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NMR spectroscopy and surface tension measurements applied to the study of self-association of casopitant mesylate, a novel NK1 antagonist

Stefano Provera[a,*,1], Stefania Beato[b,1], Zadeo Cimarosti[c,1], Lucilla Turco[a,1], Andrea Casazza[b,2], Grazia Caivano[b,1], Carla Marchioro[a,1]

[a] Analytical Chemistry, GlaxoSmithKline Medicines Research Centre, Via Fleming 4, 37135 Verona, Italy
[b] Pharmaceutical Development, GlaxoSmithKline Medicines Research Centre, Via Fleming 4, 37135 Verona, Italy
[c] Chemical Development, GlaxoSmithKline Medicines Research Centre, Via Fleming 4, 37135 Verona, Italy

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ABSTRACT

The aggregation behaviour of casopitant mesylate, a new NK1 antagonist drug, was investigated by means of NMR spectroscopy and surface tension measurements. The critical micelle concentration (CMC) in glycine buffer at pH 3.5 was determined by analyzing the ^{1}H NMR chemical shifts variation and the surface tension in function of the concentration in a series of solutions. The temperature dependence of the CMC was also evaluated by NMR spectroscopy as well as the thermodynamic parameters contributing to the aggregation discussed. Surface tension measurements were conducted as well in the formulation conditions, e.g. in the presence of sodium chloride.

1. Introduction

Neurokinin receptors have been of particular interest in the last decade as potential novel therapeutic targets for several disorders such as migraine, arthritis, asthma, inflammatory bowel disease, overactive bladder, as well as CNS disorders such as psychosis, emesis, and Parkinson's disease. In particular, a large body of pre-clinical and clinical evidence has been generated to support the efficacy of selective NK1 receptor antagonists in the treatment of anxiety and depressive disorders. As part of a wide drug discovery programme within GSK, novel series of NK1 receptor antagonists have been designed to maximize affinity for the NK1 receptor-binding site while retaining suitable physicochemical characteristics to ensure excellent pharmacokinetic and pharmacodynamic properties in vivo. An appropriate optimization strategy of the N-phenylpiperazine series available in house, allowed the identification of a new class of potent and selective NK1 receptor antagonist exhibiting remarkable drug-like properties [1]. In particular, casopitant represents one of the most potent in vitro and in vivo NK1 receptor antagonists ever identified. The mesylate salt of casopitant was then selected for full development. The structure of casopitant exhibits the presence of two hydrophobic aromatic rings attached to a relatively more flexible and polar moiety, constituted by the N-acetylpiperazine, on the basic nitrogen ($pK_a = 6.3$) of which the salification occurs.

Molecules that exhibit both a hydrophobic and a hydrophilic part are defined amphiphiles. The most common class of molecules which belong to this class are surfactants, that are constituted by a hydrophobic hydrocarbon chain and a hydrophilic head. Besides, essentially all molecules that lack polar groups on more than one side show some degree of hydrophobicity in aqueous media. A consequence of the hydrophobicity of organic molecules in aqueous solutions is their tendency to exhibit aggregation [2]. Qualitatively, the larger the hydrophobic moiety, the stronger is the tendency towards association. Several drugs possessing rigid aromatic or heteroaromatic ring systems to which charge-bearing nitrogen atom is attached are capable to aggregate in a continuous (open) association pattern [3]. A measure of the tendency towards association is given by the critical micelle concentration (CMC) which is defined as that value of the solute concentration at which just half the total solute is present in the free monomeric form. Drugs with surface active properties are known to cause morphological changes of the blood cells in the process of drug-induced hemolysis, their safety and tolerability upon injection after intravenous administration being related to the critical micelle concentration [4].

Many techniques can be used to study self-association: surface tension, osmotic coefficient, conductivity, light scattering, etc. All of them show a discontinuity in the plot physical property *versus* concentration from which the CMC value can be derived. NMR

* Corresponding author. Tel.: +39 045 8219860; fax: +39 045 8218072.
E-mail address: stefano.provera@aptuit.it (S. Provera).
[1] Present address: Aptuit Verona s.r.l., Via Fleming 4, 37135, Verona, Italy.
[2] Present address: Chiesi farmaceutici S.p.A., Via Palermo 26/A, 43122, Parma, Italy.

