INCLUSION COMPLEX OF THIOTRIAZINONE WITH α-CYCLODEXTRIN-RAMAN SPECTROSCOPY, DSC, PRELIMINARY ANTIMICROBIAL AND ANTIFUNGAL STUDY

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Thiotriazinone (TTZ) is used as an intermediate of cephalosporin, but very few things are known about its activity. The purpose of this study was to form a complex of Thiotriazinone-α-cyclodextrine and to investigate its antimicrobial activity, in order to use this "*smart drug*" in animal diseases therapy. The complex was made by liophlization at a molar *ratio* TTZ-αCD=1:1. Characterization was made by Raman spectroscopy and DSC. Antimicrobial activity was tested *in vitro* on Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, *Sarcina lutea* ATCC 9341), Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa ATCC),* and fungus *(Candida albicans* ATCC 10231, *Candida glabrata ATCC MYA 2950, Candida parapsilosis* ATCC 22019). The inclusion complex showed better antibacterial effect for *S. aureus* and *S. lutea* versus thiotriazinone. It may be noted that the compounds tested showed no antibacterial activity for *E. coli* and *Pseudomonas aeruginosa.* Although the tested compounds show antifungal activity against all fungal organisms, a more pronounced action was observed especially against *C. glabrata.*

(Received December 18, 2015; Accepted March 4, 2016)

Keywords: Cyclodextrins, Inclusion complex, Thiotriazinone

1. Introduction

Thiotriazinone is used as intermediates of cephalosporins, such as Ceftriaxone Sodium. This product is used in the preparation of beta-lactam antibiotics with proven effects as antibacterial agents and human leukocyte elastase inhibitors [1]. Some authors [2] considered TTZ to be impure, in 33 out of 34 ceftriaxone generic products. We consider, as Shui et al. (2012), that is a lack of data regarding the structure and chemical activity of thiotriazinone [3].

Cyclodextrins are cyclic oligosaccharides, named α- , β- and γ- cyclodextrins depending on the number of the glucose units 6, 7 or 8 respectively. These carrier molecules are capable to form host-guest inclusion complexes with different molecules because of their hydrophobic interior and a hydrophilic exterior [4, 5].In the last 50 years, many studies were made on the properties and potential application of cyclodextrins. The main advantages of using cyclodextrine in forming a pharmaceutical product, based on a specific active substance are: decreasing the therapeutic doses, increasing the solubility of the guest; masking the taste and odour, decreasing the volatility; decreasing the toxicity, it is chip and easy to make as Anjana says since 2013[6]. These molecules are also called "*smart drugs*".

Regarding these aspects, complexation of TTZ with α-CD offers the possibility to improve its antimicrobial activity by increasing the bioavailability, the apparent solubility and dissolution rate [4]. Molecular structures were made on four isomers of TZZ which gives us information about the regions that came from non-covalent interactions [4].

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2. Experimental

2.1 Materials

The α-cyclodextrin (α-CD) was purchased from Sigma Aldrich (USA), CAS number 10016-20-3, empirical formula $C_{36}H_{60}O_{30}$, and molecular weight 972.84 g/mol.

Thiotriazinone 98%- (TTZ), was purchased from Alpha Aesar (Germany), having the following characteristics: Synonym Tetrahydro-2-methyl-3-thioxo-1,2,4-triazine-5,6-dione1,2,4-Triazin-2-methyl-6-hydroxy-3-thio-5-one, CAS number 58909-39-0, molecular weight 156.17 g/mol, melting point 168-171º C and chemical formula $C_4H_5N_3O_2S$.

2.2 Methods

2.2.1 Preparation of Thiotriazinone-α-cyclodextrine complex (TTZ- α-CD)

TTZ-α-cyclodextrine complex was made by liophlization, using a molar ratio TTZ-αCD =1:1. Parent substance was added drop wise into the clear saturated CD solution, and the mixture was stirred for 60 h at 50º C. The water was removed after freezing, under vacuum and at very low temperature.

