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Interaction between nitroheterocyclic compounds with β-cyclodextrins: Phase solubility and HPLC studies

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ABSTRACT

Chagas disease is a serious health problem for Latin America. Nitrofurazone (NF) and Hidroxymethylnitrofurazone (NFOH) are active against *Trypanosoma cruzi*. The effect of β-cyclodextrin (β-CD) and dimethyl-β-cyclodextrin (DM-β-CD) complexation on the UV absorption and retention time of nitrofurazone (NF) and its hydroxymethylated analog (NFOH) were studied in solution. The retention behavior was analyzed on a reversed phase C_{18} column and the mobile phase used was acetonitrile-water (20/80 v/v), in which cyclodextrins (β-CD or DM-β-CD) were incorporated as a mobile phase additive. The decrease in the retention times of NF (or NFOH) with increasing concentration of HP-β-CD enables the determination of the complex stability constants by HPLC. A phase-solubility study was performed, according to the method reported by Higuchi and Connors, to evaluate the changes of NF/NFOH in the complexation state, and the diagrams obtained suggested that it forms complexes with a stoichiometry of 1:1. This is an important study for the characterization of potential formulations to be used as therapeutic options for the treatment of Chagas disease.

1. Introduction

Chagas disease affects about one quarter of the Latin America population. According to the World Health Organization, there are about 120 million people living in risk of contracting parasitosis and 16–18 million people infected with the parasite [1]. The main problem with the treatment is the resistance of *Trypanosoma cruzi* to drugs [2].

New candidate drugs have been proposed for Chagas disease chemotherapy and nitroheterocyclic compounds have been tested as antichagasic drugs to face this serious health problem in Latin America. Nitrofurazone (NF, 5-nitro-2-furaldeyde semicarbazone, Fig. 1) was synthesized based on the knowledge that furoic acid, as well as its derivatives, demonstrates antimicrobial activity, being active against Gram-positive and Gram-negative bacteria [3]. However, its high toxicity has precluded its use in systemic infections [4]. In contrast, a report has shown that nitrofurazone is also able to destroy *T. cruzi* through the inhibition of trypanotione reductase, an enzyme found in the parasite but not in the host [5].

Hydroxymethylnitrofurazone (NFOH, Fig. 1) is one of the new candidate drugs for Chagas disease chemotherapy that showed to be, *in vitro*, very potent against *T. cruzi* [6].

In the development of pharmaceutical products, β-cyclodextrins, a category of pharmaceutical excipients, have been widely used to improve solubility, chemical stability and bioavailability of a number of poorly soluble compounds.

Cyclodextrins (CD) are cyclic oligosaccharides composed of glucopyranose units and can be represented as a truncated cone structure with a hydrophobic cavity [7]. The cavity of CDs is relatively hydrophobic compared to water, while the external faces are hydrophilic [8]. The most extraordinary characteristic of a cyclodextrin is its ability to form inclusion complexes with a variety of compounds, i.e., caging foreign molecules (guest) through their interaction with the CD cavity in aqueous solution. It has been well established that the ability of β-cyclodextrins to enhance the stability and solubility of drugs is mediated through the formation of inclusion complexes [9]. Unmodified or unsubstituted β-cyclodextrin, that is, with no substituent on the glucopyranose unit, presents poor water solubility and is parenterally unsafe due to its nephrotoxicity. Therefore, several synthetically modified and relatively safe β-cyclodextrin, such as hydroxypropyl-β-cyclodextrin, sulfobutylether-β-cyclodextrin,