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spectroscopy can also be used for this purpose, being the chemical shift concentration-dependent, particularly in case of solute–solute intermolecular interactions, with typical downfield shifts of ^{1}H resonances on dilution [5–7].

Casopitant mesylate is formulated as lyophilised powder to be reconstituted with 5 mL of pH 3 glycine buffer diluent further diluted prior to infusion and intravenous administration in 250 mL of 0.9% (w/v) sodium chloride. The CMC of casopitant mesylate reconstituted with diluent has been measured by NMR spectroscopy and by surface tension. Moreover, the effect of formulation component after further dilution prior to injection has also been investigated. Finally, the temperature dependence of the CMC was evaluated by NMR spectroscopy allowing the determination of the thermodynamic parameters contributing to the aggregation.

2. Experimental

2.1. Materials and methods

Solutions of casopitant mesylate (batch NKA-D-01-1) were prepared by dissolving an appropriate weighted amount of drug substance in glycine buffer (pH 3.5) in deuterated water (D_2O). The glycine buffer was prepared by dissolving 37.53 g of glycine in ~800 ml of deionised water, 3.28 ml of concentrated HCl (37%, w/w) has been added and made up to final volume (1000 ml) with water. Solutions used for NMR measurement were prepared using the same method replacing deionised water with deuterated water.

The lower concentration solutions were prepared by dilution. All these solutions appeared limpid. The range of concentration was 0.016–81.60 mM, corresponding to 0.01–50.40 mg/ml of casopitant free-base.

2.2. High-resolution NMR spectroscopy

^{1}H NMR spectra were collected at 25 °C using a Varian INOVA 600 NMR Spectrometer equipped with a triple resonance cold-probe.

Pre-saturation of the residual water resonance was applied using the standard *presat* sequence. The number of scans was typically 16 (64 for concentrations < 0.1 mg/ml). The ^{1}H NMR chemical shift was referenced to the glycine CH_2 resonance which was considered constant in the whole concentration range. A selected set of 10 solutions was also used to collect ^{1}H NMR spectra at different temperatures (15 °C, 35 °C, and 45 °C). The same line-broadening was applied in processing all spectra (lb = 1).

2.3. Surface tension (SFT) analyses

The SFT analyses were carried out using the Tensiometer DCAT 11. SFT measurements were conducted with the Wilhelmy Plate method [8]. This method utilises the interaction of a platinum plate with the surface being tested. In the plate method the liquid is raised until the contact between the surface or interface and the plate is registered. Three replicates were performed for each solution. All the experiments were performed at room temperature. All the experiments were carried out using the following instrumental parameters: small vessel (30 ml); motor speed 1 mm/s; surface detection 8.0 mg; 5 Hz sample/s; immersion depth 3 mm.

3. Results and discussion

3.1. ^{1}H NMR assignment

The ^{1}H NMR assignment of casopitant mesylate in the solvent used for the CMC determination could be easily done by comparison with spectra recorded in deuterated dimethylsulphoxide (DMSO-d_6) and assigned by means of 2D NMR experiments (gCOSY, ROESY, gHSQC and gHMBC). Table 1 reports a summary of the ^{1}H NMR resonances relevant for this study at two different extreme concentrations compared with the corresponding ones in the DMSO-d_6 spectra.

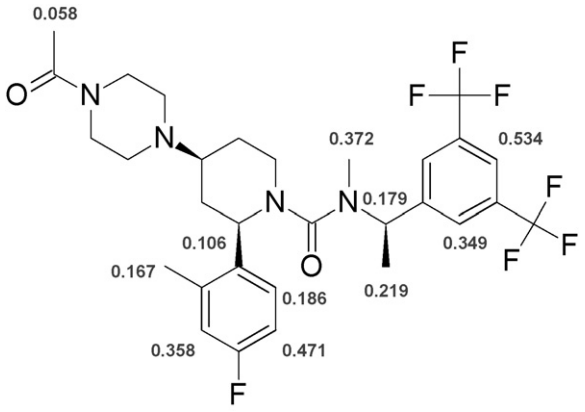


Fig. 1. Total shielding effect ($\Delta\delta$, ppm) expressed as difference between those determined in the most diluted and in the most concentrated solutions.

Most of the signals of both piperazine and piperidine rings could not be precisely determined in the D_2O glycine buffer conditions, being their signals very broad and changing upon concentration. Besides, their assignment in DMSO-d_6 is omitted here as not relevant for the purposes of this work.