2.2.2 Raman spectroscopy

A micro-Raman system (Renishaw, inVia Reflex) equipped with a 633 nm He-Ne laser (17 mW) and an optical microscope (Leica DM 2500 M) was used. The laser beam was focused through a 50× objective lens (Leica, N Plan EPI) with a numerical aperture of 0.75. Scattered light was collected in a back scattered geometry. The range of vibrational frequencies was from 100 to 3200 cm^{-1}. Three scans were accumulated for each spectrum, and the laser exposure for each scan was 10 sec. in order to achieve an adequate signal-to-noise *ratio*. The Raman measurements were performed at room temperature and atmospheric pressure. All samples were investigated in powder form and no further sample preparation was applied.

2.2.3 DSC- technique

Differential scanning calorimetry (DSC) measurements were conducted on a DSC 200 F3 Maia device (Netzsch, Germany). About 10 mg of each sample was heated in pressed and pierced aluminium crucibles. A heating rate of 10º C/min was applied. Nitrogen purge gas was used as inert atmosphere at a flow rate of 50 mL/min. The device was temperature and sensitivity calibrated with indium, according to standard procedures.

2.2.4 Antioxidant evaluation

Evaluation of antioxidant potential was achieved by means of potassium ferricyanide. Substances which have a reducing activities, reacted with potassium ferricyanide (Fe^{+3}) to form potassium ferocyanide (Fe^{+2}), which reacts with ferric ion to form a blue coloured complex, showing maximum absorbance at 700 nm. Pure thiotriazinone, α-cyclodextrin and the obtained complex (TTZ-αCD) were treated with phosphate buffer (pH 6.6) and potassium ferricyanide (1%). The mixture was incubated at 50º C for 20 minutes. After cooling, the mixture was treated with trichloroacetic acid (10%) and centrifuged and the supernatant was treated with $FeCl_3$ (0.1%).

Reducing capacity was calculated according to a formula using absorbance and the solutions concentration with an absorbance what's exactly above and below 0.5. (table 1.)

Table1. IC 50 values

Samples	IC_{50}
thiotriazinone	2.3798 ± 0.25
cyclodextrine	-
Complex (*TTZ*- α-CD)	1.9018 ± 0.32

2.2.5 In vitro activity

Antimicrobial activity was evaluated by a useful method for initial screening that uses of agar diffusion [3, 7].Tests were made on Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, *Sarcina lutea* ATCC 9341), Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa ATCC),* and fungus *(Candida albicans* ATCC 10231, *Candida glabrata ATCC MYA 2950, Candida parapsilosis* ATCC 22019*),* inoculated in soft *Mueller Hinton* agar (for bacteria) and *Sabouraud maltose* agar medium (for fungus).

Bacterial strains were incubated for 24h at 37°C, and fungal strains for 48h at 24°C. TTZ-CD complex was tested in comparison with parent substances, by filling stainless steel cylinders with an inner diameter of 6 mm with 100 µl (0.01 g/ml). The result represents the average diameters recorded in three plates. The antibacterial activity of the investigated compounds was compared with the inhibition zone obtained with a disc ampicillin 25 mg and 30 mg chloramphenicol disc placed simultaneously with the samples. Antifungal activity was compared to a 100 mg nystatin disc.

3. Results and discussion

3.1 Raman results

Raman spectroscopy is an appropriate technique for the efficient investigation of the cyclodextrin host-guest inclusion complexes formation with different molecules.

The Raman spectra of the α-cyclodextrin, thiotriazinone, their guest–host complex and 1:1 α-cyclodextrin- thiotriazinone physical mixture in the spectral range 100 – 3200 cm^{-1} are presented in figure 1. In the case of physical mixture, the Raman spectrum is nearly the sum of the individual spectra of the thiotriazinone and α-cyclodextrin molecules.

