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Research Article

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Effect of some novel metallocefepime on neurotransmitter catalyzing enzymes

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ABSTRACT

The effect of some prepared cefepime metal complexes with Cr(III), Mn(II), Fe(III), Co(II), Cu(II), Zn(II), Cd(II), Hg(II) and mixed metals (Fe,Ni) or (Cu,Fe) on the activity and kinetics of purified acetyl cholinesterase (AChE) and monoamine oxidase (MAO-B&A) were investigated. The brain AChE and MAO-A & B from Egyptian Mediterranean buffalo (Bas Buballus) was purified by ammonium sulphate precipitation, Sephadex G-25, and Sephadex G-100. The effect of these prepared metallo cefepime on the activity of pure AChE and also (MAO-B&A) were carried out in vitro by using standard colorimetric assays. The results revealed that AChE was inhibited by Mn(II) ,Cu(II) with different molar ratios(Cu:Cefepime) [(1:3)&(4:1)] ,Zn(II) and mixed metal [Fe(III),Ni(II)] cefepime complexes. Among the target compounds, Mn-cefepime complex showed the highest inhibitory activity 66.6% towards AChE with IC_{50} 945µM. The Lineweaver–Burk plot of the inhibition of AChE by the investigated inhibitors indicates a pattern of inhibition of uncompetitive type , On the other hand, Zn-cefepime complex could be accounted as non-competitive inhibitors to AChE, where it caused decrease in V_{max}– value and did not alter the K_m Also, the inhibition of MAO-B by Cr(III), Cu(II), Mn(II) and mixed metals [Fe(III),Ni(II)] &[Fe(III),Cu(II)] cefepime complexes was uncompetitive type. Mn-cefepime complex showed the highest inhibitory activity 96.36% towards MAO-A, while mixed metal complexes [Fe(III),Ni(II)] &[Fe(III),Cu(II)] cefepime complexes gave the highest inhibitory activity 75.39% towards MAO-B . The K_i and IC_{50} values for metallo cefepime inhibitors were evaluated. Therefore, some of synthesized metallocefepime can be used for preventing neurodegenerative diseases such as Alzheimer´s disease.

Keywords: Metallocefepime, metal complexes, AChE, MAO, Alzheimer´s disease.

INTRODUCTION

Acetylcholinesterase (AChE) is one of the important brain enzymes that influence the neurotransmitter (acetylcholine) levels in which the low level of ACh in the brain may led to neurodegenerative disorder Alzheimer's disease (AD) [1-6]. Fe, Cu and Zn metals play a role in pathogenesis of AD [7], high levels of Cu and Fe in brain catalyze the production of reactive oxygen species (ROS), which further elicit oxidative stress contributing to the AD pathogenesis [1,8]. Thus, metal chelators act as therapeutic approach for halting AD pathogenesis. The mainly used AChE inhibitors for treatment of AD are donepezil, rivastigmine and galantamine drugs, which could only enable a palliative treatment instead of preventing the neurodegeneration [9-10].

Monoamine oxidase (MAO) has two isoforms, A and B, which differ in their selectivity for substrates, inhibitors, and cellular localization and that are encoded by two separated genes [11]. MAO-A preferentially oxidizes serotonin, adrenaline, noradrenaline and is irreversibly inhibited by low concentration of clorgyline. Its defects have been linked to depression and abnormally aggressive behavior. MAO-B selectively oxidizes β-phenylethylamine and benzylamine and is reversibly inhibited by low concentration of deprenyl [12-13]. Both isoforms oxidize dopamine [14-15]. MAO-B expression in the brain increases with aging and may be linked to some disorders such as Alzheimer's and Parkinson's disease [16,17].

Parkinson's disease is often treated with L-DOPA, the precursor amino acid to dopamine; the addition of MAO-B inhibitor such as deprenyl dramatically increases its neuroprotective effects [18-20]. New inhibitors for MAO-B or for both isoforms were investigated such as N-methyl-2-phenylmaleimide, dehydroepiandrosterone [21], anthocyanins from berry fruits [22], and indole and benzofuran derivatives [23].

