Cyclodextrins as inhibitors of the precipitation of riboflavin-5'-phosphate due to presence of zinc chloride: a NMR investigation

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ABSTRACT

Several cyclodextrins (CDs) were probed in order to counteract the precipitation of riboflavin-5'-phosphate (or flavin mononucleotide, FMN-P) due to the presence of divalent cations, by exploiting Nuclear Magnetic Resonance (NMR) spectroscopy both for quantitative analyses and stereochemical characterizations. Among CDs, β-cyclodextrin (β-CD) showed the best solubilizing power in virtue of the formation of a 1 to 2 FMN-P/β-CD complex, the stereochemistry of which was ascertained by ROESY (Rotating-frame Overhauser Enhanced SpectroscopY) measurements.

Dedicated to Prof. Carlo Bertucci, Bologna, on the occasion of his 70th birthday.

KEYWORDS: Nuclear Magnetic Resonance; Riboflavin 5'-phosphate; Cyclodextrins; Complexation Phenomena; Solubility

1. Introduction

The role of cyclodextrins (CDs) as solubilizing agents is well recognized [1-4]. These cyclic oligosaccharides have a highly pre-organized structure endowed with an external hydrophilic surface, which is responsible for their solubility in aqueous medium, and a hydrophobic cavity inside which lipophilic molecules or molecular portions are included and hence driven in the aqueous medium. The solubilizing power of the host depends on several factors, such as the intrinsic aqueous solubility of the cyclodextrin, its structure and the suited fit between the host cavity and the guest sizes, which affect the strength of the drug-cyclodextrin interaction. In order to obtain more soluble cyclodextrins, simultaneously improving their ability to interact with a greater number of guest compounds, several β-CD derivatives have been developed for pharmaceutical uses, such as trimethylated β-cyclodextrin (TRIMEβ) and (2-hydroxypropyl)-β-cyclodextrin (HPβ-CD).

A recent investigation [5] dealing with vitamins formulations for parenteral nutrition has pointed out the propensity of riboflavin-5'-phosphate (FMN-P) to precipitate in the presence of divalent cations (Ca^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}). These effects were negligible at 0.1 mM concentration of the vitamin, but became remarkable with the rise of concentration. Even though the co-presence of selected vitamins could minimize precipitation [5,6], the use of alternative solubility promoters could be of interest for the development of homogeneous pharmaceutical formulations. Taking into account that both native and derivatized cyclodextrins have been employed for the solubilisation of riboflavin [7-9], we decided to probe the use of cyclodextrins as resolubilizing agents for contrasting the precipitation of FMN-P due the co-presence of divalent cations (Zn^{2+} in particular). To this aim we took into consideration native cyclodextrins (α-CD, β-CD and γ-CD, Fig. 1) and β-CD derivatives (HPβ-CD and TRIMEβ, Fig. 1), which find widespread applications in pharmaceutical field [3,10-12]. This investigation was performed by exploiting the remarkable potentialities of NMR both in the quantitative analyses of solubilisation processes and in the assessment of stereochemical features of supramolecular aggregates FMN-P/CDs, mainly relying on the use of DOSY (Diffusion Ordered SpectroscopY) [13-15] spectroscopy for the detection of complexation phenomena and ROESY [16] for the definition of the stereochemistry of the complexes formed in solution

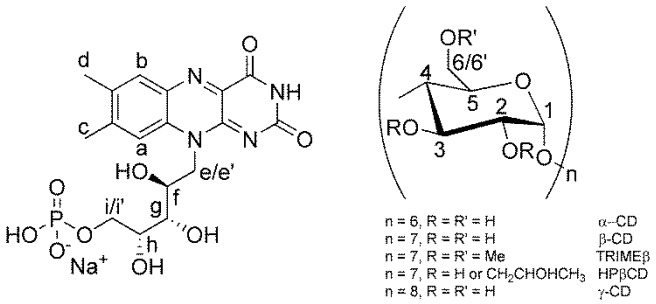

Fig. 1. Structure of FMN-P and CDs with numbering scheme for NMR analysis.