3.2. Measurement of the CMC by NMR spectroscopy

A significant shift in the aqueous media was observed for all aromatic signals as well as for the methyl signals. These shifts followed the expected trend when the self-association occurs, being all signals progressively shielded as a consequence of the intermolecular interaction involving especially the aromatic hydrophobic groups. The total shielding effect ($\Delta\delta$, ppm) that was observed for some of the signals, expressed as difference between those determined in the most diluted and in the most concentrated solutions, is summarized in Fig. 1.

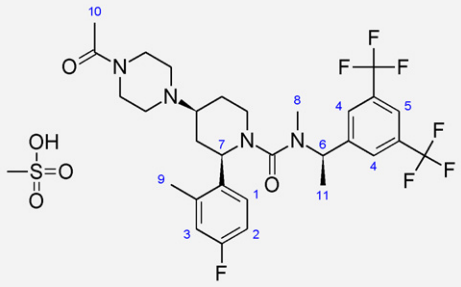
The shielding, particularly large for the aromatic protons (Fig. 2), allowed us assuming that the aromatic rings of the casopitant molecules that constitute the aggregate are placed in the inner hydrophobic part of the micelle, while the N-acetylpiperazine ring is somehow representing the hydrophilic external surface of the micelle itself. The forces that are involved in the aggregation are then those typical of π-staking.

Different plots can be drawn in order to visualize the chemical shift dependence on concentration and used to evaluate the CMC with different mathematical treatments. The graph that better describes the occurrence of a macroscopic change in the molecular order in solution was obtained by plotting the chemical shift variation ($\Delta\delta$, ppm) *versus* the reciprocal of the concentration (l/mol) (Fig. 3).

In this plot no significant chemical shift variation was observed in the solutions at concentration ≤1 mg/ml (≤1.619 mM), while a linear trend was observed in the concentration range *ca.* 50–3 mg/ml (81.595–4.799 mM). Thus, assumption could be made that the intercepts of these lines on the *x* axis corresponded to the 1/CMC value, the effective concentration where micelles formation takes place. The signal of H10 was excluded from all calculations because it was less affected by the aggregation, being placed in the hydrophilic part of the micelle and not in the hydrophobic core.

The extrapolation of the intercept from the lines, obtained on 10 different signals of casopitant, allowed us calculating

Table 1
Partial ^{1}H NMR assignment in DMSO-d_6 and in two different extreme concentrations in D_2O glycine buffer (pH 3.5). Chemical shifts are referenced to residual solvent line (DMSO-d_5, 2.50 ppm) and to the glycine CH_2 resonance (3.590 ppm), respectively.



Casopitant mesylate	DMSO-d_6 (δ, ppm)	D_2O Gly pH 3.5 81.6 mM(δ, ppm)	D_2O Gly pH 3.5 0.016 mM (δ, ppm)
5	8.00	7.499	8.033
4	7.69	7.349	7.698
1	7.24	7.111	7.297
3	6.96	6.626	6.984
2	6.83	6.426	6.897
6	5.32	5.196	5.375
7	4.20	4.253	4.359
8	2.74	2.461	2.833
9	2.37	2.235	2.402
10	2.02	2.099	2.157
11	1.47	1.297	1.516

