PRELIMINARY PREFORMULATION STUDIES FOR A NEW NORFLOXACIN RUTHENIUM (III) COMPLEX WITH BIOLOGICAL ACTIVITY

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Abstract

Most of the newly synthesized compounds with biological activity show a low solubility in water, which is inconvenient for the pharmaceutical formulation process. The present study is intended to evaluate the possibilities to improve the solubility of a new biologically active complex formed by Ru(III) with an antimicrobial drug, norfloxacin. Three strategies have been used in order to achieve an enhancement of solubility: pH adjustment/salt formation, use of co-solvents, forming of cyclodextrin inclusion complexes. The solubility profile of $Ru(nf)_2Cl_3(DMSO)\cdot H_2O$ was studied in the pH range 4.4 – 9.4 and shows that its solubility is almost unaffected in acidic medium, but increases in basic solutions due to the soluble salt formation. Solubility was increased also in binary systems water – biocompatible organic co-solvent (propylene glycol, polyethylene glycol 400), as well in a ternary system water – propylene glycol – ethanol. In the experiments using cyclodextrins, the phase-solubility diagrams indicated an enhancement of the complex solubility in the presence of beta-cyclodextrin, as well as its derivative, sulfobutylether-beta-cyclodextrin.

Rezumat

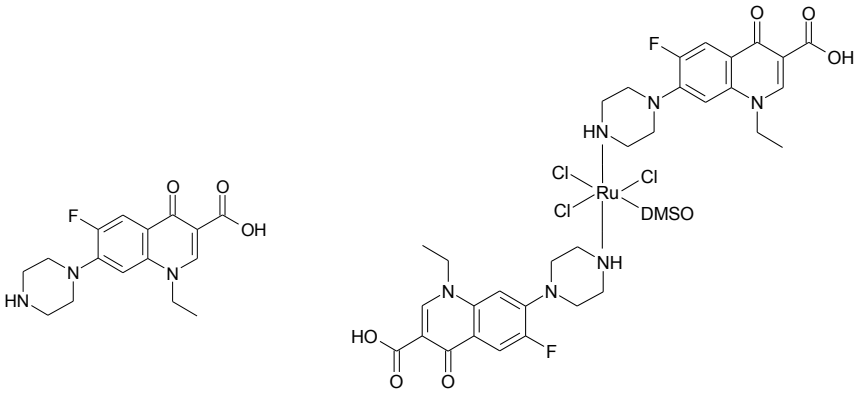
Cei mai mulţi dintre compuşii nou sintetizaţi cu activitate biologică prezintă o solubilitate scăzută în apă, ceea ce constituie un inconvenient în procesul de formulare farmaceutică. Studiul de faţă are ca scop evaluarea posibilităţilor de îmbunătăţire a solubilităţii unui nou complex biologic activ format de Ru(III) cu agentul antimicrobian norfloxacin. Au fost folosite trei strategii cu scopul de a obţine o îmbunătăţire a solubilităţii: ajustarea pH-ului/formarea de săruri, utilizarea co-solvenţilor şi formarea complecşilor de incluziune cu ciclodextrină. Profilul solubilităţii complexului $Ru(nf)_2Cl_3(DMSO)\cdot H_2O$, studiat în intervalul pH 4,4 - 9,4, arată că solubilitatea acestuia nu este afectată în mediu acid, însă creşte în mediu bazic datorită formării de sare solubilă. Solubilitatea a fost crescută, de asemenea, în sisteme binare sau ternare apă–cosolvent organic biocompatibil (propilenglicol, polietilenglicol 400, glicerol, etanol). În cazul

utilizării ciclodextrinelor, diagramele de fază de solubilitate au indicat o îmbunătăţire a solubilităţii complexului atât în prezenţa beta-ciclodextrinei, cât şi a derivatului acesteia, sulfobutileter-beta-ciclodextrină.

Keywords: ruthenium (III)-norfloxacin complex, solubility enhancement

Introduction

Norfloxacin [1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazin-1-yl)-quinoline-3-carboxylic acid] (nf, Figure 1a) is a typical fluoroquinolone antimicrobial agent which acts by inhibiting DNA gyrase and topoisomerase IV, two enzymes involved in bacterial DNA synthesis [6, 8].



(**a**) (**b**)

Figure 1
Structure of norfloxacin (a) and its complex with Ru(III) (b)

The acid-base properties and the coordinating capacity of norfloxacin are the most exploited properties in various chemical studies.

Norfloxacin molecule is a zwitterion: the weak acidic function is due to the carboxylic group (pKa 6.30) and the weak basic function, to the piperazinyl ring (pKa 8.38) [16].