Cefepime is a parenteral cephalosporin that has been described as a fourth generation broad-spectrum antibiotic [24-25]. It is active against some bacteria that are resistant to other antibiotics and it is used to treat gram-negative and gram-positive bacteria especially those causing infections in the lungs, kidneys, bladder, skin, and abdomen [26-27]. Cefepime interacts with transition metal(II) ions to give [M(cefepime)Cl_2] complexes (M= Mn(II), Co(II), Ni(II), Cu(II) and Zn(II)) which were characterized by physicochemical and spectroscopic methods. The cefepime metal complexes have been screened for antibacterial activity against several bacteria and showed activity less than that of free cefepime [27-30]. The aim of this work is to develop a new cefepime metal complexes as AChE or MAO inhibitors for treatment of Alzheimer disease. Otherwise, if some of these compounds act as activators, they may be used as anticancer drugs since they induce apoptosis through increasing the damage of lipid, protein, and DNA.

EXPERIMENTAL SECTION

2.1 Chemicals: Acetylthiocholine iodide (ACTI), 5, 5‘- dithiobis 2-nitrobenzoic acid (DTNB), Sephadex G-25,Sephadex G-100, and diethyl aminoethyl-cellulose (DEAE-cellulose), benzyle amine, P-Tyramine cefepime and metal chlorides were purchased from Sigma Chemical Company (St. Louis, Mo., USA).

2.2 Synthesis of solid cefepime metal complexes:
The simple metal–cefepime complexes were prepared by mixing the molar amount of the metal salts Cr(III), Mn(II),Fe(III), Co(II),Ni(II),Cu(II), Zn(II), Cd(II) and Hg(II) as chloride dissolved in 10 ml water with the calculated amount of the cefepime , while the hetero cefepime metal complexes Fe(III) M(II), where [M(II)=Ni(II) and Cu(II)] were prepared by dissolving 1mmol of Fe(III) and 1mmol Ni(II) chloride or Cu(II) in 10ml , the resulting solution was then added to cefepime (1mmol in10ml). The mixture was refluxed for about 5 min. The complexes were precipitated and were filtered, then washed several times with a mixture of EtOH-H_2O and dried in a desiccator over anhydrous $CaCl_2$ .The metal ion contents were determined by complexmetric titration procedures [31]. The halogen content was determined by titration with standard $Hg(NO_3)_2$ solution using diphenyl carbazone indicator [32]. The analytical data, fundamental infrared and electronic spectra of the prepared cefepime metal complexes represented in Table 1 and the proposed structures of synthesized metal complexes illustrated in Figure 1.

2.3 Preparation of Crude Acetyl cholinesterase Enzyme Extract:
Fresh brain tissue was obtained from male *Egyptian Mediterranean buffalo* (*Bos Buballus*) immediately after slaughter and washed with ice-cold phosphate buffer (pH 7.6, 0.1 M), containing NaCl (0.2M) and Na_2EDTA (0.001M). Then, the brain tissue was stored under ice-cold toluene for three days in a refrigerator. The brain tissue was washed again with ice-cold phosphate buffer and distilled water three times. Then, the brain tissue (300g) was homogenized with four volumes cold 0.1 M phosphate buffer (pH7.6) containing NaCl (0.2M), Na2 EDTA (0.001M), 5mM protease inhibitor cocktail and 0.5% Triton X-100 using a Polytron (Tekmar model, TR 10, Germany). The homogenate was centrifuged at 6000 rpm for 30 minutes using cooling centrifuge at 4 °C (Hitachi, Germany). The clear supernatant was collected and used as a crude preparation of AChE [33].

2.4 Partial Purification Procedure of Brain Acetyl cholinesterase:
All the procedures of purification were carried out at 2-4 °C in a cold room. The collected crude extract of AChE was submitted to purification process as follow, solid ammonium sulphate was added (17.6g/100ml) slowly with constant stirring to supernatant collected from the previous step to obtain 30% saturation .The solution was

centrifuged immediately at 6000 r.p.m at 4°C for 20min. Ammonium sulphate (27g/100ml) was added to the supernatant to bring 70%. This solution was stored at 4°C for 3h and then centrifuged at 6000 r.p.m at 4°C for 20 min to obtain the precipitate, then making a sucrose dialysis process for the precipitate collected from the previous step (24h) for passing them to sephadex G-25 gel column (15x1cm) previously equilibrated with sodium phosphate buffer (0.1M, pH7.6).The enzyme was eluted with the same equilibration buffer .The fractions showing acetylcholinesterase activity were polled together [33].