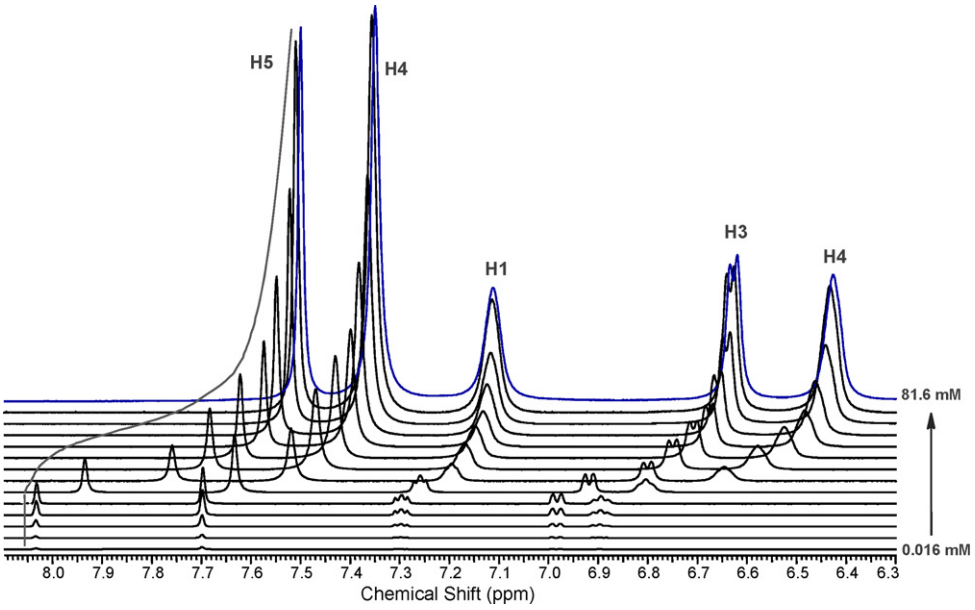


Fig. 2. Shielding trend for the aromatic protons of casopitant mesylate in the spectra collected at variable concentration.

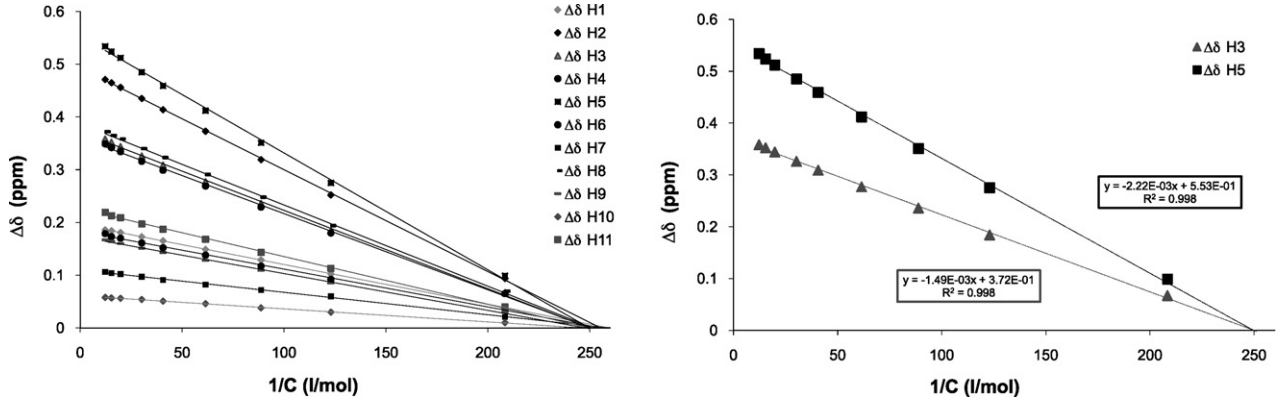


Fig. 3. Chemical shift variation ($\Delta\delta$, ppm) in function of the reciprocal of the concentration (l/mol) as measured for all resonances of interest (left), and linear regression for two representative resonances (right).

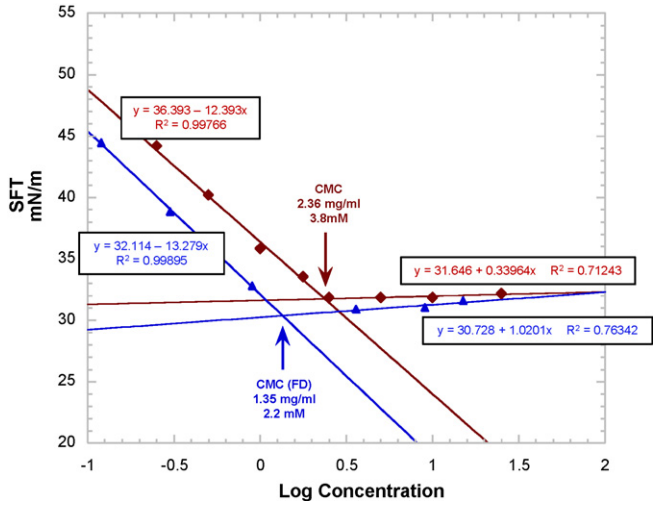


Fig. 4. Plot of CMC *versus* Log concentration for casopitant mesylate (▲) and casopitant for injection (■) at pH 3.5. The slope of the line extrapolated for the points at concentrations higher then CMC in the presence of salt results slightly increased, possibly due to the larger ionic strength in solution.

an average CMC at 25 °C of 3.96 ± 0.07 mM, corresponding to 2.82 ± 0.05 mg/ml of casopitant mesylate.