On the other hand, most commonly norfloxacin coordinates metallic cations in complexes as a bidentate ligand [21], through the pyridone and carboxylate oxygen atoms. Considerable more rarely, through the nitrogen atoms in the piperazine ring norfloxacin acts as a monodentate ligand in complexes with Ag(I) [9], [15] and Au(III) [15], [11] or a bidentate ligand, with the piperazine ring in boat conformation, stabilized by chelation [4].

In reaction with Ru(III) and dimethyl-sulfoxide (DMSO), norfloxacin (nf) is a monodentate ligand and coordinates Ru(III) through the

terminal piperazinyl nitrogen atom thus generating a complex, $Ru(nf)_2Cl_3(DMSO)\cdot H_2O$ (Fig. 1b), with biological potential as a good antimicrobial agent on *Staphylococcus aureus* methicillin resistant (MRSA), *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Bacillus subtilis* [22] and antitumor activity [5].

Development of different methods to enhance solubility of poorly water soluble drugs is an innovative approach, which solves the problems of solubility and dissolution rate as limiting factors in drug bioavailability and provides a quick onset of the therapeutical action. Ensuring efficient solubilization of poorly soluble substances is a challenging task for researchers, especially for pharmaceutical scientists.

The aim of this work was to investigate some of the well-established pre-formulation strategies to enhance solubility and bioavailability of the new Ru(III)-nf complex. The most commonly used techniques to solubilize poorly soluble drugs are pH adjustment/salt formation, co-solvency and cyclodextrin complexation.

Given the structure of the Ru(III)-norfloxacin (Ru(III)-nf) complex, in which most part of norfloxacin molecule remains unchanged, solubilization by salt formation is an effective method of increasing solubility [18], applied especially for the development of liquid formulations for parenteral administration [20].

The solubility of a poor water soluble drug using organic co-solvents is a widespread formulation approach [12, 17]. The most frequently used low toxicity co-solvents for parenteral use are propylene glycol, ethanol, glycerin and polyethylene glycol [19].

Solubilization through complex formation often refers to the use of cyclodextrins for enhancing the solubility. Hydrophilic cyclodextrins (methyl, hydroxyl-propyl, sulfo-alkylated and sulfated derivatives of natural cyclodextrins), with high water solubility and low renal toxicity are preferred for pharmaceutical use [3], [13], 19].

Materials and Methods

The Ru(III)-nf complex was prepared according to previously reported procedure [1]: a DMSO solution of ligand and $RuCl_3$ in a 2:1 molar ratio was heated under reflux for 6 h. The solution was turned into dark-brown. After cooling, a solution 2 M of NaCl has been added in order to obtain the solid product. The brown residue was filtered off and washed several times with distilled water and dried in air.

Water solubility of the newly synthesized Ru(III)-nf complex is very low (S=0.105 mg/mL), for which reason a solubilization enhancement

technique is required in order to provide a sufficient amount of active substance to be available at the absorption site.

A. Solubility determination in aqueous phosphate buffers with different pH values

NaCl (Merck KgA, Germany), NaH_2PO_4 (Merck KgA, Germany) and NaOH (Merck KgA, Germany), used for buffer preparation were of analytical grade.

50 mM phosphate buffer solutions were prepared using $NaH_2PO_4 \cdot 2H_2O$ and NaOH 1 M solution with 100 mM NaCl added. The pH values (4.4; 5.4; 6.4; 7.4; 8.4 and 9.4) were adjusted under potentiometrical control, using a Consort C830 potentiometer.

Solubility was determined by placing an excess quantity of solid complex in a vial along with the solvent (aqueous phosphate buffer). The tightly closed vials were vigorously shacked for 30 seconds, maintained under sonication for 30 minutes at 30°C in a SonoSwiss thermostated ultrasonic bath, then stirred on a Heidolph Vibramax 100 stirrer for 24 hours at 30±2ºC. The solutions were filtered through a 0.45 μm filter and the filtrate was analyzed for drug content on an UV/Vis spectrophotometer (Jasco–V650) after appropriate dilutions, whenever it was necessary. Solubility was calculated using molar absorptivity values for each pH values, using the absorbance of a solution prepared by hundredfold dilution with buffer of a solution 1mg/mL of complex dissolved in DMSO.

B. Solubility determination in co-solvent mixtures

Dioxane and polyethylene glycol 400 (PEG 400), were purchased from Merck KgA, Germany. The HPLC grade ethanol (ETOH), glycerin (GLY) and propylene glycol (PG) were obtained from Sigma-Aldrich Chemie GmbH, Germany.

