METHOTREXATE AS COORDINATION COMPLEX LIGAND: STUDY OF INTERACTION WITH Zn(II)

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A new metal complex containing Zn(II) as complex generator and active substance methotrexate (MTX) as ligand was obtained. The coordinative compound was characterized by means of thermogravimetric analysis, complexometry and elemental analysis.It was proved that in the experimental conditions a compound was obtained with molar ration Zn(II):MTX = 1:2. As the thermal stability may be crucial to assessing applicability of new compounds, the thermal behavior of the complex is discussed in detail comparative with the one of the ligand. The spectroscopic data suggest that the methotrexate acts as ligand toward the metal cation of Zn. The TG-DTG and DSC curves show for the Zn(II) complex the dehydration process occurs in two consecutive steps and the thermal decomposition of the anhydrous compound takes place in a continuous process composed of two significant steps. This fact is sustained by the different E_a values function of α, the result being interpreted as overlapping reactions with a complex mechanism.

1. Introduction

Methotrexate (MTX) is a drug that belongstothe group of chemotherapy drugs known as anti-metabolites [1] and the mechanism of action consists inthe inhibition of producing DNA and RNA by preventingmalignant cells from using folate[2]. The chemical structure of MTX is presented in Figure 1:
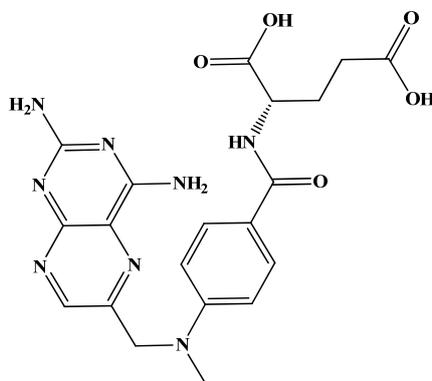
Fig.1. Chemical structure of MTX

MTX is one of the most intensely studied therapeutic agent used in the treatment ofvarious tumors, autoimmune diseases, as well blood malignancies [3]. MTX is considered a key-molecule in the treatment of cancers, such as breast cancer,osteogenic sarcoma, lymphatic leukemia,

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myeloid leukemia, lung cancer, choriocarcinoma, bladder carcinoma and brain medulloblastoma[4,5].

Beyond from the usual use as ananticancer chemotherapeutic agent, MTX it alsoinvolved in the treatment of several other diseases such as multiple sclerosis, Crohn's disease, rheumatoid arthritis and psoriasis. The use of MTX in the treatment of rheumatoid arthritis is considered nowadays to be an efficient standard therapy [6].

Zinc is nowadays considered a dietary essential trace element that plays important structural roles in many proteins and enzymes [7] and mononuclear Zn(II) coordination compounds show interesting antidiabetic activities [8,9]. Bioactive molecules, such as pharmaceuticals, interact with metal ions forming salts or coordinative compounds with different structures and geometries. This domain is a highly-actualdomain of study, mainlyby the fact that in vivo, essential trace elements interact with pharmaceutical ligands that appear in living systems. It is known that the cations can bind to enzymes, proteins and other biological ligands [9]. Numerous molecules, including heterocyclic compounds that contain nitrogen, sulphur and carboxyl moieties have been synthesized [10-12] and used as ligands in synthesis of coordination compounds [13,14]. In our recently studies [15] we described the synthesis of a coordination compound of Zn(II) with acetaminophen and it's spectroscopic and TG-DTG-HF characterization.

Thermal analysis is one of the most frequently used methods in pharmaceutical field applied from testing of new compounds with potential biological activity[16-19], crystal structure [15], and evaluation of possible physico-chemical incompatibility between the active substance and different pharmaceutical excipients [20-22], to quality control of the purity of final products using the kinetic triplet [23,24].