2. Materials and methods

2.1. Materials

Riboflavin-5'-phosphate, zinc chloride, α-cyclodextrin, β-cyclodextrin, γ-cyclodextrin, (2,3,6-tri-*O*-methyl)-β-cyclodextrin (TRIMEβ), and (2-hydroxypropyl)-β-cyclodextrin (HPβ-CD) were purchased from Sigma-Aldrich.

2.2. NMR measurements

NMR measurements were performed in D_2O on a spectrometer operating at 600 MHz for ^1H nuclei. The temperature was controlled to ±0.1 ˚C. The 2D NMR spectra were obtained by using standard sequences with the minimum spectral widths required. The 2D ROESY experiments were performed by employing a mixing time of 300 ms. The pulse delay was maintained at 1 s; 256 increments of 16 scans and 2K data points each were collected. DOSY experiments were carried out by using a stimulated echo sequence with self-compensating gradient schemes, a spectral width of 6600 Hz and 64K data points. Gradient strength was varied in 20 steps (16 transients each), while values of the diffusion delay and the gradient pulse duration were optimized to obtain an approximately 90–95% decrease in the resonance intensity at the largest gradient amplitude. The baselines of all arrayed spectra were corrected prior to processing the data. The data were processed with the DOSY macro (involving the determination of the resonance heights of all the signals above a pre-established threshold and the fitting of the decay curve for each resonance to a Gaussian function) to obtain pseudo two dimensional spectra with NMR chemical shifts along one axis and calculated diffusion coefficients along the other. ^1H NMR quantitative spectra were recorded by using a 45° pulse width (5.5 μs), with 15 s relaxation delay. The concentration of FMN-P was calculated based on selected FMN-P resonances by the qEstimate software (Agilent) and by comparing their integrated areas to those of a sample of FMN-P at known concentration.

A substitution degree of 0.99 was found for HPβ-CD on the basis of the comparison between the integrated area of the proton units of the glucosidic and 2-hydroxypropyl moieties, the first one being determined in anomeric protons spectral region and the latter one in methyl protons region.

3. Results and discussion

Preliminarily, the effect of the presence of the cyclodextrins on FMN-P protons chemical environment was evaluated by comparing the ^{1}H NMR spectra of pure FMN-P and its mixtures with CDs (1:1 ratio, 15 mM), in completely homogeneous solutions and, hence, in the absence of divalent cations. Table 1 collects the complexation shifts ($\Delta\delta = \delta_{mixture} - \delta_{free}$) for aromatic protons H_a and H_b and methyl protons H_c and H_d (Fig. 1).

Resonances of aromatic and methyl nuclei underwent high-frequencies shifts due to the presence of cyclodextrins (Table 1), the magnitude of which was significantly dependent on the macrocycle size, in accordance with an inclusion hypothesis: the cyclodextrin constituted by 6 glucopyranose units (α-CD) and the 8-unit γ-CD led to lower shifts of the FMN-P resonances in comparison to the 7-unit β-CD. Furthermore, the complexation shifts seemed to depend significantly on the polarity of the groups on the large and narrow edges: the TRIMEβ caused no significant complexation shifts, thus pointing out the role of hydrogen bond interaction involving the OH groups at the cyclodextrin rims. HPβ-CD led to enhanced variations (Table 1), however 2 equivalents of HPβ-CD were needed to reach the same effect given by one equivalent of β-CD.

Table 1

Complexation shifts ($\Delta\delta = \delta_{mixture} - \delta_{free}$, ppm) of selected protons of FMN-P (15 mM, 600 MHz, D_2O, 25 °C) in solutions FMN-P/CD (where not specified, the molar ratio FMN-P/CD is 1:1)

	Δδ			
	H_a	H_b	H_c	H_d
FMN-P/α-CD	+ 0.02	+ 0.03	+ 0.01	+ 0.01
FMN-P/β-CD	+ 0.06	+ 0.11	+ 0.04	+ 0.03
FMN-P/TRIMEβ	+ 0.01	+ 0.02	0.00	+ 0.01
FMN-P/HPβ	+ 0.04	+ 0.07	+ 0.02	+ 0.02
FMN-P/HPβ 1:2	+ 0.07	+ 0.12	+ 0.04	+ 0.03
FMN-P/γ-CD	+ 0.01	+ 0.03	+ 0.01	+ 0.01