All chemicals were of analytical grade.

Preparation of co-solvent mixtures and evaluation of DR

The new synthetised Ru(III)-nf complex has a very low solubility which can be improved by addition of an organic co-solvent to water. This process is known as cosolvency and the solvent used to increase solubility is known as co-solvent.

In practice, to approximate the solubility of compounds in aqueous co-solvent systems, the dielectric requirement (DR) value is necessary. For this reason water and different co-solvent mixtures were prepared.

In the preliminary studies we used dioxane as test co-solvent. The selection of dioxane as test co-solvent is based on the extreme value of dielectric constant for the two solvents in pure state (ε_{water}=78 and

$\varepsilon_{dioxane}$=2). This co-solvent system is a versatile model for studying drug solubility.

For DR value determination, three different co-solvent systems water/dioxane were prepared. The solubility of the Ru(III)-nf complex in each system was evaluated by using the shake-flask method: 10 mL of each co-solvent system was added to an excess of solid complex, using 10 mL volumetric flasks (all determinations were performed in triplicate). The vials were vigorously shacked for 30 seconds, and maintained under sonication for 30 minutes, at 50°C, by means of a SonoSwiss thermostated ultrasonic bath. The samples were placed for 24 hours on a Heidolph Vibramax 100 stirrer, at 200 rpm. The samples were furthermore centrifuged and the supernatant was filtered through a cellulose-esther membrane with 0.45 µm average pore size.

Ru(III)-nf concentrations were determined using the absorbances measured at 282 nm (Figure 2) after appropriate dilutions with water/dioxane mixture and the previously constructed calibration curve (in the 1-20 µg/mL range). An UV-Vis Jasco – V650 apparatus was used.

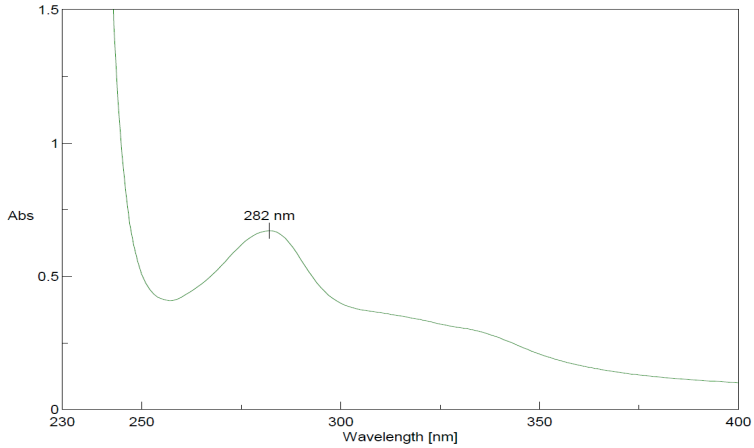


Figure 2

UV absorption spectrum of a 10 µg/mL standard solution of Ru(III)-nf complex in 1/1 (v/v) water/dioxane mixture

The value of ε_{app} for a binary system was evaluated according to the equation (1):

$$x_1\varepsilon_1 + x_2\varepsilon_2 = \varepsilon_{app} \quad (1)$$

where: x_1, x_2 are the molar ratios of the solvents (water, and dioxane, respectively), ε_1, ε_2 are the dielectric constants of the solvents.

The dielectric requirement (DR) for Ru(III)-nf complex represents the apparent dielectric constant (ε_{app}) of the system corresponding to components ratio that provides its maximum solubility.

Solubility determinations

It is important to note that for the same DR value, the solubility remains the same regardless of co-solvent used.

Because dioxane is highly toxic for pharmaceutical use, it was substituted with the most frequently used low-toxicity co-solvents: polypropylene glycol (ε=25), polyethylene glycol 400 (ε=19), glycerol (ε=43) and ethanol (ε=32). The water:co-solvent ratio in the resulting systems was calculated according to equation (1), in order to obtain a system where the DR value is respected.

The solubility of Ru(III)-nf complex in each biocompatible system was established by using the same experimental protocol used for evaluation of DR value.

All of the experiments were carried out in triplicate.

C. Solubility determination in the presence of cyclodextrins

Beta-cyclodextrin (β-CD) was purchased from Fluka (Sigma-Aldrich Chemie GmbH, Germany) and sulfobutylether-beta-ciclodextrin (SBE-β-CD) from Cydex, USA. The phosphate buffer solutions (pH 4.4; 7.4 and 9.4) were prepared following the above described procedure.

