The Environmental Effects of Lead Concentrations on Protein and DNA Structures in Epileptic Patients from an Infrared Spectroscopic Study

Jane Anastassopoulou[1,*], Maria Kyriakidou[2], Pavlos Nisianakis[3], George Papatheodorou[4], Michail Rallis[5] and Theophile Theophanides[2]

[1]*International Anticancer Research Institute, 1st km Kapandritiou-Kalamou Road, P.O. Box 22, Kapandriti, 19014 Attiki, Greece*

[2]*National Technical University, Chemical Engineering School, Radiation Chemistry & Biospectroscopy, Zografou Campus, 15780 Athens, Greece*

[3]*Center of Biological Research of Armed Forces, 6 Tax. Veliou, P. Penteli, 15236 Attiki, Greece*

[4]*401 Army General Hospital, 138 Mesogion Ave., 11525 Athens, Greece*

[5]*Department of Pharmacy, Pharmaceutical Technology, School of Health Sciences, National and Kapodistrian University of Athens, Panepistimiopolis, 15784 Athens, Greece*

Abstract: Fourier transform infrared (FT-IR) and inductively coupled plasma mass spectrometry (ICP-MS) elementary analysis were used to investigate the environmental effects of lead blood serum levels on the life metal ions (Cu^{2+} and Zn^{2+}), protein secondary structure and DNA structure in epileptic patients. By increasing the lead concentration an increased intensity of the band at 1744 cm^{-1} was observed due to induced oxidative stress. The shifts of the amide I and amide II bands of the peptide group, -CONH- from 1655 cm^{-1} and 1550 cm^{-1}, respectively, to lower frequencies is due to the change of protein molecular structure from α-helix to β-sheets. An important change in the spectral region between 1200-900 cm^{-1}, where the phosphates and phosphate-ribose groups of DNA and RNA are absorbing, is suggesting an attack on the DNA backbone as a function of the increase of lead concentration. The characteristic band at 1170 cm^{-1} could be used as a "marker band" for the damaged DNA backbone structure upon lead exposure. The ICP-MS elementary analysis showed a decrease of the ratio [Cu/Zn] by increasing the lead levels in blood serum is linked to oxidative stress and is confirming the FT-IR data.

Keywords: Infrared spectroscopy, ICP-MS, Environmental pollution, Lead, Epilepsy, oxidative stress.

INTRODUCTION

Analysis of human tissues and organs have shown that many of metal chemical elements, called the life metals, are "essential" for the human body. The metals are natural chemical elements and they are essential in living processes since the origin of life. The metals constitute almost 80% of all the chemical elements of the Periodic Table and can be divided in three categories: 1) The life metals that are necessary for our health in the cells that are the metal ions found in every human cell, which are sodium (Na^+), potassium (K^+), magnesium (Mg^{+2}) and calcium (Ca^{2+}). In this category are included also some transition metal ions, such as, iron in haemoglobin, copper in copper-proteins, vanadium, chrome, manganese, zinc, cobalt, molybdenum found in various enzymes. Both excess and deficiency of these metals from normal natural concentrations can cause pathological changes and diseases. 2) There are the metals that have been used in pharmaceuticals for several diseases, such as the antitumor coordination complex, cis-platinum, i.e. cis-$Pt(NH_3)_2Cl_2$, discovered in 1965 by Barnett Rosenberg, gold complexes for arthritis, etc. 3) There are the very toxic metals, such as arsenic (As), mercury (Hg), cadmium (Cd) and lead (Pb), which are in the environment of our planet and the biological molecules can react with them and cause changes in their structures, which can lead to damage to our health. There is a clear evidence since Ancient Roman times that exposure to lead has shown a toxic affinity to human tissues and nervous system. Lead (Pb) is not an essential nutrient to plants and animals and is released to the environment from anthropogenic activities, cars, industries, etc. and enters to our bodies by breathing, drinking and eating. Although Pb as fuel additive has been eliminated in many countries, it is still used in Pb-acid batteries, and Pb-containing paints and other products. Many studies have shown that exposure to Pb, even at low concentrations, was associated with various non-reversible neurological disorders, such as abnormal

*Address correspondence to this author at the International Anticancer Research Institute, 1st km Kapandritiou-Kalamou Road, P.O. Box 22, Kapandriti, 19014 Attiki, Greece; Tel: (+30) 6973013308;
E-mail: ianastas@central.ntua.gr, i.anastassopoulou@gmail.com

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behavior, psychosis and epilepsy [1-4]. Pb is neurotoxic and promotes the haemoglobin oxidation to methaemoglobin, accelerating the formation of superoxide anion radicals, ($O_2^{-\cdot}$) [5].