2.5 Protein Assay:
Protein was assayed in the brain using bovine serum albumin as a standard protein [34].

2.6. Acetylcholinesterase Activity Assay:
The AChE activity assay was carried out using an acetylthiocholine iodide substrate, based on colorimetric method [35].Cefepime metal complexes were dissolved in DMSO. In ELISA plate (Bio Tec. USA), 151µl of phosphate buffer (pH 8.0) was directly put in ELISA blank well and 131µl of phosphate buffer (pH 8.0) was directly put in ELISA activity wells. Then to the blank and activity wells, 5µl of substrate

ACTI (75 mM) was added and then 20µl of enzyme was added in Activity ELISA wells only. The plate was preincubated for 15 min at 37ºC before the addition of the second substrate (0.32 mM) DTNB, then 60µl of DTNB was added in both the blank and activity wells. Finally, absorbance was measured at 405 nm every two minutes.

Specific Activity= [A] x [Total volume in cuvette (µl)] / ([Molar extinction coefficient of DTNB] x [Volume of brain extract (µl)] x [Protein concentration (mg/ml)])

Where: -Specific activity = moles of substrate hydrolyzed /minute / mg of protein.

A = change in O. D. per minute = Slope

Molar extinction coefficient of DTNB = 1.36×10^4

Volume of brain extract = 20µl

Volume in cuvette = 20µl [vol. of brain extract] +131µl [vol. of phosphate buffer, pH 8.0] + 60 µl of DTNB + 5µl of Acetylthiocholine Iodide = 216 µl.

2.7 Determination of AChE kinetic parameters:
The type of enzyme inhibition exerted by the synthesized metal complexes can be determined from the kinetic studies by using different substrate concentrations (7.5-75mM). The data were plotted by the method of Lineweaver–Burk to reveal the mechanism of inhibition. Plots of 1/rate versus the inhibitor concentrations gave an estimate of K_i, the dissociation constant of inhibitor to AChE [36]. The inhibition was calculated by using the following equation: % inhibition= {[Activity of control – Activity in presences of 1 mg/ml inhibitor]/ activity of control}X 100.

2.8 Preparation of brain MAO-B &A:
Fresh brain tissue was obtained from male *Egyptian Mediterranean buffalo* (*Bos Buballus*) immediately after slaughter .The brain was rinsed thoroughly in cold saline (0.9% NaCl), then homogenized in four volumes(W/V) of 0.25 M sucrose, 0.1 M sodium phosphate buffer (pH 7.4) in a Teflon glass homogenizer. The homogenates were centrifuged at 6000 r.p.m for 10min. The supernatant fraction was divided into 3ml portions in small screw-cap vials and kept frozen for later assaying of MAO [37].

2.9 Assay of MAO-B &A:
MAO activity toward benzylamine(MAO-B) and P-Tyramine (MAO-A) was determined by taking 150 µl of the complex and and 300 µl brain homogenate solution,the mixture was incubated 45 min, then take 150 µl of previous mixture, 133 µl potassium phosphate buffer(pH 7.6) and 667 µl of benzylamine or P-Tyramine(500µM) [38]. The reaction was started by the addition of benzylamine or P-Tyramine and the progress of the reaction (formation of benzaldehyde) was monitored at 250 nm. Initial velocities as ΔA/min were measured from the time scanning of the

reactions at 250 nm, ε (M^{-1} cm^{-1}) 12,500.The maximum velocity was expressed as micromole per milligram protein per minute.

MAO activity (U/l) =ΔA x total volume x 1000/ 32.2 x sample volume x 0.5.

2.10 Determination MAO-B kinetic parameters:

The effects of different concentrations from Cr(III),Mn(II),Cu(II) and the two mixed metals cefepime complexes on MAO-B activities were determined. The enzyme MAO-B was preincubated with each complex for 45 min at 30 °C and then MAO-B activity was assayed and compared with the control in which the enzyme was replaced by an identical volume of buffer. The remaining MAO-B activities were expressed as percentages of control basal activity and plotted against concentration. In case of inhibition, the IC_{50} is calculated by nonlinear regression, which is defined as the concentration necessary to give 50 % enzyme inhibition [39]. Steady state kinetic constants (Michaelis constant, K_m, and maximum velocity, V_{max}) were determined from studies on the effects of different substrate concentration (3.3–50 μM benzylamine) on the initial velocity of MAO-B in the absence and in the presence of each complex. Lineweaver–Burk plot [36] was used to determine K_i and the kinetic parameters of MAO-B