In this paper, we present the synthesis and characterization of a new coordination compound, obtainedby the reaction ofan antineoplastic antimetabolite with immunosuppressant properties (MTX) as ligand, which containstwo carboxylate groups, two amino groups and several heterocyclic nitrogenswith $ZnCl_2$as complex generator. This study was undertaken to report the synthesis of the new metal complex and its characterization using thermogravimetric techniques (TG/DTG), supported by differential scanning calorimetry (DSC) and infrared spectroscopy (FTIR).

As the thermal stability may be crucial to assessing applicability of new compounds, the thermal behavior of the ligand and the complex with Zn(II) is discussed in detail. Kinetic analysis under non-isothermal conditions was also performed to determine the kinetic triplet (activation energy, pre-exponential factor and reaction order) of the new complex comparative with the kinetic parameter's values characteristic to the pure active substance, methotrexate (MTX).

2. Materials and methods

Active substance used as ligand (methotrexate trihydrate, MTX), was purchased from Calbiochem, Japan (lot No. D00135520) and has a pharmaceutical grade (HPLC purity >99.7%).

All chemical used were of the analytical reagent grade and of highest purity available. They included anhydrous $ZnCl_2$from Sigma-Aldrich and bi-distilled water which was used in all preparations.

The composition of the metal complex (C, H, N and Cl) was obtained by means of elemental analysis using aVario El Cube analyzer. The metal content from complex (namely Zn^{2+}) was determined by complexonometric titration with EDTA, at pH $\sim$ 10 in buffer solution (NH_3/NH_4Cl), in the presence of Eriochrome Black T as indicator, by a standard analytical procedure.

Thin-layer chromatography was carried out on silica gel-coated plates $60F_{254}$ Merck using hexane:ethylacetate:methanol 1:3:4 (v/v) as eluant. The FTIR spectra of MTX and metallic complex with Zn were obtained on the JASCO PLATINUM ATR-QL DIAMOND on 4000-500 cm^{-1} spectral range with a resolution of 1cm^{-1}, with 20 acquisitions for each sample and in KBr pellet on a Jasco FT/IR-410 spectrophotometer for the product of thermal decomposition of Zn(II) complex, respectively, on 4000-400 cm^{-1} spectral range.

TG measurements were carried out on a Netzsch thermal analyzer TG 209 F3. The experiments were performed under air flow (100 mL·min^{-1}) at heating rates of 5, 7, 10 and 15 °C·min^{-1}; the sample mass was cca 10.0 mg using ceramic crucibles.

Calorimetric measurements were carried out with the Netzsch DSC 200 F3 calorimeter (Germany) operating in the dynamic mode. The dynamic scans were performed at the heating rate of 10 °C·min^{-1} from room temperature to the maximum of 400 °C under air atmosphere (30 mL min^{-1}) using aluminium-sealed crucibles. The DSC was calibrated with indium and zinc. The mass of the samples was approx. 6.0 mg and an empty aluminium crucible was used as a reference.

Synthesis of Zn(II) complex with MTX

The metal complex was obtained by the reaction of $ZnCl_2$ with MTX in bi-distilled water. A solution containing anhydrous zinc chloride (0.1364g, 1 mmol) in water (10 mL) was added dropwise (2h time) to an aqueous solution of MTX (0.9089g, 2 mmol / 400 mL bi-distilled water) under intense stirring and heating at 70 °C. The mixture was kept at 70 °C for 48h, and then was cooled down at 25 °C. After cooling, approx. 300 mL of solvent (water) was removed under reduced pressure (40 °C / 10 mmHg). The concentrated solution was allowed to slowly evaporate at room temperature and after 2 weeks acrystalline pale-light yellow solid precipitated, which was filtered off under reduced pressure, washed with distilled water (3x5 mL) and dried for 48 h at 40 °C.