Epilepsy is a common chronic brain disease, which affects an increasing amount of populations of all ages worldwide [3] and is the third risk of death after strokes and Alzheimer. Epilepsy is characterized by spontaneous recurred epileptic seizures leading to neuronal death and neurogenesis [4]. Epilepsy can be divided into idiopathic and secondary or symptomatic. There are many heterogenic factors that induce the disease.

Fourier transform infrared (FT-IR) spectroscopy has gained some attention from the medical community as a valuable tool in a non-destructive (non-invasive) characterization and identification of the molecular features of the very complex systems such as the human tissues and liquids. FT-IR is a very easy technical tool, sensitive and cost effective early diagnostic method. The FT-IR spectra are produced when infrared light falls and interacts with matter. It depends on the vibration of atoms in the molecules or biomolecules, where infrared light is absorbed from these molecules, the biological molecules in the present case [6]. The FT-IR spectra provide a wealth of information not only about the changes that take place in the molecular structures but also about the environment of the biological molecules in healthy cells. The FT-IR spectra are very characteristic of the molecules and their structures in addition to the "fingerprint" region of the characteristic changes induced to these biological molecules during the disease progression [7]. Based on mid-infrared spectra in the region 4000-400 cm^{-1} and results of the literature [6-13] on normal and malignant tissues showed that the FT-IR spectra could be used in clinical trials for diagnosis of cancers and other diseases or several disorders [7,14,15]. FT-IR spectra of excellent quality can be taken easily of tissues and other body liquids without any pre-preparation of the samples, such as coloring or subtraction of the metal ions with EDTA today. This is a major advantage of the application of ATR-FT-IR spectroscopic technique for an early diagnosis of neurodegenerative diseases much faster than any histopathological assessment of diseases by using a minimum amount of sample (10μg) and preparation required in order to obtain very good infrared spectra for a diagnosis [6-15]. In the present work we used FT-IR spectroscopy as well as ICP-MS elementary analysis in order to investigate protein and DNA structural changes upon lead presence and its concentrations.

MATERIALS AND METHODS

Samples

For the study it was used serum from 15 epileptic patients (age 18-24 years) and 10 healthy donors (age 18-24 years) for comparison purposes. Immediately after the whole blood collection, serum was separated with centrifugation from plasma and it was freeze- dried in order to obtain the ATR- FT-IR spectra and ICP-MS elementary analysis.

Statement of Ethics

The samples were taken according to Helsinki rules and the Greek low of ethics for *ex-vivo* clinical research studies.

Infrared Spectroscopic Method

The ATR-FT-IR spectra were recorded with Nicolet 6700 thermoscientific spectrometer coupled with Attenuated total reflection (ATR) crystal (See Figure **1**).

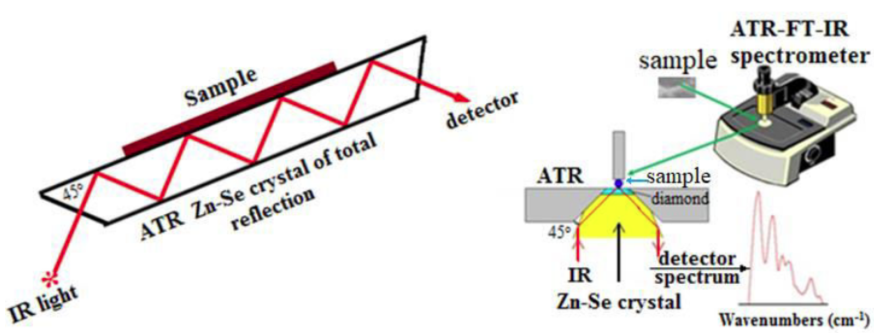


Figure 1: Schematic presentation of attenuated total reflection (ATR)- Fourier Transform Infrared technique. The Infrared light passes through a zinc-selenide (Zn-Se) crystal and after multiplication of the reflections (insert) the beam is collected by a detector