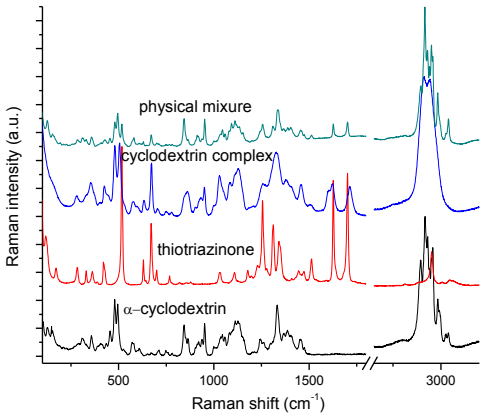


Fig. 1. The Raman spectra of the α-cyclodextrin, thiotriazinone, their guest–host complex and 1:1 α-cyclodextrin- thiotriazinone physical mixture in the spectral range 100 – 3200 cm^{-1}

The 1550–1800 cm^{-1}region deserves special attention since no interfering bands from cyclodextrin are present. In the Raman spectra of the guest–host complex, the 1624 (stretching vibration of the C=N groups) and 1712 cm^{-1} (stretching vibration of the C=O groups) peaks corresponding to free thiotriazinone are reduced and shifted (with 2cm^{-1} and -12 cm^{-1}, respectively). Also, a broadening of these bands is observed in comparison to the bands of the pure thiotriazinone, indicating the existence of the guest-host interactions.

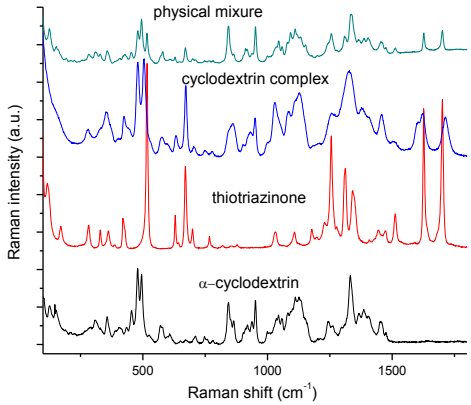


Fig. 2. The Raman spectra of the α-cyclodextrin, thiotriazinone, their guest–host complex and 1:1 α-cyclodextrin- thiotriazinone physical mixture in the spectral range 1550 – 1800 cm^{-1}.

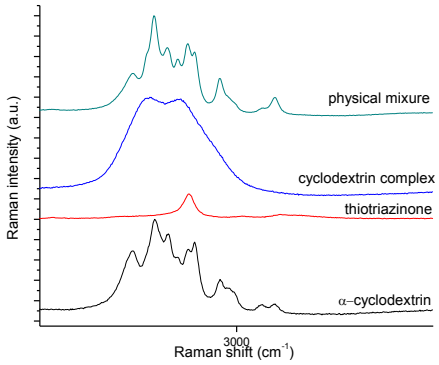


Fig. 3. The Raman spectra of the α-cyclodextrin, thiotriazinone, their guest–host complex and 1:1 α-cyclodextrin- thiotriazinone physical mixture in the spectral range 2850 – 3050 cm^{-1}.

In the region 2850-3050 cm^{-1}, which comprises C-H stretching modes, the bands profile is changed relative to the pure cyclodextrin spectrum.

3.2 DSC results

DSC is a useful technique in determining the occurrence of host–guest solid state interactions. Such interactions are identified from differences between inclusion compounds and pure substances that occur by phase transformations during heating.

The guest molecule exhibits an intense and sharp melting profile with a peak value at 261ºC. The decrease in intensity or disappearance of the guest molecule melting profile is a general evidence of complexation [8]. This aspect is also indicated in figure 4, on the DSC heating curve of the inclusion complex, where a significantly lower intensity may be observed for the guest molecules melting profile, accompanied by its displacement towards a lower temperature value (230º C). α – CD exhibits a typical three stage endothermic process (76º C, 106º C and 131º C) due to loss of crystallized water molecules from its cavity. This dehydration profile may also be observed on the physical mixture (PM) heating curve. However, for the inclusion complex, the α–CD dehydration peaks coalesee into a single endothermic peak, which is significantly reduced in intensity. These differences also indicate inclusion complex formation. Furthermore, although the PM melting profile is also shifted towards a lower temperature value (235º C), comparable to that of the guest molecule in the inclusion compound. The former exhibits a higher intensity, similar to that of the uncomplexed guest molecule. This is an indication that specific physical host-guest