In consideration of complexation shifts data, we then employed β-CD in the resolubilization of FMN-P in presence of zinc chloride. HPβ-CD was also tested because β-CD is not suitable for all pharmaceutical applications, an important exception being represented by parenteral administration [17]. Precipitation and resolubilization processes were detected by quantitative NMR on the basis of

the changes of the integrated areas of selected FMN-P resonances, by using as standard a solution of pure FMN-P at known concentration. Because of the limited solubility of β-CD in water (18.5 mg/mL), we selected a starting concentration of 1 mM for FMN-P, in order to allow the use of an excess of CD. During the first experiments, we found that precipitation of FMN-P due to the presence of the metal was a low rate reaction, therefore, in the following tests, we performed quantitative analysis after 24 hours from samples preparation, in order to allow them to equilibrate. In our previous work [5], we have already shown that at 1 mM concentration of FMN-P, one equivalent of zinc chloride causes the precipitation of about 40% of the vitamin and simultaneously leads to coalescence of the signals of aromatic protons H_a and H_b (Fig. 2b). The addition of 5 equivalents of HPβ-CD caused no significant effects, while the use of 5 eq. of β-CD (Fig. 2c) or 15 eq. of HPβ-CD allowed solubilisation of 10% of the vitamin. Finally, the addition of 15 eq. of β-CD (Fig. 2d) or 30 eq. of HPβ-CD caused resolubilization of the vitamin up to 90% of the original concentration. Thus, we can conclude that β-CD is an efficient solubilizing agent with respect to FMN-P; HPβ-CD is also a good solubilizing agent, but these tests confirmed that it should be used in double amount to obtain the same effects of β-CD.

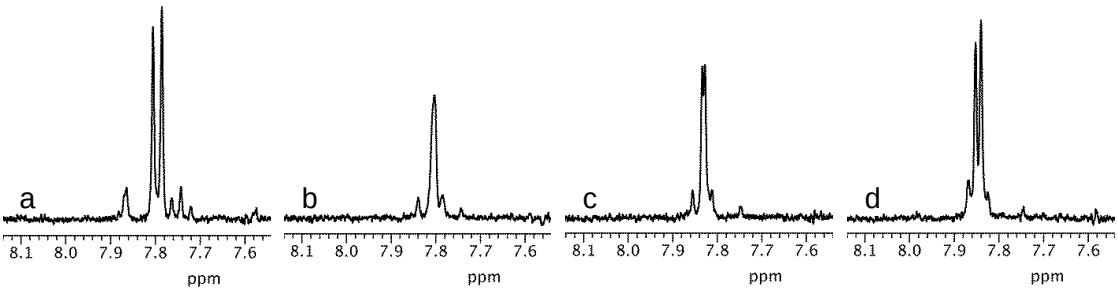

Fig. 2. ^{1}H NMR (600 MHz, D_2O, 25 °C) spectral regions of aromatic protons of FMN-P (1 mM) in the following solutions: a) FMN-P; b) FMN-P/$ZnCl_2$ 1:1; c) FMN-P/$ZnCl_2$ 1:1 + 5 eq. of β-CD; d) FMN-P/$ZnCl_2$ 1:1 + 15 eq. of β-CD

Once established that β-CD shows the best complexation efficiency with respect to FMN-P and represents a good solubilizing agent, we went deeply into the analysis of the nature of FMN-P/β-CD complex. Preliminary indications were afforded by the analysis of cyclodextrin resonances, among which the ones of H_3 and H_5 protons, which are respectively located in the large and small areas of the cavity, showed remarkable complexation shifts ($\Delta\delta$ = -0.04 ppm for H_3 and $\Delta\delta$ = -0.03 ppm for H_5). Regarding FMN-P, significant chemical shifts changes occurred for the methyl substituted ring (Table 1), in addition to shifts for protons $H_{e/e'}$ (+0.06 ppm) and H_f (+0.03 ppm), which are in proximity of the isoalloxazine ring, whereas ribityl side chain protons of FMN-P were nearly unaffected by the presence of the cyclodextrin. Above data were in favour of the hypothesis of an inclusion complex.