Table 1: Digestion Conditions (Milestone Start D): After Cooling, Dilution to 100 mL was Performed

	Temp (°C)	Time (min)	Power (W)
1	200	20	1200
2	200	200	1200

By using the ATR apparatus the IR light passes through a Zn-Se crystal and after multiplication of the internal reflections of the sample the beam reflections were collected by a detector and transformed by Fourier Transform to a spectrum. The diamond crystal of the instrument increases the ratio of signal to noise and thus minimizes the size of the sample. Modern infrared spectrometers are equipped with Attenuated Total Reflection apparatus and diamond crystal, which allows the detection of even very small sample amounts. A very small amount of the freeze- dried serum was added on the ATR crystal's surface (Figure **1**). Each plot consisted of 120 co-added spectra at a spectral resolution of 4 cm^{-1} and the OMNIC 7.2α software was used for data analysis.

Metal Chemical Elementary Determination

The elementary concentrations of the serum of essential and toxic metals were determined by using ICP-MS (Thermo Fisher Scientific, iCAP Qc) in KED (kinetic energy discrimination) mode with internal standards for biological samples. The protocol standards were used for the multi- element analysis and the metals ^{39}K, ^{23}Na, ^{24}Mg, ^{43}Ca, ^{95}Mo, ^{57}Fe, ^{78}Se, ^{66}Zn, ^{63}Cu and ^{208}Pb were analyzed.

For each patient 0.1-0.2 g of dry serum were diluted in 4 mL HNO_3, 1 mL H_2O_2, 4 mL water in a digestion vessel. The digestion conditions are shown in Table **1**.