interactions occurred, however exterior of the α–CD cavity in the PM. A similar aspect was reported in the literature by Corciova et al., 2014 [9]. By determining the melting heat values of the inclusion complex (24.53 J/g) and that of pure guest molecule (216.4 J/g) and applying equation (1)[10, 11]there was calculated an inclusion efficiency value of 88.66%.ΔH_{ic} represents the melting heat value of the inclusion compound and ΔH_{pg} represents that of the pure guest compound.

Equation (1):

$$\% \text{ Inclusion} = 100\ (1 - \Delta H_{ic}/\Delta H_{pg})$$

ΔH_{ic} = inclusion compound melting heat value
ΔH_{pg} = pure guest compound melting heat value

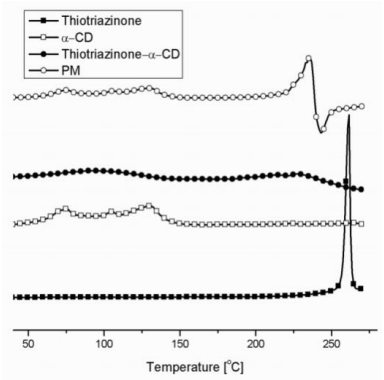


Fig.4. DSC heating curves of the studied structures.

3.3 Absorbance study

For cyclodextrin, because absorbance values are below 0.5, IC50 cannot be calculated. Comparing IC50 values of TTZ-CD complex with thiotriazinone is observed proxies, but slightly increased values for the complex, which may prove a better antioxidant activity after complex formation. The biggest difference was observed at the concentration of 10 mg/ml, when the absorbance value of TTZ-CD complex is 6.1 compared to 3.8 for non-complexed thiotriazinone (figure 5).

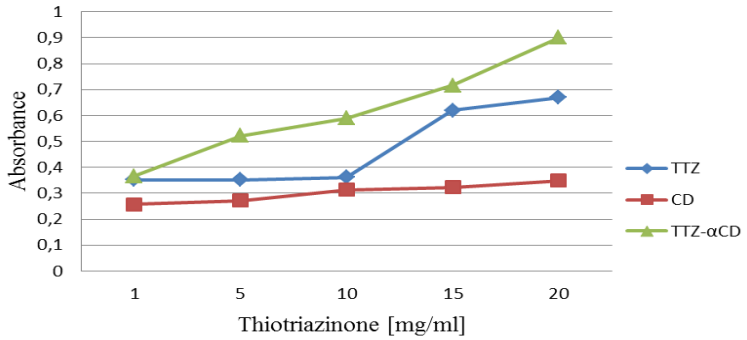
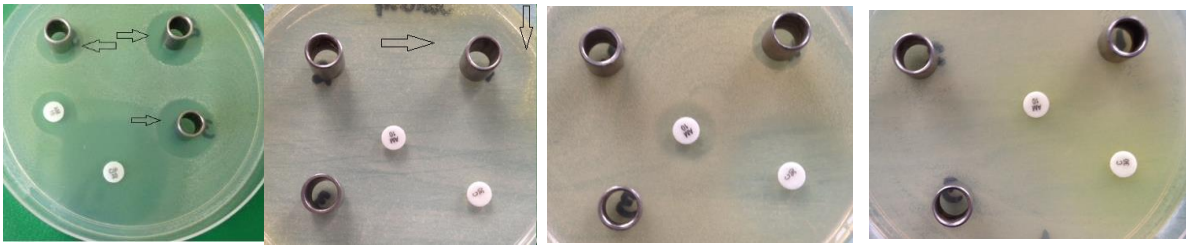


Fig.5. Absorbance variation depending on the concentration samples.