$[Zn(MTX)_2(OH_2)_2]^{2+}$ 2Cl$^-$ metal complex
Crystalline pale-light yellow solid (0.9963 g, 0.891mmol, yield 89.1%), TLC one spot;
Chemical formula: $C_{40}H_{52}Cl_2N_{16}O_{14}Zn$; molar mass: 1117.23 g/mol;
FTIR (UATR, cm^{-1}): 3216 (large), 2751(large), 2602(large, intense), 1616(intense), 1590(intense), 1499, 1438, 1372, 1251, 1043, 831, 805, 732, 602.
Elemental Analysis (Calc.%/Found%): C (43.00%/42.18%); H (4.69%/5.02%); Cl(6.35%/6.28%); N (20.06%/19.92%); Zn (5.85%/4.89%)

3. Results and discussion

Synthesis of metal complex

The synthesis of the complex was carried out in aqueous medium, by treating the Zn(II) salt with ligand (MTX) in a molar ratio 1:2. An increased amount of bi-distilled water and heating at 70 °C were chosen in order to assure that the formation of metal complex occurs in solution and not in a suspension, being known the fact that MTX possess a limited solubility in water.

The formation of the coordinative compound was monitored by comparative TLC, when spots corresponding to MTX (free ligand) and reaction mixture were applied on the chromatographic plate and eluted as previously stated. After 48h of stirring at 70 °C, the disappearance of the spot (R_f=0.76) corresponding to free ligand was observed.

Comparative thermal analysis of ligand and metal complex

The comparative TG/DTG and DSC curves of methotrexate (MTX) and Zn(II) complex are depicted in Figs. 2 and 3 (the heating rate is 10 °C·min^{-1}). By the analysis of TG/DTG curve, it can be observed that there are three main successive steps of mass loss MTX from 25 to 1000 °C, which correspond to the intense endothermic processes in the DSC curve. Both in the TG/DTG curve and DSC curve show that MTX is thermally unstable as a trihydrate, losing three molecules of water up to 87 °C; anhydrous MTX is, however, stable up to 200 °C. A process with an endothermal nature can be identified by the analysis of the DSC curve at 198 °C, which correspond to the melting of the anhydrous MTX. Anhydrous MTX presents an increased thermal stability up to 200 °C, when thermal degradation of MTX begins as a continuous process up to 800 °C (Figs. 2 and 4)
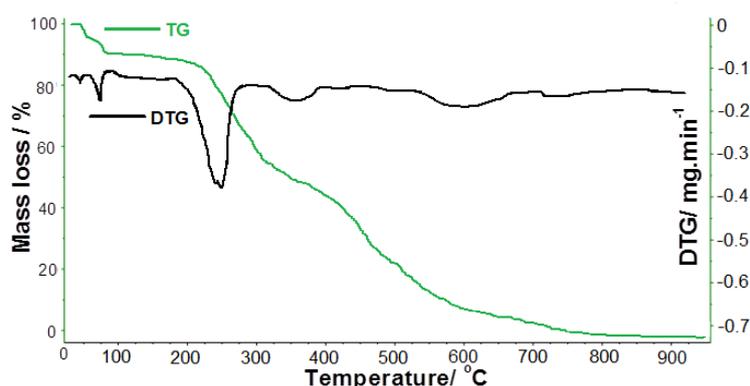
Fig. 2. The thermoanalytical curves TG/DTG obtained in air at β=10 °C·min⁻¹ for MTX.

Thermal behavior of Zn(II) metal complex is relatively similar to the one of free ligand, with several differences. The first mass loss on the thermogravimetric curve occurring in the 70-137 °C range corresponds to the loss of 2 moles water (calculated mass loss =3.87 %; experimental mass loss=3.22 %). The dehydration is accompanied by a weak endothermic peak on the DSC curve (DSC_{peak}=76 °C) according to the loss of two water molecules. This fact may point a relatively weak bonding of water molecules in the complex structure. According to literature [15] lattice water is removed at temperatures below 100 °C, whereas coordinated water is eliminated within 110-275 °C temperature range. As a conclusion to this, the two water molecules should correspond to the lattice water, not to coordination water. This process is followed by another endothermic process which takes place with another mass loss, which corresponds to another 2 moles water (calculated mass loss=3.34 %; experimental mass loss=4.09 %). Due to the fact that this mass loss occurs at temperatures higher than the ones previously observed (T_{onset}=110 °C and DSC_{peak}=143 °C), it can be concluded that this water is involved in the coordination geometry of the complex.