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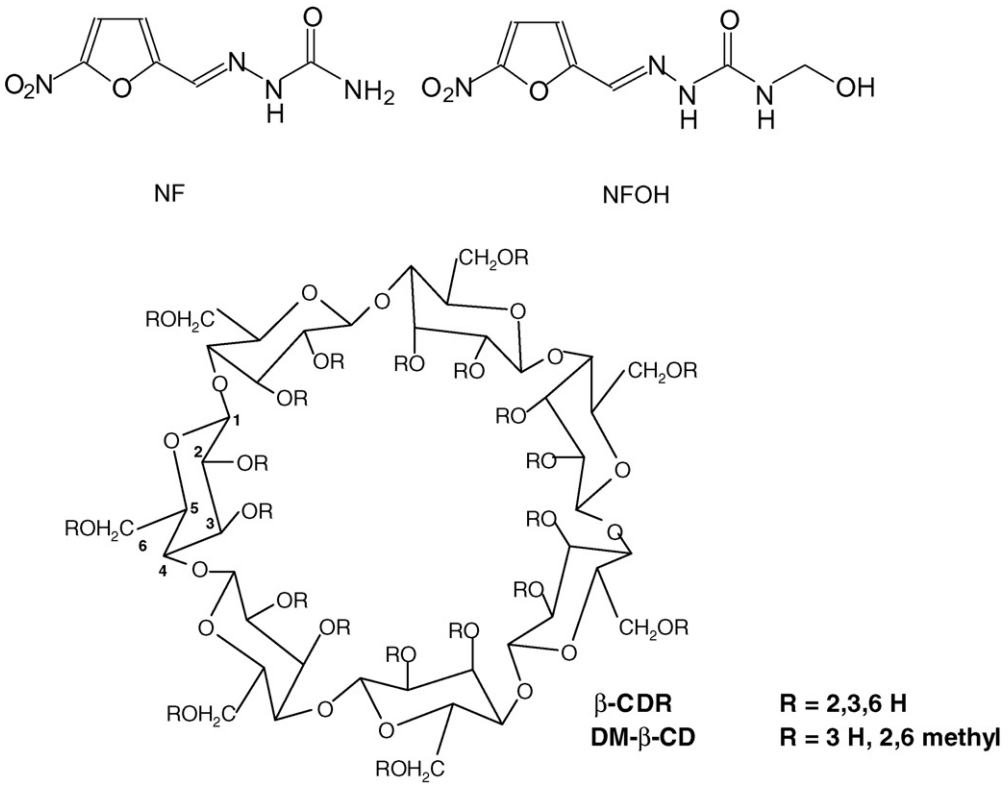

Fig. 1. Chemical structure of NF, NFOH and schematic representation of β-cyclodextrins.

dimethyl-β-cyclodextrin, have been produced and used in parenteral formulations [10,11].

In a previous work, we have investigated the interaction between NF [12] and NFOH [13] with 2-hydroxypropyl-β-CD. These studies showed that the formation of the inclusion complex changed the solubility, release profile and the photo stability of both NF and NFOH.

The aim of the present study was to characterize the inclusion complex formed between NF or NFOH and cyclodextrins (β-CD and DM-β-CD) through the study of the HPLC retention behavior of NF or NFOH in presence of cyclodextrins and through phase-solubility isotherms studies. This is an elementary study for the characterization of a potential formulation to be used as a therapeutic option for Chagas disease.

2. Experimental

2.1. Reagents and chemicals

Nitrofurazone and Hydroxymethylnitrofurazone were synthesized as previously described [6]; β-CD and randomly methylated-β-CD (degree of substitution ~1.7–1.9) were obtained as a gift from Roquette. HPLC-grade acetonitrile (ACN) was obtained from J.T. Baker and deionized water at 18 mΩ from a Waters ultra pure water system. The solutions were filtered trough 0.22 μm Millipore® nylon membrane filter (Belford, USA).

2.2. Effect of cyclodextrin in NF/NFOH retention time by HPLC

The chromatographic experiments were performed using a Shimadzu SCL-10VP controller pump, a Shimadzu SIL-10AD VP auto injector, a UV–vis SPD-10A VP detector (detection: 260 nm for NFOH) and Class-VP 6.12 as software. A reversed phase Phenomonex Gemini C_{18}, 5 μm, 10 cm × 0.46 cm was employed. The mobile phase used for these studies was acetonitrile–water (20/80 v/v), in which cyclodextrin was dissolved (0, 5, 10, 15, 20, 30 mM). The whole solution was filtered through a 0.2 μm pore size nylon membrane filter. The mobile phase was pumped at a flow rate of 1.0 mL/min. The chromatographic experiments were carried out at 25 °C. The NF/NFOH concentration in the injected solution was 60 μM and the injection volume was 0.2 mL in all experiments.

The retention behavior of NF/NFOH is governed by the drug partition coefficients between the mobile and stationary phases. In the presence of cyclodextrins, there is an additional contribution to the drug retention behavior due to the complexation process.