3.4 Antimicrobial activity

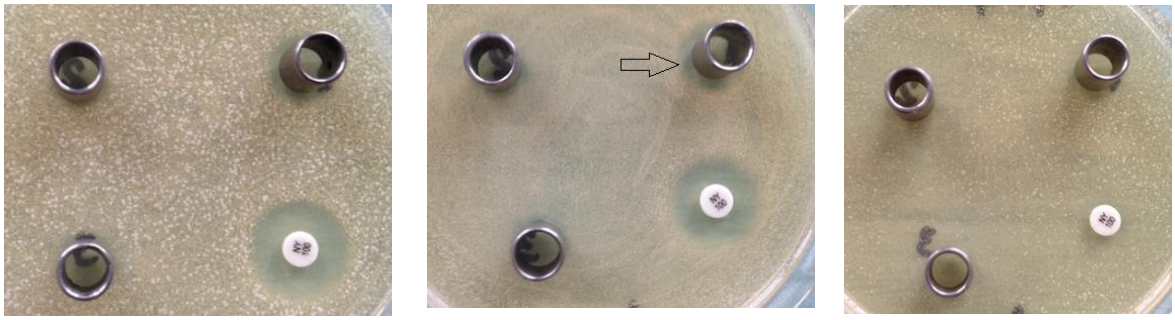
The diameters of the inhibition zones (in mm), corresponding to the test samples are shown in Figure 6a, 6b and 7. The inclusion complex showed better antibacterial effect for *S. aureus* and *S. lutea* versus thiotriazinone. It may be noted that the compounds tested showed no antibacterial activity for *E. coli* and *Pseudomonas aeruginosa.*

Although the tested compounds show antifungal activity against all three fungal organisms, a more pronounced action was observed especially against *C. glabrata.*



Staphylococcus aureus | *Sarcina lutea* | *Escherichia coli* | *Pseudomonas aeruginosa*

a) Bacteria



Candida albicans | *Candida glabrata* | *Candida parapsilosis*

b) Fungus

Fig. 6. The inhibition zones, corresponding to the test samples
a) Bactria b) Fungus

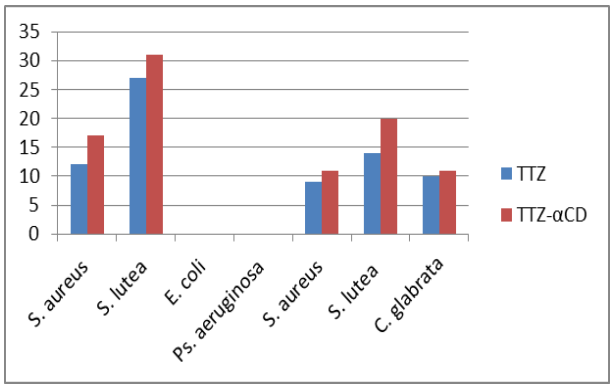


Fig.7.The effect of complexation against different bacteria and fungus

4. Conclusions

Complexation of TTZ with α-CD improve its antimicrobial activity for, *Staphylococcus aureus* and *Sarcina lutea,* and have no action against *Escherichia coli* and *Pseudomonas aeruginosa.*

Complexation of TTZ with α-CD improve its antifungal activity against all three fungal organisms belongs to genus *Candida*, especially for *Candida glabrata.*

The absorbance value of TTZ-CD complex is double (6.1 compared to 3.8) for non complexed thiotriazinone.

By Raman spectroscopy, the spectra of the α-cyclodextrin, thiotriazinone, their guest–host complex and 1:1 α-cyclodextrin- thiotriazinone physical mixture in the spectral range 1550 – 1800 cm^{-1}, show an efficient inclusion complex who indicates the existence of the interactions, who improve the quality of complex.

By DSC technique, was proved and calculated an inclusion efficiency value of 88.66%.

Acknowledgements

This study was suported by the project POSDRU/159/109/S/132765funded by European Union and Romanian Goverment.

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