After the water elimination, a decomposition process with a continuous mass loss takes place until 1000 °C. The anhydrous zinc complex is stable up to 260 °C. The solid residue obtained during thermal decomposition of complex is in agreement with formation of ZnO (calculated mass loss=7.29%, experimental mass loss=8.04%). The thermal analysis results of the new Zn(II) complex with MTX, determined in non-isothermal conditions are summarized in Table 1.

Table 1. Thermoanalytical data of the MTX and its metallic complex with Zn(II)

Substance	TG/DTG curves		DSC curves		Nature of the process
	T_{onset}/°C	$T_{peak\ DTG}$/°C	T_{onset}/°C	$T_{peak\ DSC}$/°C	
MTX	47	51	49	65	dehydratation
	53	87	78	120	dehydratation
	-	-	190	198	Melting
	200	218	220	250; 268	decomposition
Zn(II) complex	70	83	55	76	dehydratation
	137	145	110	143	dehydratation
	260	433; 520	225	280; 320	Decomposition
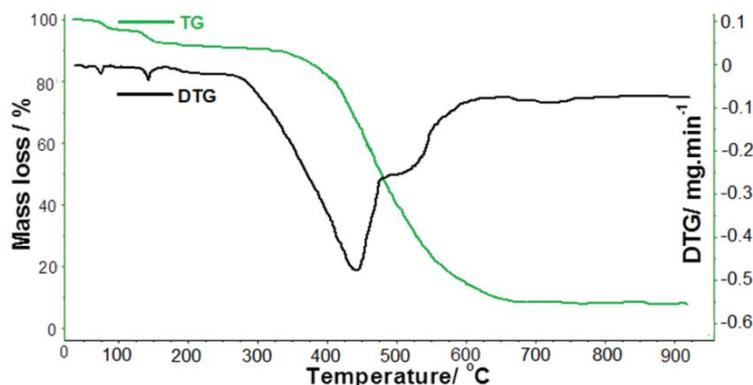
Fig. 3. The thermoanalytical curves TG/DTG obtained in air at β=10 °C·min^{-1} for complex with Zn(II).
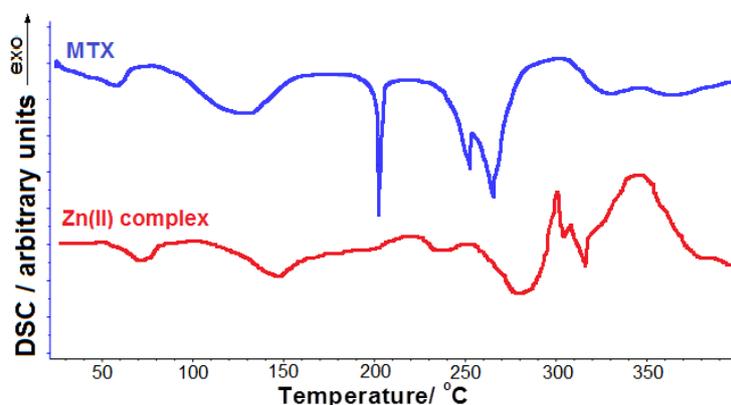
Fig. 4. The comparative DSC curves obtained in nitrogen at β=10 °C·min^{-1}.

UATR-FTIR analysis of MTX and $[Zn(MTX)_2(OH_2)_2]^{2+}$ 2Cl$^-$ metal complex

The UATR-FTIR spectrum of pure methotrexate (MTX) drawn up on 4000-500 cm^{-1} (Fig.5) is very similar with the one previously mentioned in our studies [25]. According to this, a characteristic absorption band as a large signal at 3451 cm^{-1} (O–H stretching from carboxyl groups superposed with the O-H stretching from three molecules of crystallization water) can be observed. Other important bands consist in signals at 3080 cm^{-1} (primary amine N–H stretching) andat 1670-1600 cm^{-1} assigned to C=O stretching (–C=O stretching from carboxylic group and C=O stretching from amidic group). The bands corresponding to N-H bending from amidic group appear in the 1550–1500 cm^{-1} spectral range, partly overlapping with the aromatic –C=C stretching. Other prominent bands, such as 1400-1200 cm^{-1} correspond to –C–O stretching from carboxylic group, 930 cm^{-1} to O–H bending out of plane and 820 cm^{-1} to*para* substitution.