The capacity factors for NF/NFOH were monitored in the presence of increasing concentration of cyclodextrin. The stability constant of the complex, *K*, was determined in triplicate, using Eq. (1) [14]:

$$\frac{1}{k'} = \frac{1}{k'_s} + \frac{K[\mathrm{CD}]^x}{k'_s} \quad (1)$$

where k' is the capacity factor at each cyclodextrin concentration [CD], and k'_s is the solute capacity factor in absence of cyclodextrin. For a 1:1 stoichiometry complex, a plot of $1/k'$ versus [CD] yields a straight line and K is obtained from the slope-to-intercept ratio.

2.3. Determination of the stability constants

Excess amounts of NF/NFOH were added to 10 mL glass tubes containing different concentrations of cyclodextrin. The tubes were shaken until equilibrium was reached (32 h) at 25 °C. Then, the solutions were centrifuged at 25 °C and the concentration of NF/NFOH was spectrophotometrically determined at 260 nm using a Femto spectrophotometer. The presence of cyclodextrin did not interfere in the spectrophotometric assay of NF/NFOH. Deionized water was

Table 1
Apparent stability constants, *K*, for NF and NFOH with cyclodextrins inclusion complexes determined from HPLC study, 25 °C

Inclusion complex	Apparent stability constant (M^{-1})	Correlation coefficient, *r*	Slope	Intercept
NF:b-CD	2.0 ± 0.3	0.997	2.52	1.241
NF:DM-b-CD	16.8 ± 0.7	0.998	19.9	1.186
NFOH:b-CD	1.6 ± 0.2	0.997	1.57	0.945
NFOH:DM-b-CD	8.6 ± 0.1	0.998	8.03	0.924

used as the background during the spectrophotometric determination of NF/NFOH concentration.

When a linear relationship between the solubility of NF/NFOH and the concentration of cyclodextrin is obtained, the diagram is classified as A_L, according to Higuchi and Connors [15] and the experimental data fit equation:

$$K = \frac{\text{slope}}{S_o(1 - \text{slope})} \quad (2)$$

where S_o is the molar solubility of NF/NFOH. The stability constant of the complex formed, *K*, can be obtained from the slope of the straight line.

A very reliable method for evaluating the solubilizing potential of cyclodextrins is to determine the complexation efficiency (CE), which refers to the complexed/free cyclodextrin ratio and that can be obtained from the slope of their phase-solubility profile, according to Eq. (3) [16,17].

$$\text{CE} = S_o K_{1:1} = \frac{[\text{D/CD}]}{[\text{CD}]} = \frac{\text{slope}}{1 - \text{slope}} \quad (3)$$

where [D/CD] is the concentration of dissolved complex, [CD] is the concentration of dissolved free cyclodextrin. CE values can be used to calculate the D:CD ratio, according to Eq. (4) [16,17]:

$$\text{D} : \text{CD} = 1 : \left(1 + \frac{1}{\text{CE}}\right) \quad (4)$$

3. Results and discussion

3.1. Chromatographic determination of the stability constant

When cyclodextrins are added to the mobile phase, solute retention is driven by the drug partition between the mobile and stationary phases and the solute complexation with cyclodextrins. According to the solute retention time and the void time, capacity factors were calculated for each solute in the presence of increasing concentrations of cyclodextrins. As expected, the retention times decreases as the concentration of cyclodextrin in the mobile phase increases due to the formation of the analyte–cyclodextrin complex, which enhances the guest solubility in the mobile phase and reduces its residence time in the column [18,19]. An assay is shown as an example of NFOH in the presence of DM-β-CD (Fig. 2, variation in the retention times obtained by replication was always lower than 1%).

The above-mentioned formation constant for NF or NFOH:cyclodextrin complex was calculated according to Fig. 3(a) and (b) and Eq. (1). The linear relationship between $1/k'$ and the cyclodextrin concentration (Fig. 3) with correlation coefficient higher than 0.99 indicates that the behavior of NF/NFOH is well described by the model, assuming a 1:1 stoichiometry between the guest and cyclodextrins [20,21].

The stability constants obtained for NF/NFOH with cyclodextrins are shown in Table 1.

As can be observed in Table 1, the stability constant of NF (or NFOH) with β-CD is lower than the observed for DM-β-CD; this result is reported in literature for other drug:cyclodextrins systems [22,23]. The differences in stability constants observed between NF and NFOH with cyclodextrins can be associated with physicochemical properties of these molecules, because, hydroxymethyl derivatives as NFOH (S_o = 0.992 mg/mL and log *P* 0.19) is more hydrophilic than NF (S_o = 0.657 mg/mL and log *P* 0.23) [6]. The same behavior was described in the literature for the inclusion complex between NF and NFOH with 2-hydroxypropyl-β-CD [12,13].