RESULTS AND DISCUSSION

3.1 Chemistry:

The IR and nujol mull electronic spectra of cefepime and its metal complexes are recorded in Table1. The lactam (C=O) band appears at 1772 cm^{-1} in the spectrum of cefepime , while the complexes show this band as shifted and overlapped with the amide carbonyl band due to formation of hydrogen bond and another bands at 1653 cm^{-1}, which is corresponding to the stretching vibrations of C=O of the amide . The band at 1632 cm^{-1} corresponding to the carboxylate asymmetrical stretching of the free ligand, is shifted to lower wavenumbers in the spectra of the complexes indicating coordination through that group [40-41]. A carboxylate ligand can bind to the metal either monodentate or bidentate, giving changes in the relative positions of the antisymmetric and symmetric stretching vibrations [42]. The presence of ν(M–N) stretching vibrations in the 423–499 cm^{-1} range for the metal complexes (absent in the free ligand) provide evidence that the moiety is bonded to the metal ion through nitrogen. These results suggest coordination by the ligand as a multidentate chelating agent via Oh, Td and square planar geometry. Bands in the 402-460 cm^{-1} region observed in the complexes, and absent in the free cefepime, are tentatively assigned to ν (M–O) vibrations. The supposed structures of cefepime metal complexes (1, 9 and14) as a representative example of synthesized metalocefepime are shown in Figure1.

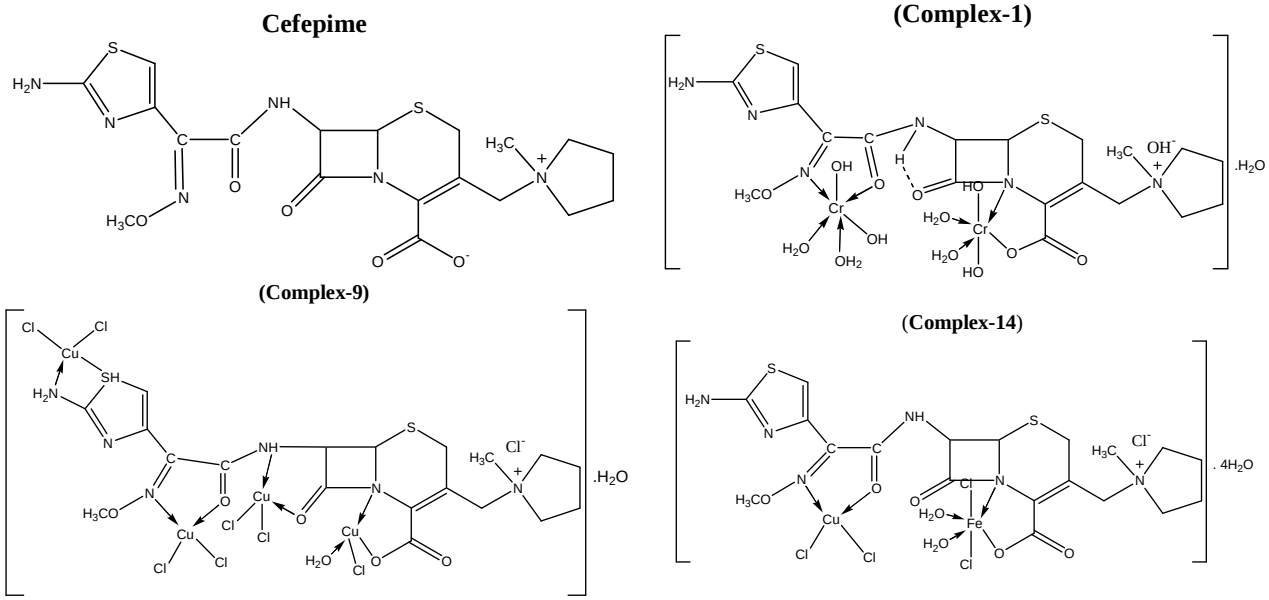


Figure (1) Proposed structures of cefepime and its metal complexes(1,9&14)

Table 1: Analytical data, Fundamental infrared , electronic spectra and geometry of the prepared cefepime metal complexes