The UATR-FTIR spectrum of Zn(II) complex of methotrexate (MTX) was drawn up in identical conditions to the one obtained for pure ligand(Fig.5). By the analysis of the FTIR spectra, the presence of the coordinated water in the structure of the complex is suggested by the characteristic vibrations ν(H$_2$O) as broad band in the 2500-3500 cm^{-1} range for chelated compounds (peak at 3216 cm-1). According to literature [26-30] the COOH group is characterised by two major ways of vibrations: ν(C=O) and ν(C-OH), the latter being represented by two bands. Once the deprotonation of the -COOH group occurs, it is expected that the -COO$^-$ group adopts a C$_{2v}$ symmetry, so ν^{as}(COO) and ν^s(COO) vibrations occurs. As expected, the analysis of the IR spectrum reveals that the deprotonation of the carboxyl group occurs in the metal complex, fact sustained by the shift of ν(C=O) from 1670 cm^{-1} (in the case of free ligand) to a considerable low frequency (1616 cm^{-1}) in the case of the Zn(II) metal complex.

An interesting fact can be observed by the analysis of the FTIR spectrum of metal complex: new bands appear in the spectral range 3200-2400 cm^{-1} as multiple broad peaks, which are overlapping to the broad signal of water. These bands with maximum at 2751 cm^{-1} and 2602

cm^{-1} can be assigned to the vibrations of –NH$_3^+$ group due to the formation of zwitter ions which appear as a consequence of presence of both the acidic (COOH) and the basic (NH$_2$) moieties from MTX molecule. According to this, the formation of metal complex is accompanied by the formation of the so-called internal salt of the ligand. The protonation of the –NH$_2$ group to –NH$_3^+$ is sustained by the apparition of previously-mentioned bands, as well by the fact that the bands corresponding to N-H bending from amidic group appear in the samespectral range as for the ones from free ligand, suggesting that no modification occurs for this moiety.The apparition of bands in the FTIR spectrum corresponding to –NH$_3^+$ group supports the fact that the –NH$_2$ group is not involved in the formation of dative bonds. All the data obtained by spectroscopic means are confirmed by the results from thermal analysis.
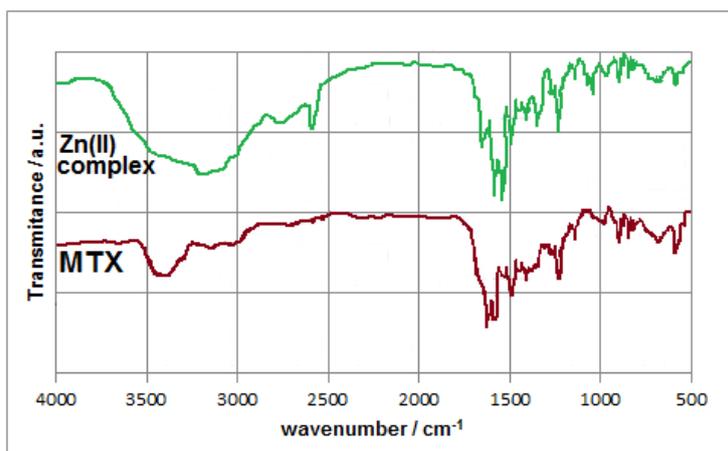
Fig. 5. Comparative UATR-FTIR spectra for MTX (free ligand) and Zn(II) complex