3.2. Determination of the stability constant by solubility isotherm

All phase-solubility diagrams of NF or NFOH with β-CD and DM-β-CD, within the concentration range studied, displayed a typical A_L type diagram (i.e., linear increase of NF or NFOH solubility with increasing cyclodextrin concentration), consistent with a 1:1 molecular complex formation for all cyclodextrins (Fig. 4). The result observed showed a linear behavior which is unequivocal for all CDs studied (r = 0.996 or better). The binding constant, *K*, of the complexes was calculated from the slopes of the linear phase-solubility plots according to the methodology described before. Results are summarized in Table 2.

As shown in Table 1 and Fig. 3 (and Table 2 and Fig. 4), the binding constant determined for both compounds (NF and NFOH) with the CDs followed the rank order: DM-β-CD > β-CD, reflecting an enhancement on binding and solubility with the presence of methylated groups, since these groups seems to be important for the binding of these compounds in the cyclodextrin cavity, as described in literature [22–24]. The correlation between the stability constant for NF/NFOH in DM-β-CD and β-CD and values determined for the interaction of these compounds with HP-β-CD [12,13] show that the association constant *K* increases in the order HP-β-CD < β-CD < DM-β-CD. The complexation ability is significantly enhanced by methylation, which enlarges the CD cavity, makes its environment more hydrophobic and favors the adaptability of the CD towards a guest, through an enhanced flexibility [25]. The same results have been reported in literature for the interaction of nitroimidazole compounds with these cyclodextrins [23].

The differences of *K* measured by HPLC and phase-solubility isotherm (for NF and NFOH) might be explained by the amount of acetonitrile present in the mobile phase of the HPLC method and the time needed by the complex formation to reach equilibrium. It is

Table 2
Apparent stability constants, *K*, for NF and NFOH with cyclodextrins inclusion complexes determined by phase-solubility techniques, 25 °C

Inclusion complex	Apparent stability constant (M^{-1})	Correlation coefficient, *r*	Slope	Intercept, S_o ($\times 10^{-3}$)	CE	Drug:CD ratio
NF:β-CD	27.0 ± 2.5	0.996	0.0296	1.128	0.028	1:36
NF:DM-β-CD	72.6 ± 1.5	0.998	0.0859	1.294	0.094	1:11
NFOH:β-CD	24.7 ± 2.8	0.998	0.0342	1.434	0.035	1:29
NFOH:DM-β-CD	58.6 ± 1.6	0.997	0.0783	1.450	0.084	1:12