Three sets of six solutions each, with increasing concentrations of cyclodextrin were prepared (1 to 5 mM for β-CD, respectively, 1 to 25 mM for SBE-β-CD) using the phosphate buffer solutions as solvents.

For the solubility studies we used a Vortex-Genie®2 (Scientific Industries) shaker, an Elma ultrasound bath and a Jasco–V650 UV/Vis spectrophotometer.

The experiments were conducted in triplicate.

Excess amounts of Ru(III)-nf complex (approx. 5 mg) were added to 5.0 mL aqueous solutions of increasing concentrations of cyclodextrins and shaken for 24 hours at 25±0.5°C. After 2 hours the samples were filtered through a 0.45 μm Nylon filter membrane (Whatman® PuradiscTM) and the absorbance at λ 280 nm (pH 4.4), 272 (pH 7.4) and 271 nm (pH 9.4) was measured, in order to determine the concentration of the dissolved Ru(III)-nf complex. This concentration was estimated using the molar absorptivity of the complex at the pH values of 4.4; 7.4 and 9.4 respectively.

Results and Discussion

A. Solubility determination in aqueous phosphate buffers with different pH values

Solubility of the complex was determined in aqueous phosphate buffer solution with pH ranging between 4.4 and 9.4. Data for solubility

variation depending on the pH of aqueous solution are presented in Table I and in Figure 3.

The solubility profile of Ru(III)-nf complex is quite different from that of norfloxacin. Norfloxacin as an acid-base ampholyte exhibits a „U"–shape pH–solubility profile, characterized by high solubility at pH values below 5 and above 10, with minimum solubility close to the neutrality pH, in the proximity of the isoelectric point [2, 14].

Table I

Solubility of the Ru(III)-nf complex at different pH values

pH	ε ($L \cdot mol^{-1} \cdot cm^{-1}$)	λ (nm)	Solubility (mg/mL)
4.4	44103	277	0.102
5.4	45435	277	0.182
6.4	47104	273	0.152
7.4	50444	272	0.325
8.4	57914	272	1.215
9.4	68038	272	1.372

For Ru(III)-nf complex it can be observed a marked constant enhancement of solubility toward alkaline pH as a consequence of salt formation, the basic piperazine nitrogen atom being involved in Ru(III) coordination.

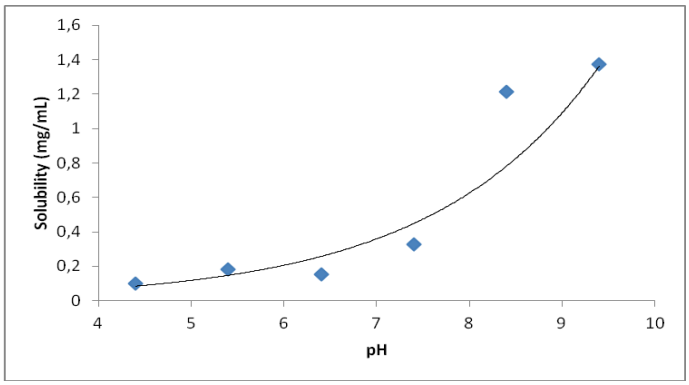


Figure 3

Ru(III)-nf complex solubility profile as a function of pH

B. Solubility determination in co-solvent mixtures

The dielectric requirement for Ru(III)-nf complex was determined in a binary mixture water/dioxane with different compositions (Table II).

The value of ε_{app} corresponding to the maximum solubility of the Ru(III)-nf complex was considered as DR.

Table II

Evaluation of ε_{app} and DR value by using different water/dioxane mixtures

H_2O (mL)	**Dioxane (mL)**	**ε_{app}/DR**	**Solubility (mg/mL)**
2.0	2.0	64.761	1.361
1.0	3.0	**48.556**	**1.951**
1.0	9.0	28.231	1.336

The solubility in the mixtures with ε_{app} 48.556 (ratio water/dioxane = 1/3) is maximum. Because the significant difference due to solubility, we have considered at DR, the value for co-solvents ratio 1/3 although it is recommended that for the biocompatible system the addition of co-solvent to be minimal.

After establishing the optimum value of $\boldsymbol{\varepsilon_{app}}$, solubility of the complex was determined in binary co-solvent mixtures water/PG, water/PEG 400, water/GLY with the same ε_{app} (Table III). The co-solvents were selected based on their compatibility with pharmaceutical formulations.