No	Complexes	M%	Cl%	$\nu_{(CO)}$ lactam	$\nu_{(CO)}$ amide	$\nu_{(COO)}$ asymmetric	$\nu_{(COO)}$ symmetric	ν_{M-N}	ν_{M-O}	ν_{M-Cl}	Electronic spectra λ(nm)
	Cefepime	-	-	1772	1653	1632	1382	-	-	-	
1	$[Cr_2$ (Cefepime)$(OH^-).4H_2O]$ H_2O	13.69	-	-	1650	1523	1355	423	403	-	225,247,280, 362,379
2	$[Mn_2$ (Cefepime)$_3$. $(OH^-)_4]$	6.67	-	-	1623	1542	1392	460	402		333,354
3	[Fe (Cefepime)$_3$.Cl_3]. $4H_2O$	3.27	6.26	-	1653	1558	1395	478	418	391	389,463,567
4	[Fe(Cefepime).Cl_4. $2H_2O$].$2H_2O$	7.62	14.51	1774	1653	1560	1361	499	457	409	309
5	$[Co_2$(Cefepime). $(OH^-)_4.H_2O]$	17.22	-	-	1634	1545	1382	464	421	-	360,473,569
6	[Ni (Cefepime). Cl_2. $H_2O]$.$5H_2O$	8.17	9.75	-	1650	1621	1391	491	445	370	324,354,605
7	[Ni (Cefepime)$_2Cl_2$].$6H_2O$	4.90	5.91	1776	1650	1623	1385	481	460	393	360
8	[Cu (Cefepime)$_3$.$(OH^-)_3$].H_2O	4.08	-	-	1657	1544	1384	460	418	-	310,329,421
9	$[Cu_4$ (Cefepime).Cl_8..H_2O]..H_2O	24.08	26.87	1769	1630	1606	1369	491	432	370	320,350,490, 580
10	[Zn (Cefepime). $H_2O.Cl_2$].$5H_2O$	9.02	9.87		1634	1539	1384	434	401	391	340
11	[Cd (Cefepime).$(OH^-)_2.H_2O$]	17.43	-	-	1650	1621	1391	465	401	-	373,466,566
12	[Hg (Cefepime).Cl_2]. $6H_2O$	14.96	5.29	-	1646	1530	1387	464	434	335	336
13	[Fe Ni (Cefepime)$_3$.Cl_5].$2H_2O$	3.13 3.29		1774	1662	1630	1359	471	428	373	335,390
14	[FeCu(Cefepime). $Cl_3.2H_2O$] .$4H_2O$	Fe 6.31 Cu 7.18	9.94	1775	1658	1628	1358	470	422	374	289,340,430

3.2 IC_{50} and AChE inhibition kinetics:

Specific activities of all investigated compounds towards AChE are summarized in Table 2. It could be seen from the table that the complexes (1,2,6,8,10,11,13,14)showed inhibitory effect toward AChE, when compared with cefepime and control , while the complexes (3,4,5,12) acts as activators . Among the target compounds, Mn-cefepime complex (complex-2) showed the highest inhibitory activity 66.6% (Figure 2) with IC_{50} 945µM. Moreover, the IC_{50} for other inhibitos was 945.31, 474.45, 932.75, 1425.59 and 91.05 µM for complexes 8,9,10,13, repectively Table 3. The Lineweaver–Burk plot of inhibitors 2,8,9,10 and 13 (Figure3) indicates uncompetitive inhibition pattern with Ki values of 1011.17, 904.52, 646.74, 1099.15 and 580.28 µmol/min/mg/protein, respectively. On the other hand, complex-10 could be accounted as non-competitive inhibitors to AChE, where it caused decrease in V_{max}– value and did not alter the K_m-value [43].

Table 2: Effect of cefepime and its metal complexes on MAO (A& B) and AChE

No	Specific Activity (µmol/mg.protein/min)		
	MAO-A	**MAO-B**	**AChEx10^{-5}**
Control DMSO	4.93±0.01	4.99±0.00	4.96±0.3
Control H_2O	0.789 ±0.00	5.26±0.025	5.54±0.4
Cefepime	0.00	28.28±0.1	6.22±0.9
1	0.197±0.00	0.179±0.00	0.25±0.1
2	0.179±0.00	1.31±0.026	2.71±0.1
3	0.591±0.00	10.32±0.024	9.38±2.1
4	0.00	47.15±0.47	6.18±1.5
5	0.789±0.00	18.02±0.01	6.89±2.7
6	12.430±0.04	4.01±0.01	4.2±0.4
7	0.00	5.19±0.042	7.42±1.7
8	0.394±0.01	16.04±0.01	1.45±0.00
9	1.381±0.48	0.098±0.06	0.45±0.00
10	0.394±0.00	15.52±0.00	3.33±1.1
11	0.986±0.00	7.76±0.01	4.47±0.41
12	0.00	14.00±0.05	4.9±2.67
13	2.565±0.00	3.02±0.09	0.218±0.21
14	3.354±0.05	1.77±0.01	3.79±3.7

