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pH-dependent phase transition of chlorpromazine micellar solutions in the physiological range

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The effects of pH and drug concentration on aggregation properties of chlorpromazine-HCl (CPZ) are examined. The critical micelle concentration (cmc) changes from 0.2 mM at pH 7.3 to 2 mM at pH 5.6 as estimated from the stearic acid spin label solubility measurements. For concentrations above the cmc CPZ micelles undergo a concentration-, temperature- and pH-dependent transition leading to phase separation. This phase transition is followed by a sudden increase of light scattering. The phase diagram pH vs. concentration is obtained by observation of the cloud point for concentrations ranging from 0.01 to 10 mM. The intramicellar environment is probed at pH ranging from 5.5 to 8.0 using a stearic acid spin label. The intramicellar compactness increases smoothly with increasing pH suggesting the weakening of polar heads repulsion due to charge decrease. The reported results indicate that pH effects are relevant and should be properly taken into account in the performance and interpretation of experiments with CPZ.

Introduction

The mechanism of action of local anesthetics and tranquilizers has been given a considerable attention in recent years and is thought to involve the interaction of these drugs with cell membrane. One aspect which is still not clear is concerned with the role of charged and uncharged species in the action of these molecules. The action of these drugs is in general strongly concentration-dependent and affects the membrane propertics differently at low and high concentrations. Attempts have been made to correlate the surface activity [1] and the charge of the drug [2,3] to its biological activity. The surfactant-like behaviour of drugs leads to different phases under different conditions. This can influence bio-availability and reactivity. Chlorpromazine is a phenothiazine tranquilizer which has been studied in connection with its action in membranes, especially in erythrocyte and nerve membranes. Despite of numerous studies [4–12] its mechanism of action remains still unknown. Chlorpromazine forms micellar systems [10,13,14] which undergo temperature- and concentration-dependent phase transitions. Little is known about these phases and critical parameters. Critical micelle concentrations (cmc) reported by different authors [1,10,11] using different techniques and experimental conditions are scattered over a range of two orders of magnitude (10^{-5} up to 10^{-3} M). Stearic acid spin labels have been widely used in membrane research, and ESR spectrum features of spin labeled systems turn out

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important in elucidating drug-membrane interactions [6,8,10,15,16].

In this work we present the results of our investigation on CPZ aggregation properties as a function of pH from both intermicellar and intramicellar points of view. We made use of the intense light scattering which follows a phase transition, in order to analyze intermicellar and intermolecular interactions. Stearic acid spin label ESR spectra were used to obtain information on the intramicellar environment. We present here a pH dependent phase diagram which can explain discrepancies among several authors and which points out to the necessity of careful pH and temperature controls during experiments. Even if information on drug's phase properties does not directly elucidate the mechanism of action, it is important in controling experimental research on drug-membrane interaction and interpreting experimental results.

Materials and Methods

Chlorpromazine hydrochloride (CPZ) and 16-doxylstearic acid spin label (16-SASL) were purchased from Sigma Co. Isotonic phosphate buffers 310 imosM, as used for preparing erythrocyte ghosts [17], were obtained by mixing appropriate amounts of 155 mM NaH_2PO_4 and 103 mM Na_2HPO_4. Hypotonic buffers, 20 imosM, were prepared from the corresponding isotonic buffer by dilution. Samples were prepared from 240 mM CPZ solutions in 10 mM or 150 mM NaH_2PO_4 (low pH buffer component). CPZ phase diagrams were obtained by observation of the cloud point for different conditions. Temperature, pH and CPZ concentration were the fundamental parameters. pH was varied by addition of 10 mM NaOH to a hypotonic phosphate buffered CPZ solution with or without 140 mM NaCl. Spin labeled samples were prepared by adding CPZ to evaporated aliquots of a 16-SASL ethanol stock solution. Unless otherwise stated, the molar ratio of CPZ to spin label was 150 : 1. The solution were diluted with sodium phosphate buffer of desired pH.

CPZ concentrations were measured with a Cary 17D spectrophotometer by diluting small aliquots of samples in the low pH buffer component, 155 mM NaH_2PO_4 ($\varepsilon_{225} = 3.2 \cdot 10^4\ M^{-1} \cdot cm^{-1}$). The final pH for each sample was measured with a Schott Mainz CG810 pH meter. ESR spectra of samples placed in 50 μl glass micropipets were obtained with a X-band Varian spectrometer equipped with a Varian variable temperature accessory. Temperature was measured with a chromel-constantan thermocouple placed just above the sample.