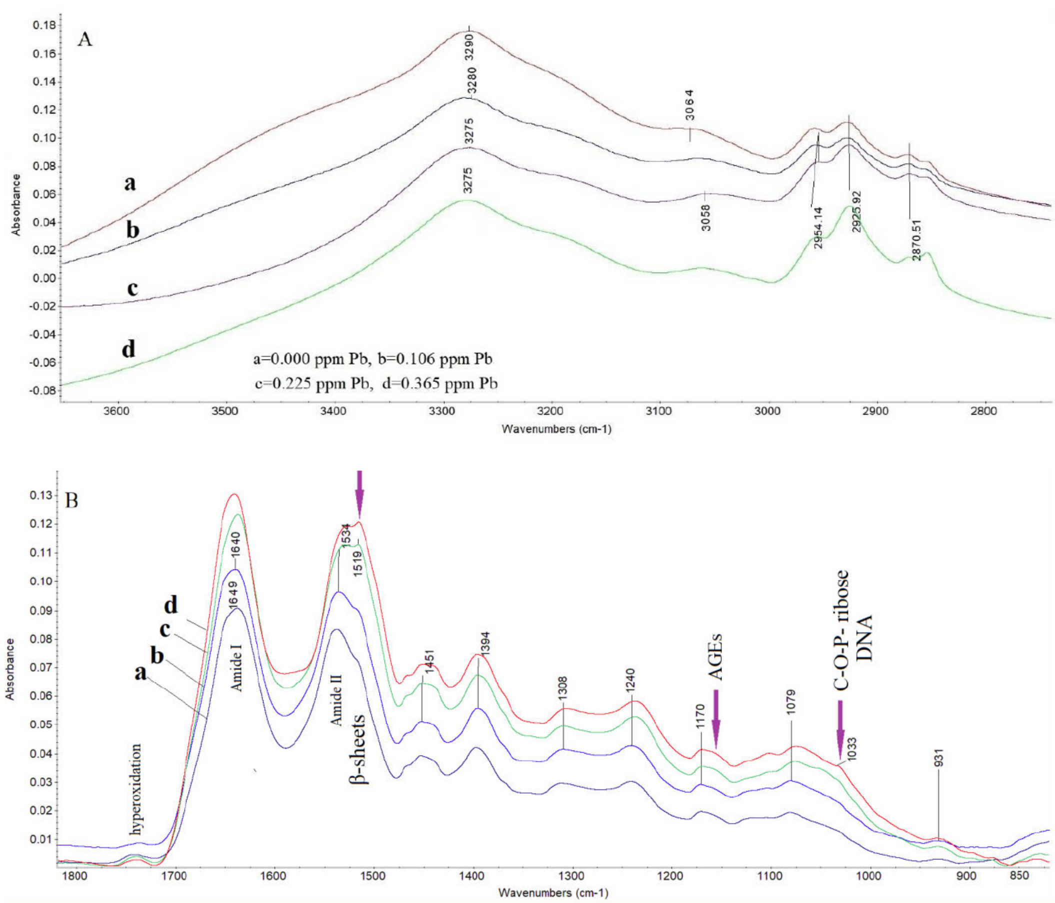


Figure 2: Representative FT-IR spectra of freeze-dried serum from epileptic patients obtained in room temperature. The curves a, b, c, d correspond to control and with lead concentrations 0.365, 0.225 and 0.117 mg/kg or in (ppm), respectively, in the spectral region A, 3600-2800 cm^{-1} and B 1800-900 cm^{-1}.

Table 2: Changes of Characteristic Signature Absorption Bands upon the Increase of Lead (Pb) Concentration (ppm) in the Spectral Region 3600-1000 cm^{-1}

Control	0.106 (ppm)	0.225 (ppm)	0.365 (ppm)	Assignments
3475	↓	↓	↓	*v*OH
3290	3280	3275	3275	*v*NH, amide A
3060	3090	3090 (br)	3090 (br)	*v*NH, amide B
	3060	3060	3060	*v*=C-H olefinic
2922	2922↑	2922↑	2922↑	$v_{as}CH_2$
2852	2852↑	2852↑	2852↑	v_sCH_2
1650 marker band		1690↑	1690↑	β-sheet↑↓
	1650 ↓			amide I,α-helix
	1630↑↑	1630↑↑	1630↑↑	β-sheet
1550 marker band	1550			Amide II,
	1534↓	1534↓	1534↓	random coil
		1514↑	1514↑	β-sheet↑↑
1240	1238	1236	1236	vPO_2^-, DNA
1170 marker band				Sugar-phosphate of DNA
1165				*v*C-O-C of glycation products
1033				*v*C-O-P, ribose DNA *v*C-O-C

RESULTS AND DISCUSSION

In Figure **2A** and **B** are shown in superimposed representative spectra of freeze-dried serum at room temperature from (a) healthy donor, (b) from epileptic patient with lead (Pb) concentration 0.106 mg/kg, (c) 0.225 mg/kg and (d) 0.365 mg/kg (ppm) in the spectral region 3600-850 cm^{-1}. Comparison between the spectra revealed intensity changes, frequency shifts and shape changes of the absorption bands (Table **2**).

In the region 3600-3000 cm^{-1} (Figure **2A**) are shown the stretching vibrations of *v*OH groups of mainly water molecules and DNA- sugars (3600-3400 cm^{-1}) and *v*NH groups (3400-3000 cm^{-1}) of proteins. It is shown in Figure **2A** that when the Pb concentration increases the intensity of the bands, which correspond to *v*OH group absorptions decreases. This finding shows that the presence of Pb^{2+} cations affects the water -OH groups and thus the hydration of the cells, as well as the -OH groups of the proteins and DNA-Sugar-OH's, which may be due to hydrogen bonding and to the effect of homeostasis of the electrolytes. Dehydration of blood cells can also be induced by carbonic anhydrase activity (CA), which catalyzes the hydration of carbon dioxide to bicarbonate and protons. CA is a zinc catalyzed enzyme and its activity in the neuro systems was described by van Goor in 1940 [16]. From ICP-MS metal elementary analysis it was found that the presence of Pb^{2+} cations affects the Zn enzyme concentration. However, in the case of zinc (Zn^{2+}) cations there was not observed a clear ratio between [Pb]/[Zn], while, in the case of copper (Cu^{2+}) there was a clear decrease of the ratio [Cu]/[Zn] ions by increasing the serum Pb concentrations. Cu^{2+} and Zn^{2+} are essential metal ions and have physiological role in humans. There is evidence suggesting that zinc and copper ions function as signaling ions in the nervous system and are released from the synaptic terminals of certain neurons [17]. Thus, this finding is important, since these metals are present in Cu-Zn superoxide dismutase (SOD). This characteristic enzyme catalyzes the dismutation of superoxide anions (O_2^-) free radicals to molecular oxygen (O_2) or hydrogen peroxide (H_2O_2) and regulates the defense immune system, according to the following reactions (1) and (2): [18].