Table 3: Kinetic parameters of AChE in absence and presence of inhibitors

Inhibitors	K_m(µM)	V_{max}(µM)	K_i(µmol/min/mg/protein)	IC_{50}(µM)
Control	20.77	0.0014		
2	18.56	0.0024	1011.17	945.31
8	112.65	0.0021	904.52	474.45
9	29.20	0.0010	646.74	932.75
10	20.25	0.0011	1099.15	1425.59
13	8.44	0.0015	580.28	91.05

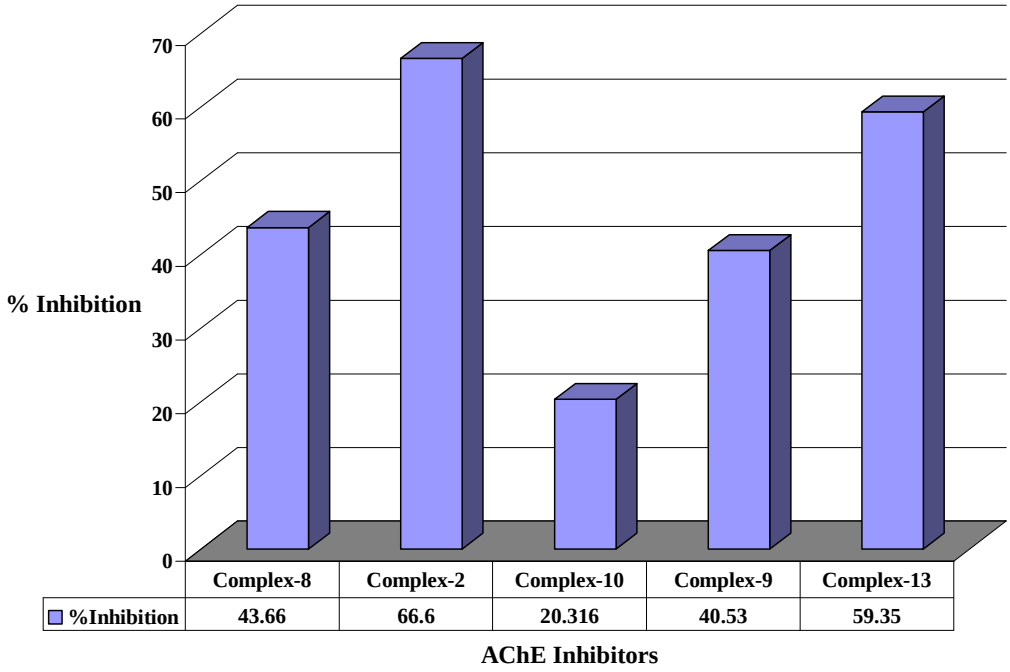


Figure (2): Percent inhibition of AChE activity in the presence of synthesized cefepime metal complexes (2, 8, 9, 10 and 13)