According to spectroscopic data, and by corroboration to thermal behaviour of complex and the results from elemental analysis, a proposed structure for the metal complex is presented in Fig.6.
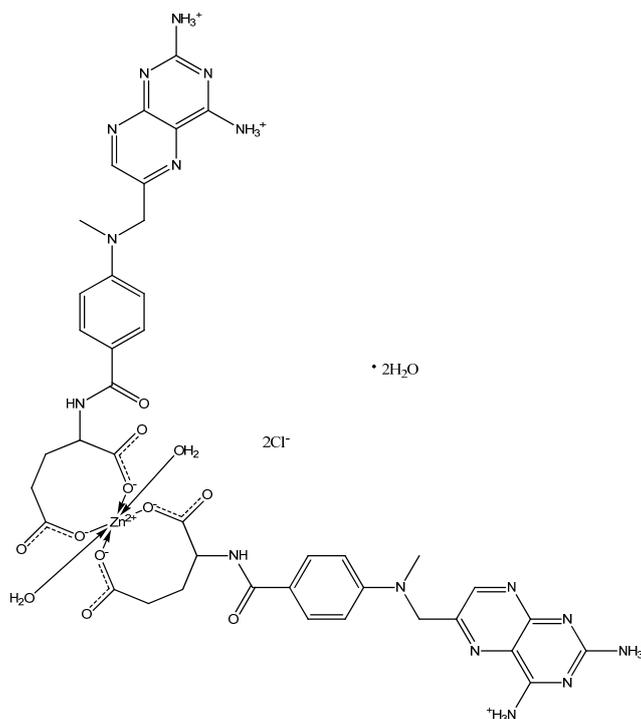
Fig. 6. A proposed structure for the synthesized Zn(II) complex

In order to determine the final decomposition (constant mass in the temperature range 632-1000 °C) product obtained from the thermal treatment of the Zn(II)-MTX at 1000 °C, an FTIR spectra was drawn up. Due to the high temperature (1000 °C), as well for the presence of oxidative atmosphere, the final product (a white amorphous powder) was suspected to be Zn(II) oxide. In order to prove its formation a comparative FTIR analysis was realised, namely the comparison of the FTIR spectra drawn up for sample with the FTIR spectrum of pure ZnO. It was proven that the decomposition product is ZnO and traces of organic fragments from MTX are no longer present (Fig. 7).
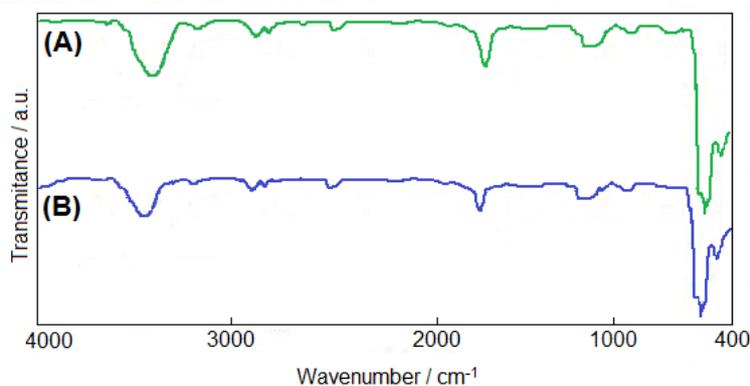
*Fig.7. FTIR spectra for pure ZnO (A) and the decomposition product
of Zn(II)-MTX complex at 1000 °C (B)*

Non-isothermal thermogravimetric studies
The kinetic investigation of the metallic complex with Zn(II) degradation was performed using non-isothermal thermogravimetric experiments at four heating rates: 7, 10, 12 and 15 °C·min^{-1}.

The thermogravimetric curve shows that the new complex is thermally stable up to 280 C, where it starts to decompose, with a significant mass loss ($\approx$80 %) up to 640 C (Fig.3). Non-isothermal thermogravimetric analysis was performed to determine the activation energy involved in the decomposition of this complex (E_a).