To gain further evidence of the occurrence of FMN-P/β-CD complexation, translational diffusion coefficient was detected, which is a size dependent parameter and can be measured by using the NMR DOSY technique. According to the Stokes-Einstein equation (Eq. (1)), which strictly holds in the spherical approximation, the diffusion coefficient is directly related to hydrodynamic radius r_H:

$$D = kT/(6\pi\eta r_H) \quad (1)$$

where k is the Boltzmann constant, T the absolute temperature, and η the solution viscosity. In the fast exchange condition of free and bound species in equilibrium, the observed parameter (D_{obs}) is the weighted average of its value in bound (D_b) and free (D_f) states (Eq. (2))

$$D_{obs} = x_b D_b + (1 - x_b) D_f \quad (2)$$

where x_b is the molar fraction of bound species. Thus, any kind of aggregation phenomenon, which increases the molecular sizes and hence r_H, is expected to bring about a decrease of the diffusion coefficient with respect to the pure components.

Here, the diffusion coefficient of FMN-P decreased from 2.9 x 10^{-10} m^2s^{-1} in the pure sample to 2.6 x 10^{-10} m^2s^{-1} in presence of one equivalent of β-CD. Analogously, the diffusion coefficient of β-CD decreased from 2.4 x 10^{-10} m^2s^{-1} to 2.2 x 10^{-10} m^2s^{-1}. Effects due to viscosity changes caused by the presence of the cyclodextrin were ruled out by adding 50 μL of DMSO/TMS solution and measuring the diffusion coefficient of TMS in the solution containing pure FMN-P and its mixture with the cyclodextrin. No changes in TMS diffusion coefficient were detected. Therefore the observed changes confirmed that an interaction between the two molecules takes place. However, a decrease of the diffusion coefficient of CD is not often observed in inclusion complex formation; in this case, it is probably due to the comparable sizes of the two compounds, but it could also be due to the fact that more than one unit of β-CD was involved in the complex formation. Thus, we decided to use Job's method [18-20] to determine the stoichiometry of FMN-P/β-CD complex. According to this method, different solution of the two compounds at constant total concentration, but different stoichiometric ratios, were analysed. Then, it is possible to obtain complexation stoichiometry by plotting observed variations of a specific parameter for one component, multiplied for its molar fraction, versus the molar fraction of the other component. In NMR spectroscopy, the chemical shift is commonly observed and we focused on proton H_3 of β-CD, which underwent the most significant complexation shifts in the mixtures. Samples analysed were 12 mM as total concentration. In the corresponding plot (Fig. 3), maximum lied in correspondence of 0.35 molar fraction of FMN-P: this was related to a prevailing 1 to 2 stoichiometry of FMN-P/β-CD complex.