Results

Chlorpromazine micellar solutions undergo a phase transition characterized by a change from a limpid to a largely turbid solution [10,13]. This phenomenon has been observed in various micellar systems [18] and can be explained by a decrease of the polar head groups hydration so that the intermicellar hydration repulsion decreases. Hydration decreases with increasing temperature and at the cloud point micelles undergo secondary aggregation and separate into two isotropic phases one of which is rich in water and the other in surfactant [18].

Since chlorpromazine is a cationic amphiphile, intermolecular and intermicellar interactions depend on the polar head charge and counter ion concentration. Therefore the phase transition described above should be pH and ionic strength dependent. Indeed, maintaining the temperature and ionic strength fixed and increasing the pH permitted us to observe this phase transition. The aggregates represent the surfactant-rich phase and can be precipitated by centrifugation. Fig. 1 shows the soluble-fraction concentration obtained after centrifugation as a function of pH, at room temperature. The sharp phase transition occurs at pH 6.9. At low pH CPZ is soluble, and the unsoluble aggregates are formed as pH increases. The cloud point is in fact observed at a pH around 6.6, slightly below the value of maximum rate of change. The pK for CPZ was determined as 8.6, for concentrations above 0.2 mM. At the pK, aggregates are present for concentrations as low as 0.02 mM at 22° C.

Critical pH for appearance of opalescence was investigated as a function of CPZ concentration, by observing the cloud point. This phenomenon is strongly dependent on CPZ concentration appear-

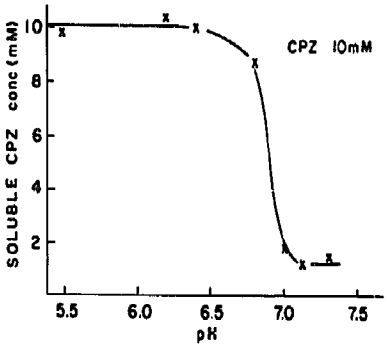

Fig. 1. Concentration of soluble fractions obtained after centrifugation of 10 mM CPZ solutions as a function of pH. Solutions were prepared in isotonic phosphate buffers. Temperature 22 °C.

ing at lower pH as CPZ concentration increases. The results for CPZ solutions in 10 mM phosphate buffer at room temperature (22 °C) are presented in Fig. 2. The solid curve separates the one phase region at lower pH values where the solutions are limpid from the two phase region at higher pH values. Concentrations below 0.01 mM do not present a cloud point. We observed also

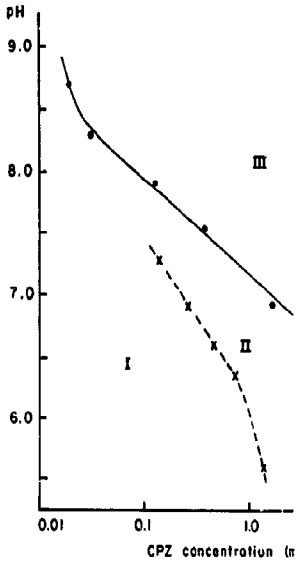

Fig. 2. Phase diagram of CPZ at 22 °C. ●, Clouding points; ×, cmc obtained from Fig. 3. Region I, monomeric amphiphile in water; region II, one-phase micellar region; region III, two-phase region.

that at low ionic strength the transition pH is ionic strength dependent. However, measurements made in the presence of 140 mM NaCl were not considerably different from those in Fig. 2, suggesting that the ionic strength effect has already attained its limit at 10 mM phosphate, at least for low drug concentrations.

The critical micelle concentration is also strongly pH dependent. The curve of cmc vs. pH which separates the monomeric phase from the ordinary micellar phase is obviously localized below the phase-separation curve in Fig. 2. The cmc at two different pH values were obtained by a procedure similar to that described by King and Marsh [19], using a stearic acid spin label. The stearic acid spin labels have low solubility in water but at low concentrations and in the absence of hydrophobic environment the narrow three line ESR spectrum characteristic of labels in aqueous solutions appears. If CPZ micelles are present in the solution this narrow spectrum is absent because the micelle-water partition coefficient is very large. Nevertheless diluting the system to concentrations below the drug's cmc the narrow spectrum reappears. We made two series of experiments in order to estimate the CPZ cmc at pH 7.3 and pH 5.6. We prepared several samples at different degrees of dilution starting from a 20 mM CPZ, 0.4 mM 16-SASL sample and measured the intensity of the low field narrow line. The results are presented in Fig. 3. At low concentrations the amount of labels in water increases linearly (slope 1, in Fig. 3). At the cmc micelle formation starts and spin labels are removed from solution to the intramicellar environment. Fig. 3 shows that this occurs between 0.1 and 0.2 mM for pH 7.3 and between 1 and 2 mM for pH 5.6. Values of cmc for different pH values are given in Fig. 2 (dashed line).