3.3. Measurement of CMC by surface tension and effect of electrolytes in formulation

The CMC for casopitant mesylate in the diluent (glycine buffer pH 3.5) has been determined by carrying out surface tension measurements on a series of solutions at different drug concentrations.

The tension is calculated using Eq. (1):

$$\delta = \frac{F}{Lx\cos\theta} \quad (1)$$

where δ is the surface or interfacial tension; F is the force acting on the balance; L is the wetted length; θ is the contact angle. The plate is made of roughened platinum and is optimally wetted so that the contact angle is virtually 0°. This means that the term $\cos\theta$ has a value of approximately 1, so that only the measured force and the length of the plate are needed to be taken into consideration.

The surface tension decreases linearly with the logarithm of the casopitant mesylate concentration up to the point where a further increase in concentration no longer has any appreciable influence on the surface tension. CMC is obtained from the intersection of the straight lines for the linear concentration-dependent section and the concentration-independent section (Fig. 4). A CMC value of 2.36 mg/ml corresponding to 3.8 mM has been obtained.

Similarly CMC for casopitant mesylate after reconstitution and dilution with saline has been measured (intravenous administration formulation). A lower CMC value has been obtained: 1.36 mg/ml corresponding to 2.2 mM. The significant reduction of the CMC with respect to the previously reported is here attributed to the effect of the presence of electrolytes (NaCl) [9].

3.4. Temperature dependence of the CMC by NMR spectroscopy

A selected number of solutions were chosen in order to verify how the CMC varies in function of the temperature. The concentration range was chosen from 81.595 to 0.479 mM as no relevant changes could be observed at lower concentrations in the set of experiments performed at 25 °C. Measurements were performed at 15 °C, 35 °C, 45 °C and the CMC was evaluated as previously described. Fig. 5 shows the difference in the slope of the lines used to measure the CMC for the four temperatures data sets, while results of CMC measured at different temperatures are reported in Table 2.

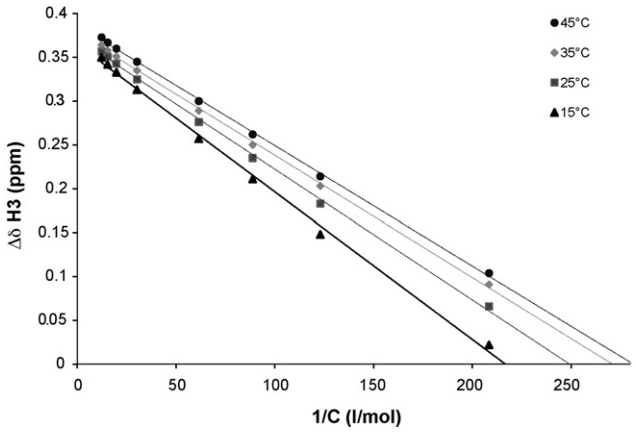


Fig. 5. Chemical shift variation ($\Delta\delta$, ppm) in function of the reciprocal of the concentration (l/mol). Example of slope variation at different temperatures for one representative resonance (H3).

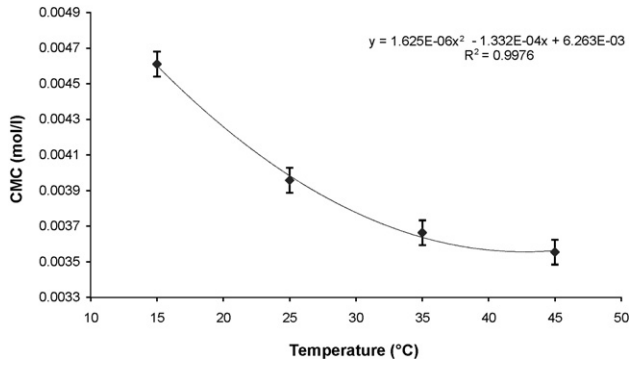


Fig. 6. CMC (mol/l) variation in function of the temperature (T, °C).