To perform the kinetic analysis of the TG experimental data, there were used to estimate the kinetic parameters a differential method, *Friedman isoconversional (Fd)* [31], respectively two integral methods, *Flynn–Wall–Ozawa (FWO)* [32], *Kissinger–Akahira–Sunose (KAS)* [33,34].

a) *Friedman method (Fd)*
Friedman method is an isoconversional one and the characteristic equation is:

$$\ln(\beta\frac{d\alpha}{dT}) = \ln[A \cdot f(\alpha)] - \frac{E}{R \cdot T}$$

(1)

For α=constant and using various heating rates, the graphical representation $\ln(\beta\frac{d\alpha}{dT})$ *vs.* (1/T) is linear and from the slopes of the straight lines (Fig. 8), the values of the activation energy (E_a) are obtained (see Table 2). According with these data, it is noted a significant variation of the activation energy depending on the conversion degrees.
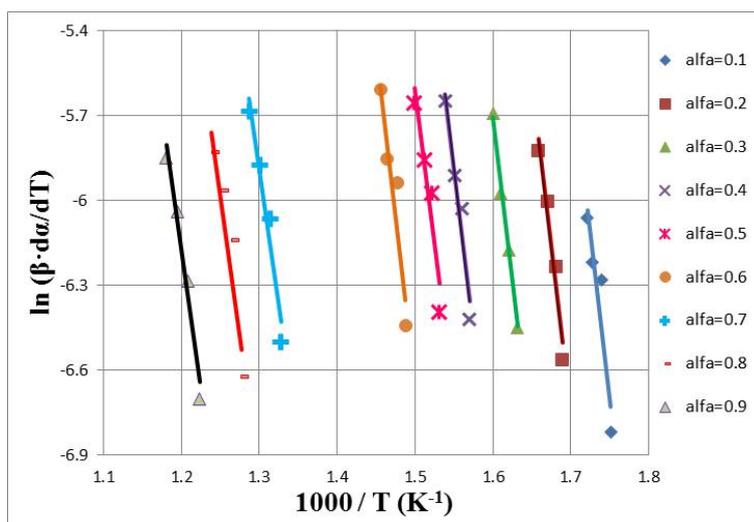
Fig. 8. Friedman's plot for metallic complex at four different heating rates

b) *Kissinger-Akahira-Sunose method (KAS)*

The KAS method is based on the equation (2) and consists on measuring the temperature corresponding to fixed values of the conversion degrees (α) for experiments at different heating rates β:

$$ln\left(\frac{\beta}{T_\alpha^2}\right) = ln\frac{A \cdot R}{E \cdot g(\alpha)} - \frac{E}{R \cdot T_\alpha} \qquad (2)$$

In Fig. 9 are presented the straight lines obtained by the graphic representation of $ln(\beta/T_\alpha^2)$ *vs.* $(1/T_\alpha)$ and the slopes of these lines are used to determine the activation energy of the analyzed process. It was noticed a change of the E_a values function of α, the result can be interpreted as a multistage reaction mechanism (see Table 2).
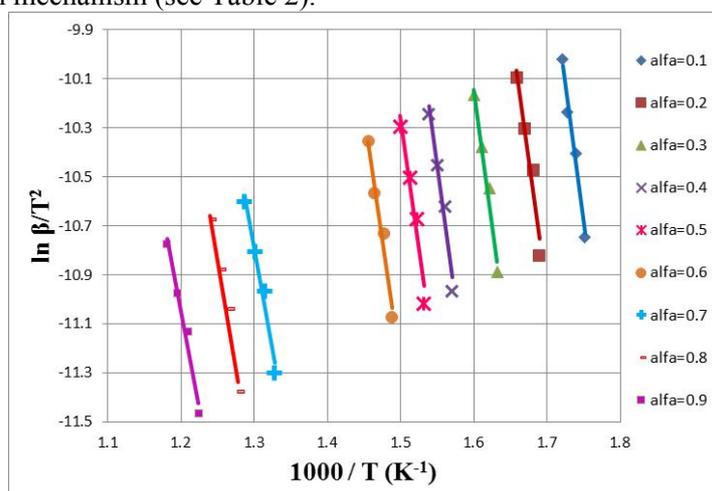
Fig. 9. The KAS isoconversional diagrams