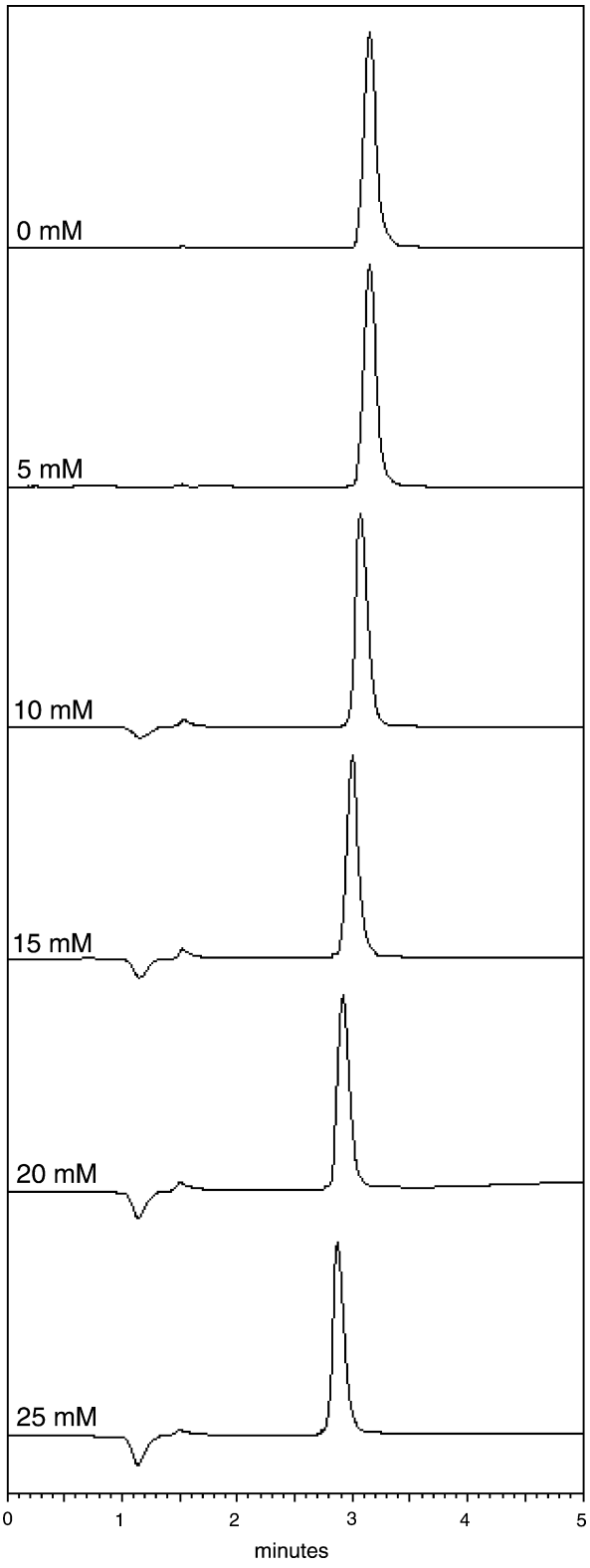

Fig. 2. Decrease in NFOH retention time in the presence of increasing concentrations of DM-β-CD (0, 5, 10, 15, 20, 25 mM) at 25 °C. Chromatographic conditions—column: Phenomenex C_{18}, 5 μm, 10 cm × 0.46 cm; mobile phase: acetonitrile/water (20/80 v/v).

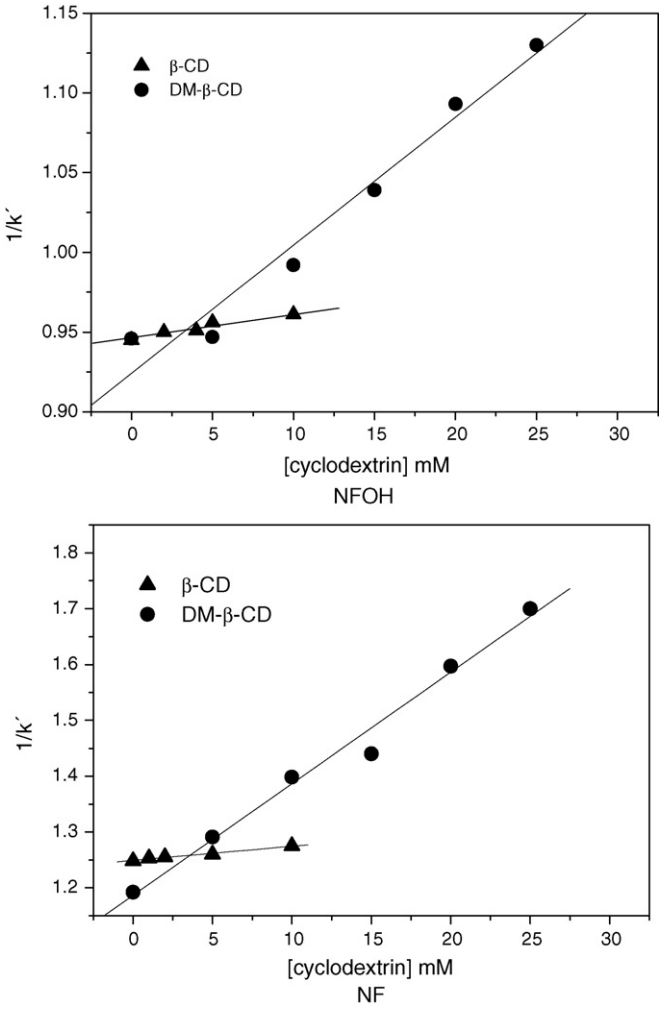

Fig. 3. Plot of 1/k' vs. [cyclodextrin] (β-CD and DM-β-CD) for NFOH and NF at 25 °C. Chromatographic conditions as described in Fig. 2.

well known that the addition of acetonitrile or methanol may have a negative effect on complex formation with cyclodextrin. For these studied systems, it seems that the addition of organic solvents led to a decrease in the binding constants. There are several factors which may contribute to this decrease: firstly, the amount of organic solvent results in a less polar mobile phase, in which the non-polar solutes become more soluble and as a consequence, the solute affinity for the hydrophobic cavity of cyclodextrins diminishes and part of the driving force for inclusion is removed. Secondly, a phenomenon of competition between the solute and the organic solvent for binding cyclodextrins may occur, even though organic solvent binds weakly to cyclodextrins [26]. However, instead the difference in size order of the K values determined by HPLC and solubility isotherm for the inclusion complex between NF/NFOH with cyclodextrins, the data present a good correlation coefficient ($r > 0.9$) when the K values determined by HPLC was plotted against the K values determined from solubility isotherm.