$$Cu^{2+}-SOD+O_2^{-\bullet} \rightarrow Cu^{+}-SOD+O_2 \quad (1)$$

$$Cu^{+}-SOD+O_2^{-\bullet} \xrightarrow{2H^+} Cu^{2+}-SOD+H_2O_2 \quad (2)$$

It was also observed that by increasing the concentration of Pb there was an increase of serum potassium (K^+) concentration compared with the healthy donors. From this extracellular efflux of K^+ there was an indication of dysfunction of ion channels.

It is known that in potassium channel control the potassium flux is associated with epilepsy [19-21]. In the infrared spectra the intensity of the band near 3060 cm^{-1} increases with Pb concentration and becomes more broad (Figure **2Ad**, 3100-3000 cm^{-1}). Deconvolution of this region shows that this band is consisting of two overlapping bands at 3090 cm^{-1} and 3060 cm^{-1}. The band at 3090 cm^{-1} indicates that some of the proteins have the configuration of amide B. The shift to lower frequencies is affected strongly from the lipophilic environment. For amide B the β-sheet protein structure predominates [8,22]. This means that the effect of the NH stretching of the peptide bond -NHCO- is stronger than the C=O, unlike in the amide A case in which it is reversed. The coexistence of both A and B conformations of proteins illustrates the prevalence of different hydrogen bonds that hold the protein strands together [12,13,22,23]. As it is known, the hydrogen bonding is important in stabilizing the protein helix and any modification implies that the physiological environment has changed [24]. We have found that these changes are very important and constitute a basic criterion in order to characterize the disease and its progression [7,8,14,15]. The band at about 3060 cm^{-1} may be assigned to stretching vibration mode of (ν=CH) terminal group with an olefinic c haracter [25]. This band is used as "marker band" and is related to oxidation of the aliphatic chain of membrane lipids [25]. Oxidative stress has been widely implicated in neuronal apoptosis that occurs in neurodegenerative disorders. Superoxide anion free radicals, produced during mitochondrial respiration is involved in the generation of several potentially damaging reactive oxygen species (ROS) including hydrogen peroxide, hydroxyl radical and also peroxynitrite that is formed spontaneously upon reaction of superoxide anion with nitric oxide [26].

The bands in the spectral region between 3000 cm^{-1} to 2870 cm^{-1} are assigned to the symmetric and antisymmetric stretching vibrations of methyl ($\nu_{as}CH_3$, ν_sCH_3) and to methylene ($\nu_{as}CH_2$, ν_sCH_2) groups of lipids and proteins [23,27-33]. A more detailed analysis shows that by increasing the Pb serum concentration the intensity antisymmetric and symmetric stretching vibration bands of methylene νCH_2 at 2922 cm^{-1} and 2852 cm^{-1} increases, while the stretching vibration symmetric and antisymmetric bands of νCH_3 are decreased. This finding was also obtained in many other diseases [7,29-32] indicating structural and conformation puckering of membranes due to more lipophilic surrounding medium [12,13]. Deconvolution of this region showed a new band at 2892 cm^{-1}, which is assigned to the presence of branched alkyl chains [33]. From these results it is suggested that during the disease through metabolic pathways, oxidative stress is taking place. By oxidative stress it is understood that there is a balance between endogenous oxidants and antioxidants and an excess amount of free radical production is produced chemically with an unpaired electron.