Appearance of opalescence under various conditions is due to intermicellar interactions and gives information about the forces which drive these interactions. On the other hand amphiphilic spin labels can probe intramicellar packing of hydrophobic molecular portion and internal viscosity. ESR line positions of disordered samples reflect effects of molecular motion. The definition of a parameter measured from the experimental spectrum which helps describing the rela-

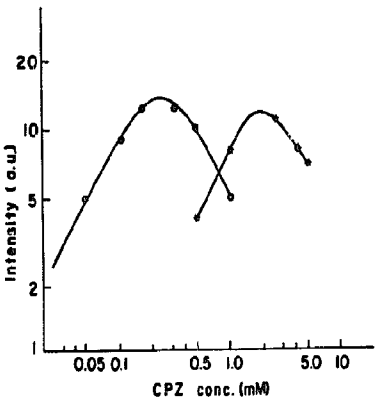

Fig. 3. Amplitude of the low-field narrow ESR line (see text) as a function of CPZ concentration. ○, pH 7.3; *, pH 5.6, isotonic phosphate buffer, at 22°C.

tive changes of a system is mathematically correct. We choose the parameter S defined by:

$$S = (A_{max} - A_{min})/(A_{zz} - (A_{xx} + A_{yy})/2)$$

where A_{max} and A_{min} are measured from the experimental spectra and A_{xx}, A_{yy} and A_{zz} are the hyperfine tensor principal values measured in the absence of molecular motion and in an environment of similar polarity [20]. It is important to bear in mind that motion and spatial order effects can be present in membrane systems and they are indistinguishable by ESR. In the present system this parameter is related to motion rather than order.

It was observed that at fixed temperature and pH the shape of the ESR spectrum is independent of the CPZ concentration (above cmc). This means that from the point of view of intramicellar interactions there is no change of the molecular motion inside the micelles or aggregates as a function of CPZ concentration. On the other hand the ESR spectra are temperature and pH dependent. Fig. 4 shows the ESR spectra of 16-SASL in 20 mM CPZ solutions at pH values above and below the transition, at room temperature and at 37°C. The pH dependence of parameter S is shown in Fig. 5. The plot reflects a transition from weakly to strongly constrained motion as pH increases. Comparing this transition with the one presented in Fig. 1 it can be noticed that the pH range is the

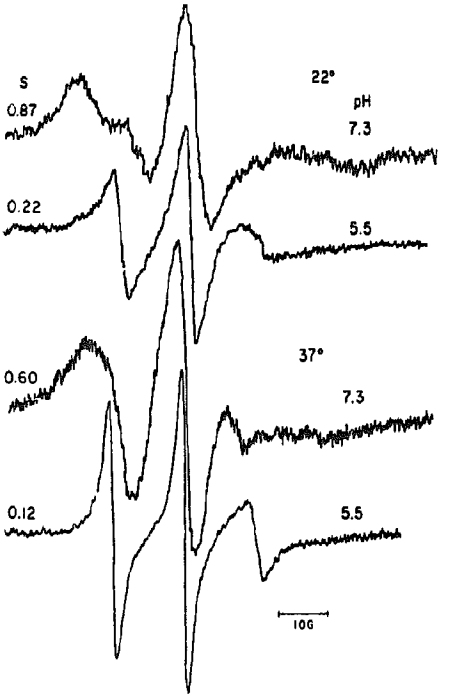

Fig. 4. ESR spectra of 16-SASL in 20 mM CPZ solutions in isotonic phosphate buffers pH 7.3 and pH 5.5, at 22°C and 37°C, respectively. Values of the parameters S related to motion (see text) appear on the left.

same but the spin label detected transition is not as sharp as the optically detected one. This can be easily explained having in mind that the spin label detects the intramicellar compactness, while the critical opalescence arises as a consequence of

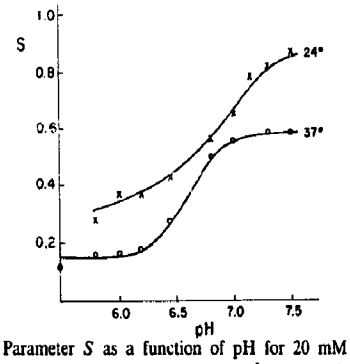

Fig. 5. Parameter S as a function of pH for 20 mM CPZ samples in isotonic phosphate buffer at 24°C (×) and 37°C (○).