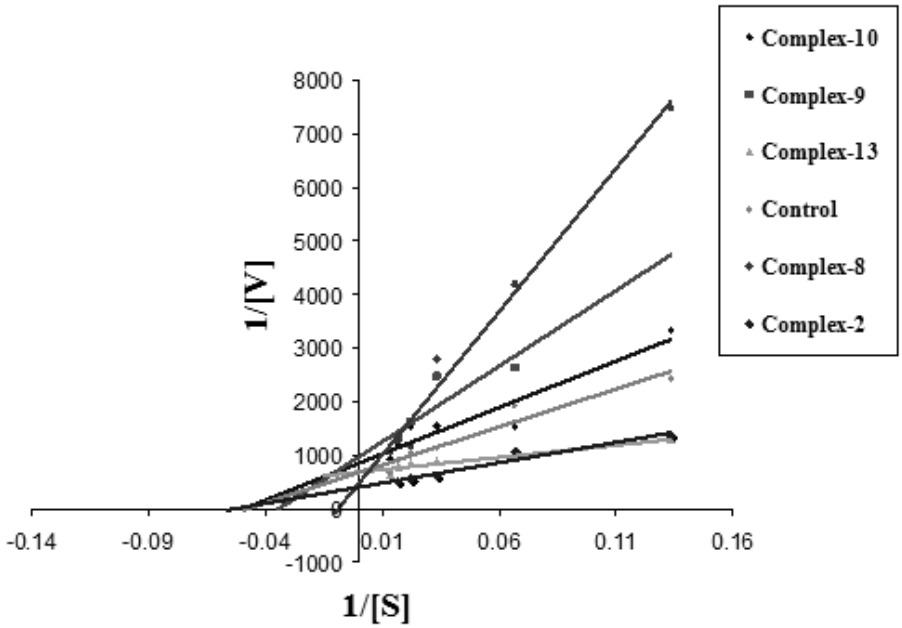


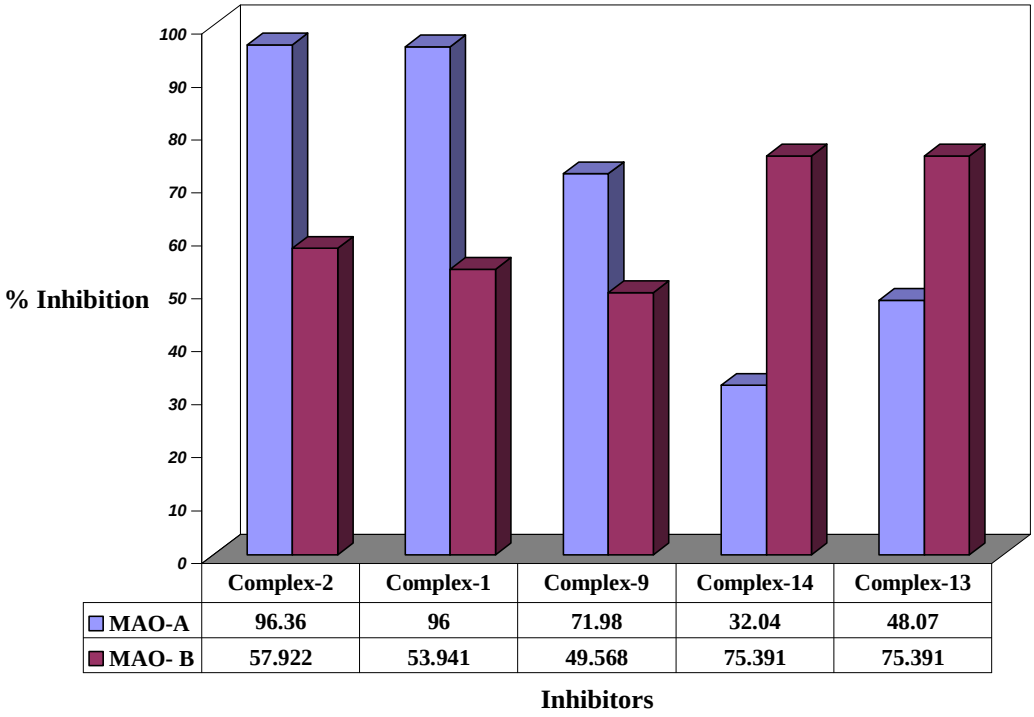
Figure (3): Double reciprocal 1/V versus 1/[S] lineweaver Burk plot for the partially purified AChE in absence and presence of synthesized cefepime metal complexes (2, 8, 9, 10 and 13). The data were analyzed by Lineweaver-Burk plot and the values of K_m and V_{max} were averaged

3.3 Inhibition of MAO-B basal activities and determination of enzyme kinetic parameters:

Specific activities of all investigated metallocefepime towards MAO(A&B) are summarized in Table2. The result revealed that the complexes (1,2,9,13,14) showed inhibitory effect toward MAO(A&B) compared with cefepime and control and the complexes (3,4,5,7,8,10,11&12) were inhibitors for MAO-A only. Among the target compounds, Mn-cefepime complex (complex-2) showed the highest inhibitory activity 96.36% (Figure 4) against MAO-A, while complexes(13&14) gave the highest inhibitory activity 75.39% against MAO-B. The Lineweaver–Burk plot of the inhibition of MAO-B by the inhibitors 1, 2,9,13 and14 (Figure 5) indicates a pattern of inhibition of uncompetitive type with Ki = 184.88, 468.36, 436.4, 137.97 and 569.64 µmol/min/mg/protein, respectively .The IC_{50} (concentration causing 50% inhibition of MAO-B) of the previous complexes were determined to be 794.62, 149.19, 713.13, 196.77 and 1112 µM, respectively. Table 4 [44-45].