These data can be plotted in a CMC *versus* temperature graph leading to a "U" shaped trend (Fig. 6) [10]; consistent with what is described in the literature for various ionic surfactants. The polynomial equation

$$\ln(\mathrm{CMC}) = a + bT + cT^2 \quad (2)$$

where the constants a, b and c are determined by the least-squared regression analyses, can be used to correlate the CMC with the temperature and to predict a minimum CMC, which corresponds to 3.5 mM at 41 °C for casopitant mesylate.

The CMC values, as determined at various temperatures were further used for the evaluation of the thermodynamic parameters of micellization. In accordance with the phase separation model [11], the standard Gibbs free energy of micelle formation, ΔG°, is given by

$$\Delta G^\circ = \gamma RT\ \ln(\mathrm{CMC}) \quad (3)$$

Table 2
Average critical micelle concentration (CMC, mM) for casopitant mesylate, measured at four different temperatures, considering data deriving from chemical shifts of ten different ^{1}H NMR resonances.

Temperature (°C)	CMC (mM)
15	4.61 ± 0.06
25	3.96 ± 0.07
35	3.66 ± 0.06
45	3.55 ± 0.09

Table 3
Thermodynamic parameters of casopitant mesylate in D_2O glycine buffer (pH 3.5) at various temperatures.

Temperature (K)	γ	ΔG° (kJ mol^{-1})	ΔH° (kJ mol^{-1})	ΔS° (kJ mol^{-1} K^{-1})	$-T\Delta S^\circ$ (kJ mol^{-1})
288	1	−12.8815	−0.7374	4.2167×10^{-2}	−12.1441
	2	−25.7631	−1.4747	8.4335×10^{-2}	−24.2885
298	1	−13.7054	−0.8134	4.3262×10^{-2}	−12.8921
	2	−27.4108	−1.6269	8.6523×10^{-2}	−25.7839
308	1	−14.3670	−0.8946	4.3742×10^{-2}	−13.4725
	2	−28.7341	−1.7892	8.7483×10^{-2}	−26.9448
318	1	−14.9142	−0.9809	4.3815×10^{-2}	−13.9332
	2	−29.8284	−1.9619	8.7631×10^{-2}	−27.8667

where R is the gas constant, γ is the degree of counterion binding (if $\gamma = 1$, then the anti-ions are completely ionized; if $\gamma = 2$, then all the anti-ions are bound to micelles) and T is the temperature in the Kelvin scale.

The enthalpy of micellization can be obtained by applying the Gibbs–Helmholtz relation equation (4):

$$\Delta H^\circ = \gamma T^2 \frac{\partial(\Delta G^\circ/T)}{\partial T} = \gamma R T^2 \partial \ln \frac{(\mathrm{CMC})}{\partial T} \quad (4)$$

The enthalpy of micelle formation is then calculated numerically by substituting Eq. (2) into Eq. (4):

$$\Delta H^\circ = \gamma R T^2 (b + 2cT) \quad (5)$$

The entropy in the micellization process can be estimated from the calculated enthalpy and free energy values as:

$$\Delta S^\circ = \frac{(\Delta H^\circ - \Delta G^\circ)}{T} \quad (6)$$

The thermodynamic parameters of micellization obtained by applying above procedure are described in Table 3.

In the whole temperature range studied, the aggregation process is slightly exothermic ($\Delta H < 0$) and the observed trend is in agreement with an increase of the enthalpic contribution to the aggregation with the increase of the temperature. The free energy of micellization is negative over the whole range of measured temperatures measured in this study. This is in agreement with a spontaneous process of aggregation for casopitant mesylate in the aqueous media used for this study. It is finally evident that in the aggregation of casopitant mesylate, the micellization phenomenon is dominated by the entropic contribution (which counts for 93–94% of ΔG°).

4. Conclusions

The present study allows confirming that casopitant mesylate is a surface-active substance. The aggregation has been studied by both NMR spectroscopy and surface tension, allowing the determination of the critical micelle concentration. The two different techniques showed consistent results. The CMC was also determined by NMR spectroscopy at different temperatures, allowing the evaluation of the thermodynamic contribution to the phenomenon. Finally, surface tension measurement provided the confirmation of a relevant decrease of the CMC in the presence of electrolytes (formulation conditions), allowing a correct prediction of the tolerability of the compound formulated for intravenous administration.

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