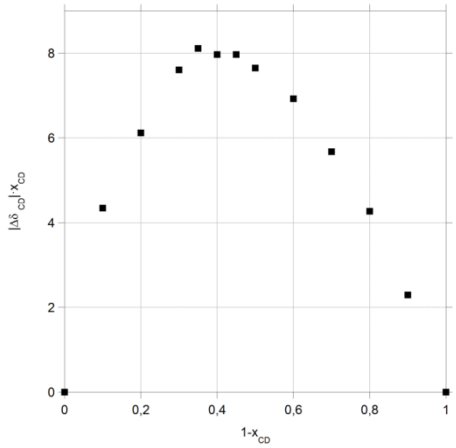

Fig. 3. Job's plots obtained from data analysis: H_3 proton of β-CD

Finally, we employed the NMR 2D ROESY technique, in order to obtain proximity constraints between FMN-P and β-CD protons due to intermolecular dipole-dipole interactions. In the 2D ROESY map of FMN-P/β-CD sample, we observed intermolecular ROEs generated by the methyl and aromatic protons of FMN-P at the frequencies of cyclodextrins protons (Fig. 4). In particular, both methyl protons H_d and H_c gave ROEs at the frequencies of internal protons H_3 and H_5 of the cyclodextrins, which were almost comparable in magnitude in the case of H_d, whereas the ROE H_c-H_3 was markedly more intense of the ROE H_c-H_5 (Fig. 4a). Analogously, proton H_b, which was adjacent to methyl H_d, showed intermolecular ROE at the frequency of H_3, greater than that detected in the case of H_a (Fig. 4b). Therefore, the analysis of ROEs confirmed the inclusion hypothesis; furthermore, it put in evidence how the aromatic moiety of FMN-P was deeply included into the hydrophobic cavity of β-CD with the H_d nuclei located near the narrow edge and the proton H_a located near the larger edge. Nuclei belonging to the ribityl side chain did not show any intermolecular ROE, indicating they were not involved in the inclusion complex formation, as expected from the presence of highly polar hydroxyl groups; this, however, does not exclude that hydroxyl groups themselves could interact, via hydrogen bonds, with hydroxyl groups of the same, or another, CD molecule.

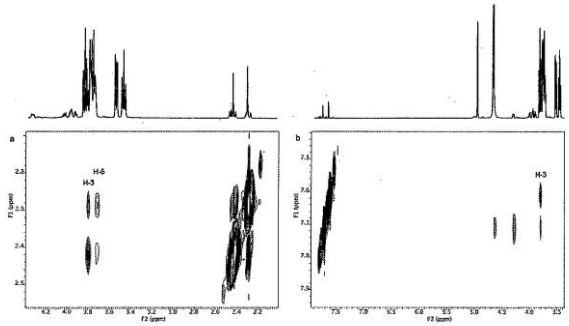

Fig. 4. Partial 2D ROESY (600 MHz, D_2O, 25 °C, mix. time 300 ms) spectrum of FMN-P/βCD 1:1 15 mM solution highlighting methyl (a) and aromatic protons (b) of FMN-P intermolecular ROEs.

Thus, the most reliable model for the FMN-P/β-CD 1:2 complex could be represented by the inclusion of the aromatic ring of the isoalloxazine structure into the hydrophobic cavity of β-CD (Fig. 5), as demonstrated by ROEs, and, reasonably, the contemporary non-inclusion interaction, via hydrogen bonds, between the ribityl side chain of FMN-P and the hydroxyl groups of a second molecule of CD.

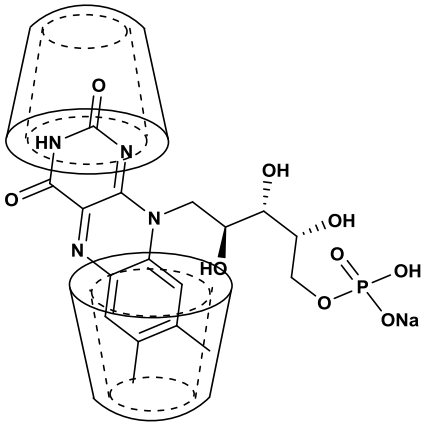

Fig. 5. Representation of the 1:2 complex FMN-P/βCD 1:1 according to ROE data.

4. Conclusions

Our results confirmed the leading role of NMR spectroscopy in the analysis of the molecular basis of host-guest supramolecular aggregation phenomena, on which rely the use of cyclodextrins as solubility promoter excipients. β-CD and HPβ-CD have shown the best complexation ability towards FMN-P and the comparison with CDs having a different number of glucopyranose units has demonstrated that the interaction process essentially depends on the sizes of the CDs cavities, as well as the polarity of groups located on the wide and narrow edges of the host molecule. Riboflavin-5'-phosphate, therefore, is included into the lipophilic cavities of β-CD and hence subtracted to the interaction with divalent cations, which is responsible for its precipitation.

Acknowledgements

The work was supported by University of Pisa (PRA 2016 "Functional Materials").

Appendix A. Supplementary Data

^{1}H NMR spectra of FMN-P, β-CD and FMN-P/β-CD.

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