Table 4: Kinetic parameters of MAO-B in absence and presence of inhibitors

Inhibitors	K_m(µM)	V_{max}(µM)	K_i(µmol/min/mg/protein)	IC_{50}(µM)
Control	1.72	24.44		
1	6.88	10.28	184.88	794.62
2	1.75	18.51	468.36	149.19
9	3.12	11.26	436.4	713.13
13	7.32	6.01	137.97	196.77
14	2.04	12.33	569.64	1112



Figure(4) Percent inhibition of MAO (A&B) activity in the presence of synthesized cefepime metal complexes (1, 2, 9, 13 and 14)

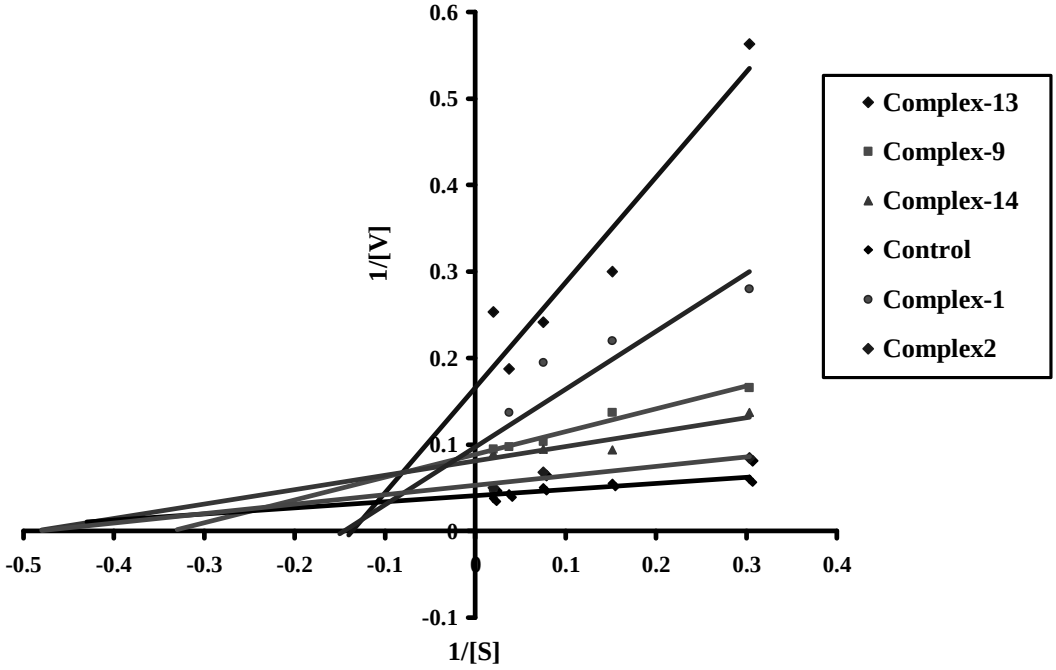


Figure (5): Double reciprocal 1/V versus 1/[S] lineweaver Burk plot for MAO-B in absence and presence of synthesized cefepime metal complexes (1, 2, 9, 13 and 14) at concentration of 1 mg/ml. The data were analyzed by Lineweaver-Burk plot and the values of K_m and V_{max} were averaged

CONCLUSION

Metals were proved to be a good chelating agent and antibiotics were considered as useful ligands in the formation of metalloantibiotics. Physical, chemical and biological changes in cefepime antibiotic were seen after combining with metals as metallocefepime, which act as therapeutic approach for halting AD pathogenesis in comparison with cefepime only. Thus , metal chelators make improvement in the parent to act as an inhibitor. Among the fourteen metal cefepime complexes, the Mn, Cu (4:1) and mixed metal Fe Cu cefepime complexes were found to be inhibitory against both AChE and MAO (A&B), while Cu (1:3) & Zn (1:1) complexes act as AChE inhibitor and Cr (2:1) and mixed metal Fe Cu complexes as MAO-B inhibitors, so these synthesized metallocefepime can be used as Alzheimer disease therapeutic agent.

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