipoles should have taken part in the whole phenomenon.

Several pieces of evidence point toward possible relevant effects that the membrane dipolar arrangement might exert on the activity of significant membrane proteins like channels, enzymes and receptors. It has been shown that depending on the orientation of the phosphocholine group, its associated dipole potential may enhance or reduce existing electric potentials, triggering conformational changes in membrane proteins or facilitating protein insertion into membranes [39]. Lately, membrane dipoles were shown to affect the membrane insertion and folding of a model

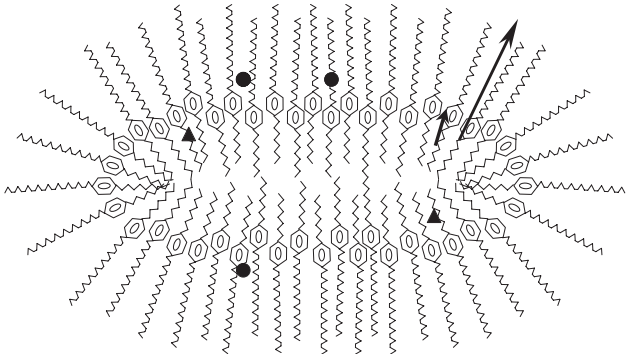

Fig. 5. Hypothetical orientation of terpenes' dipole vectors in the dipolar environment of Triton X-100 micelles. Mixed micelles of Triton X-100 and terpene. The scheme following the oblate ellipsoid model proposed by Robson and Dennis [35] for Triton X-100 with a 2:1 axial ratio and a classical hydrophobic/hydrophilic interface; all the oxyethylene units are in the hydrophilic region. The last one extends one oxyethylene chain length (1.6 nm) beyond the hydrophobic core, making the radius of the whole micelle (about 4.4 nm). Circles: CDX molecules; triangles: merocyanine molecules; thin long arrow: Triton X-100 dipole; thick short arrow: terpene dipole. The arrows point to the negative end of the dipole.

amphiphilic peptide [40]. Also the activity of PLA2, an enzyme with relevant participation in signal transduction pathways [41], was shown to be modulated by the membrane polarization induced by some sphingolipid derivatives with proven anti-exitotoxic effects in vivo (Ref. [42] and refs. therein). Moreover, some drugs that interact with membranes without binding to specific receptors (e.g. anaesthetics), to be pharmacologically active, require not only to partition into membranes and to modify membrane organization, but also to change the membrane dipolar potential [43]. This fact explained why some anaesthetics can disobey Meyer-Overton rule [44]. In this context and taken together the results shown in the present work, we suggest that the effects of some terpenes on $GABA_A$-R, demonstrated previously [13,17], may be due in part to the changes in the accessibility of the ligand (a BZD) to the receptor site because of its sequestration within a membrane hydrophobic compartment. Moreover, our results might suggest that the effects of terpenes on $GABA_A$-R activity described by Mohammandi et al. [14] might be related to subtle changes in the dipolar organization of the receptor environment. This hypothesis is currently being investigated in our laboratory.

4. Conclusions

The effect of terpenes on chlorodiazepoxide (CDX)–membrane binding are unspecific. Several pieces of evidence point to the fact that these compounds affect general membrane properties such as "fluidity" [10,12,13,17]. In the present work, it is shown that membrane polarity and dipolar potential (which can be found among general membrane properties, too) are also affected. On the other hand, terpenes may affect "unspecifically" not only benzodiazepines (BZDs) partitioning toward the membrane (unspecific BZD–membrane binding) but also BZD binding to specific membrane receptors [13,17,45]. The mechanism of the later effect might be comparable with the effects of pinene and limonene on the membrane-bound enzyme cytochrome *c* oxidase [10] and may be related with the effects of terpenes on the kind of membrane properties that determine the conformation and the activity of integral proteins [46].

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