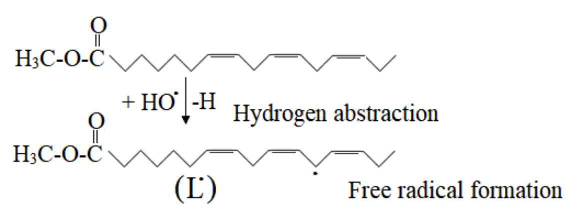
Among the free radicals, the oxygen molecule (O_2), its superoxide anion (O_2^-) and the hydroxyl radicals ($HO^\cdot$) have been found to play an important role in the progress of disease, since they react rapidly and destroy many important biological molecules (DNA, proteins and membranes). The mechanism of hydroxyl free radical ($HO^\cdot$) production was proposed originally through the Haber-Weiss and Fenton reactions [34,35]. However, the hydrogen peroxide molecules which are formed according to the reaction (2) could react with the bivalent iron cations (Fe^{2+}) of the hemoproteins or with monovalent copper cations (Cu^+) from SOD or other copper proteins, according to the following Fenton or Haber-Weiss reactions (3) and (4), respectively:

$$Fe^{2+} + H_2O_2 \rightarrow F^{3+} + HO\cdot + OH^- \quad (3)$$

$$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + HO\cdot + OH^- \quad (4)$$

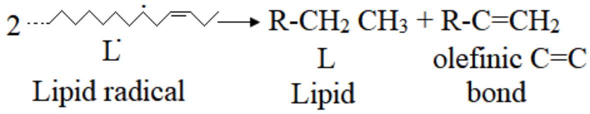
In addition, the toxic bivalent metal ions of transition metals, e.g. Co^{2+}, Ni^{2+}, Cr^{2+} are also producing hydroxyl free radicals ($HO^\bullet$) according to the above reactions (3) and (4).

The reactions (3) and (4) lead to a higher metal oxidation state (Cu^+ to Cu^{2+}, Fe^{2+} to Fe^{3+}), hydroxyl free radicals ($HO^\cdot$) and hydroxyl anions (OH^-). Moreover, the hydroxyl free radicals that are produced from the above reactions (3), (4) are very oxidizing agents with E=2.8 V and can oxidize metal ions or react with lipids by hydrogen abstraction and free radical formation in lipids as follows: [25].



(5)

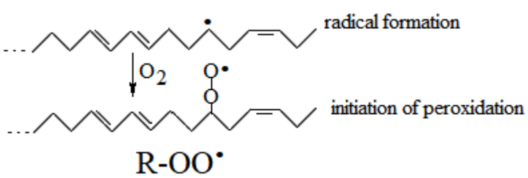
Once the lipid radicals ($L^\cdot$) are formed through reaction (5) they react with each other following the well-known dismutation reaction, which reproduces the initial lipid molecule together with one more molecule with one less hydrogen atom leading to the generation of one terminal double bond as follows:



(6)

The reaction (6) explains well the observed in FT-IR spectra increasing of the intensity of the olefinic band at 3060 cm^{-1}, which is assigned to the stretching vibration of *v*=C-H group.

The band at about 1743 cm^{-1} is characteristic for the oxidative stress and is assigned to the aldehyde (CHO) double C=O stretching carbonyl bond [31]. Since the human organism lives under aerobic conditions the oxygen (O_2), which is a double free radical ($^{\bullet}O{=}O^{\bullet}$) could react rapidly with the above carbon-centered formed free radical ($L^{\cdot}$) to generate a lipid hydroperoxyl radical (R-OO$^{\bullet}$) according to the reaction (7):

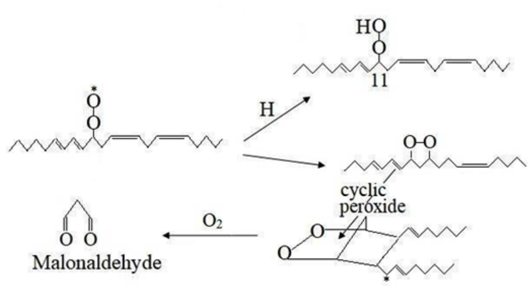


(7)