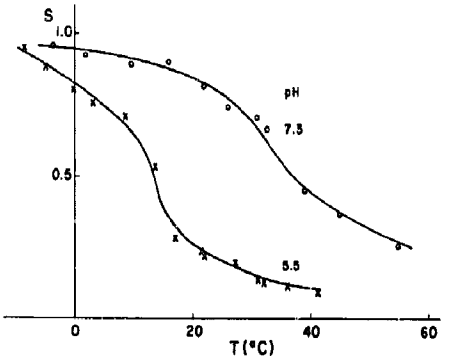

Fig. 6. Parameter S as a function of temperature for 20 mM CPZ samples in isotonic phosphate buffer pH 5.5 (×) and pH 7.3 (○). For pH 5.5 the solutions were limpid at the whole temperature range. For pH 7.3 the solutions were turbid above 5°C.

intermicellar interactions. The internal micellar environment becomes continuous more compact as the micellar charge decreases (pH increases) but aggregation starts at a critical charge value.

Parameter S temperature dependence is presented in Fig. 6 for two fixed pH values. The temperature dependence of parameter S is smooth and does not detect aggregation. The sample at pH 5.5 remains limpid throughout the whole temperature range, while at pH 7.3 the critical opalescence arises at about 5°C without a considerable change of S. We can conclude that aggregation takes place without a considerable change of internal micellar mobility.

Discussion and Conclusions

Chlorpromazine micellar properties have a strong pH dependence. In particular, the phase transition observed in many ionic and nonionic amphiphile systems, characterized by a sudden increase of light scattering when temperature increases, was observed to have also a strong pH dependence. We studied the critical pH for this transition at fixed temperature. At room temperature the critical pH depends on CPZ concentration as shown in Fig. 2. The curve of critical micelle concentration which separates the monomeric phase from the ordinary micellar phase is localized below the phase-separation curve. It can be observed from Fig. 2 that at pH 7.3 the one phase region characteristic of ordinary micelles occurs only in a narrow CPZ concentration range, from 0.2 to 0.6 mM; decreasing the pH increases the range of this one phase micellar system. On the contrary the increase in pH seems to reduce this range.

Attempts to characterize self association of phenothiazine monomers as a stacking process and to analyze further growth of CPZ micellar systems have been made [13,21], but the increase in aggregate size giving the cloud effect is still insufficiently characterized. In particular the unsoluble aggregates may be formed via stacking of monomers of via micelle aggregation. At high concentrations and below the transition pH, the ESR spectra are characteristic of spin labels dispersed in a hydrophobic micellar environment indicating the presence of micelles and suggesting that micelle aggregation is the process leading to opalescence. Stability of micellar systems is the result of a delicate balance of several forces. The repulsive electrostatic interaction due to the polar head charge of ionic amphiphiles is one of these forces. At the pK 50% molecules have a positive charge and 50% are uncharged. The ratio of uncharged to charged molecules decreases as pH decreases so that at low pH the repulsive intermicellar interaction is strong enough to prevent aggregation. If micellar charge decreases as a result of increasing pH the intermicellar attractive forces may dominate over repulsive electrostatic forces and aggregation takes place. At low drug concentrations it is difficult to obtain information on the aggregation process because the spin label method loses sensitivity and the cmc curve comes close to the phase separation boundary.

The spin label study of intramicellar environment yielded results which are consistent with the decrease of micellar charge with increasing pH. At low pH, the repulsive interaction of charged heads on micellar surface imposes a soft structure to the intramicellar hydrophobic medium. Repulsive forces decrease as micellar charge decreases and the hydrophobic environment becomes more compact restricting the spin label mobility.

Although expected, the pH dependence of CPZ properties does not seem to be taken into account in most experimental investigations [4–13]. Authors usually use low molarity buffers or no

buffer at all, forgetting that addition of CPZ lowers the pH. We observed that this lowering is quite significant when low concentration buffers are used. Assertion that the drug forms cloudy solutions which become transparent as concentration increases [8,13] cannot be explained as a drug concentration effect on the basis of the results in Fig. 2. The phenomenon is actually explained as a pH effect since the increase of drug concentration leads to decrease of pH. Since pH is important not only in case of CPZ micellar properties but also in studying every biological system, those works lacking pH control may need reinterpretation.

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