Table III

Evaluation of Ru(III)-nf complex solubility in different binary co-solvent mixtures

H_2O (mL)	**PG (mL)**	**ε_{app}**	**Solubility (mg/mL)**
10	1.9	48.556	1.887
H_2O (mL)	**PEG (mL)**	**ε_{app}**	**Solubility (mg/mL)**
10.0	0.5	48.556	2.012
H_2O (mL)	**GLY (mL)**	**ε_{app}**	**Solubility (mg/mL)**
25	1.2	48.556	1.803

Solubility of the tested complex was studied also in ternary systems using PG/PEG 400/ETOH as co-solvents, mixed in volume ratios so that the DR to be respected (Table IV).

Table IV

Evaluation of Ru(III)-nf complex solubility in a ternary co-solvent mixture

H_2O (mL)	**PG (mL)**	**ETOH (mL)**	**Solubility (mg/mL)**
5	10	10	1.643

The solubility process of the Ru(III)-nf complex studied in different water/co-solvent mixtures does not depend on the co-solvent nature. Experimental data confirmed that, for equal DR values, the solubilities were comparable. It was also revealed that for all systems analyzed the co-solvents used do not interfere with the analyzed complex.

Using various co-solvent systems, it was observed an enhancement of the complex solubility similar to the enhancement in aqueous solution at the pH value 9.4. Highest solubility was recorded in the binary co-solvent mixture water/PEG 400 in volume ratio 1/0.05.

C. Solubility enhancement using cyclodextrins

All phase-solubility diagrams of Ru(III)-nf complex with β- CD and SBE-β-CD within the concentration and pH range studied displayed a typical A_L type diagram (i.e., linear initial increase of complex solubility with increasing CD concentration), according to the Higuchi and Connors classification [7]. Figure 4 shows the phase-solubility diagram obtained at pH 7.4 and 24°C with SBE-β-CD. Apparent stability constants were estimated using parameters of the linear part of the solubility diagrams (slope and intercept), on the assumption that 1:1 complexes were initially formed:

$$K_f = \frac{slope}{intercept(1 - slope)}$$

Results are summarized in Table V.

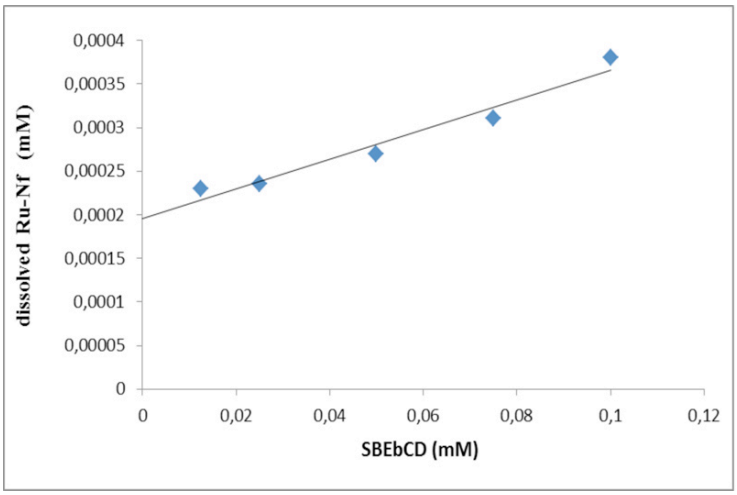


Figure 4

Phase-solubility diagram for the $Ru(nf)_2(DMSO)Cl_3 \cdot H_2O$ – SBE-β-CD inclusion complex, pH 7.4

Table V
K_f at different pH values

Type of cyclodextrin	K_f		
	pH 4.4	**pH 7.4**	**pH 9.4**
β-CD	39.29	-13.88	4.23
SBE-β−CD	17.16	8.67	6.88

Norfloxacin is easily included in beta-cyclodextrin and its derivatives [10], the molecular modeling studies indicate that the molecule enters the cyclodextrin cavity with its less polar part, i.e. piperazine ring. Though, the stability of the inclusion complex of $Ru(nf)_2(DMSO)Cl_3 \cdot H_2O$ is less than expected, probably because of the hindrance generated by the ruthenium coordination. The stability of the studied complexes seems to improve whenever hydrogen bond formation is possible, i.e. with SBE-β-CD, with an obvious tendency to decrease with higher pH values.

Conclusions

All three strategies act with good results in solubility enhancement for $Ru(nf)_2(DMSO)Cl_3 \cdot H_2O$ complex. The preliminary studies indicate that the most successful technique seems to be pH adjustment/salt formation and co-solvency, which result in ten to twenty times increase in complex solubility. Cyclodextrins have an interesting effect: the solubility increases not only by inclusion, but even by external interactions.

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