A more accurate method to determine the solubilization efficiency of cyclodextrins is to measure the complexation efficiency (CE), i.e., the concentration ratio between cyclodextrin in a complex and free cyclodextrin, and to study the influence of different pharmaceutical excipients on the solubilization [17,27].

From Eqs. (3) and (4) the CE values and drug:cyclodextrin ratios for the NF/cyclodextrins and NFOH/cyclodextrins inclusion complex were determined, as shown in Table 2 [17,27]. The high CE

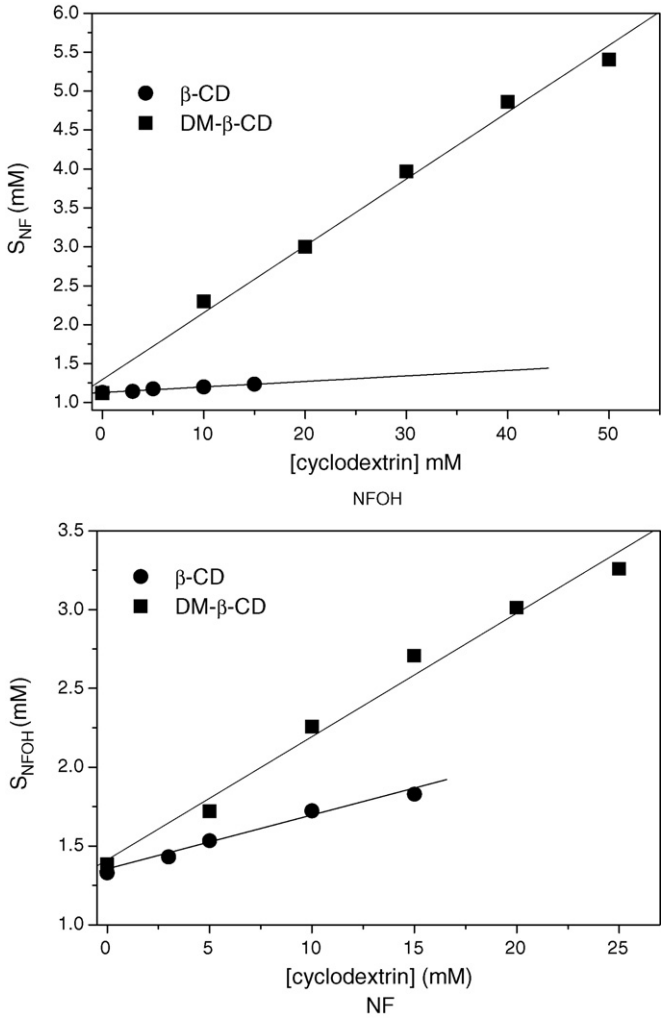

Fig. 4. Phase-solubility diagram of NF and NFOH with cyclodextrins (β-CD and DM-β-CD) at 25 °C.

values obtained for DM-β-CD indicate that this cyclodextrin is a better solubilizer than β-CD, as described by the authors [17,27].

In addition, drug:cyclodextrin ratios of 1:12 and 1:11 were calculated for NF and NFOH:DM-β-CD inclusion complex, respectively indicating that, on a 1:1 (NF/NFOH:DM-β-CD) complexation, just one out of the number of DM-β-CD molecules (12 for NF and 11 for NFOH) was forming an inclusion complex with drug [17,27].

4. Conclusions

This study showed the physicochemical characterization for the inclusion complex between NF, NFOH and two different β-cyclodextrins (β-CD and DM-β-CD). The results indicated that stable drug complexes were prepared at 1:1 molar ratio and that the complexation is driven by the physical chemical properties of the drug molecules. The characterization was investigated by the analysis of stability constants determined by two different methods (solubility isotherm and by HPLC). This study provides perspectives for future experiments using this inclusion complex of NF/NFOH with cyclodextrins in order to verify its therapeutic efficacy.

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