c) *Flynn–Wall–Ozawa (FWO) method*

The FWO isoconversional method is an integral one and requires the temperature measurement at certain conversions *α* for experiments performed at different heating rates *β*. For obtained the basic equation of this method (proposed by *Ozawa* [32] for determining the activation energy), it was used the Doyle's approximation [35] for p(*x*) which is a function known as the Arrhenius integral:

$$\ln\beta = \ln\frac{AE}{R \cdot g(\alpha)} - 5.331 - 1.052 \cdot \frac{E}{R \cdot T} \qquad (3)$$

The calculated results from the application of this isoconversional method in the 10–90% conversion range are listed in Table 2 and the graphical representation of ln*β vs*. the reciprocal of the temperature for the particular values of *α* is presented in Fig. 10. All the three methods are model-free methods because their applications do not require the explicit form of the conversion function.
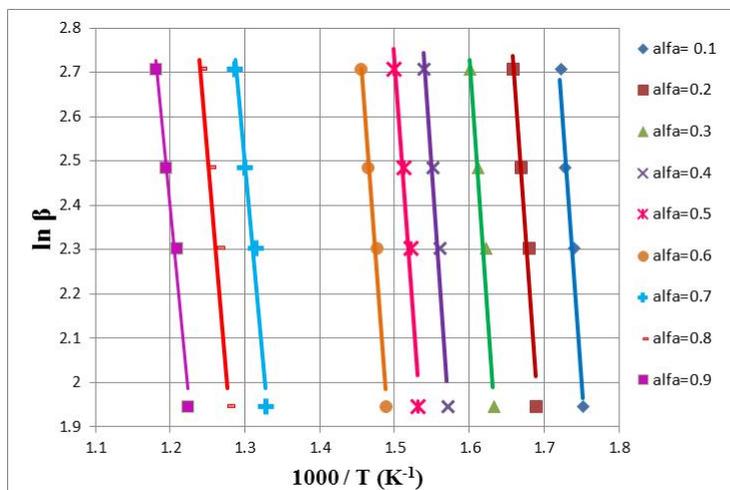
Fig. 10. The Flynn–Wall–Ozawa isoconversional diagrams

*Table 2. Values of the activation energy obtained by the Friedman (Fd), Flynn–Wall–Ozawa (FWO),
Kissinger–Akahira–Sunose (KAS) for the analyzed complex*

E_a kJ·mol^{-1}	Conversion degree α								
	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
FR	192.3 ±1.6	194.1 ±1.8	196.8 ±1.6	194.4 ±0.9	189.2 ±1.8	193.3 ±1.9	160.9 ±3.2	168.1 ±2.3	161.1 ±3.8
KAS	188.7 ±1.2	185.5 ±1.5	186.4 ±1.9	185.7 ±1.8	180.0 ±2.3	173.6 ±2.6	137.6 ±2.5	149.1 ±3.9	128.4 ±2.9
FWO	188.5 ±1.0	185.8 ±0.5	187.0 ±1.4	186.7 ±2.8	181.5 ±2.8	175.8 ±1.6	142.8 ±1.5	154.3 ±2.9	135.2 ±1.8

4. Conclusions

From thermogravimetric analysis, complexometry and elemental analysis data, a general formula could be established for the new synthesized complex in this work. As the thermal stability may be crucial to assessing applicability of new compounds, the thermal behavior of the complex is discussed in detail comparative with the one of the ligand.

The spectroscopic data suggest that the methotrexate acts as ligand toward the metal cation of Zn. The TG-DTG and DSC curves show for the Zn(II) complex the dehydration process occurs in two consecutive steps and the thermal decomposition of the anhydrous compound takes place in a continuous process composed of two significant steps. This fact is sustained by the different E_a values function of α, the result being interpreted as overlapping reactions with a complex mechanism.

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