The produced peroxyl radicals by chain reactions undergo formation of tetroxide intermediates: [36].

$$2R\text{-}OO^{\bullet} \longrightarrow R\text{-}OOOO\text{-}R \qquad (8)$$

The decomposition of these intermediates yields hydroperoxides, which finally decompose to form aldehyde according to the reaction (9):



(9)

Moreover, the formed peroxyl radicals, (C-O-O$^{\cdot}$) uptake very fast mobile hydrogen (H) atoms from compounds (donors) in the cell environment, such as adjacent lipids, thiols, etc, and could produce finally hydroperoxyl groups (–C-O-OH), which are non-ionic. In general, according to free radical chemistry the lipid radicals could give degradation products, dimers or other combined products, which change the permeability of the cell membrane [7,8]. The calculated by ICP-MS reduction of selenium (Se) concentration in serum of epileptic patients confirms the reduction of the antioxidant defense systems of the patients.

The high intensity amide I band at 1650 cm^{-1}, is assigned to *v*C=O of the peptide bond (-NHCO-) of proteins [32,33,37]. This is a "marker band" for α-helical peptide bonds. This band splits upon deconvolution into three bands at 1690 cm^{-1}, 1650 cm^{-1} and 1633 cm^{-1} due to the presence of β-sheets (↓↑, anti-parallel structure), α-helix and β-sheets (↑↑, parallel structure), respectively, indicating that the secondary structure of proteins changed from α-helix to β-sheet upon the disease's progress. Nevertheless, the band at 1690 cm^{-1} in combination with the band at 3090 cm^{-1} are very characteristic for the β-sheet formation and amyloid protein identification [10-13,29-33,37]. From these bands in combination with the increase of the intensity of the stretching vibration band of *v*CH_2 it was suggested that proteins must be dissolved in more lipophilic environment in order to satisfy the specific lipophilic effect of membranes [8,15,25]. Moreover, these structural changes are associated with misfolding of the proteins leading to amyloid protein formation. Experimental clinical data showed that amyloid peptides (amyloidosis) represent a major actor in neurodegenerative diseases, such as Alzheimer's, Parkinson's and epilepsy [38].

The next intense band at about 1550 cm^{-1} (Figure **2B**) is assigned to the vibration of the amide II of the proteins, which is mainly due to *v*C-N stretching and δNH out-o-plane bending and the band is attributed to the β-turns of the protein and it suggests that the collagen has α-helix configuration. This band also is shifting to lower frequencies at 1530 cm^{-1} upon increasing the Pb concentration, indicating the presence of β-sheets (↑↑, parallel structure) conformation of proteins. The presence in the spectra of both antiparallel and parallel β-sheets conformations of proteins confirms the formation of aggregates due to the lipophilic environment as it was suggested from the absorption spectra in the lipophilic region 3000-2850 cm^{-1} [39].

Noticeable shape and intensity changes were also observed in the spectral region at 1250-900 cm^{-1}, where are found the absorption vibrational modes of the phosphates and sugar-phosphate groups of DNA and RNA. A shift to lower wavenumbers is observed for the phosphate band at 1170 cm^{-1}. From the latter band it is suggested that Pb^{2+} may bind the DNA backbone sugar-phosphate by attacking most likely the phosphate group oxygens. Infrared changes in the region 1250-900 cm^{-1} are good indicators for structural changes of nucleotide bases in backbone of DNA. Thus, the band at 1170 cm^{-1} could be used as a "marker band" for DNA backbone damage upon lead presence in the environment of DNA [15,39]. The patern of the spectra in the region 1100-1200 cm^{-1} shows an increas in band intensity of 1165 cm^{-1}, which could correspond to advanced glycation end-products (AGEs). This band is an indicator of protein glycation and oxidation [7] concerning the inhibition of CuZn-SOD antioxidant activity, due to the presence of Pb^{2+}, as it was mentioned above.

CONCLUSIONS

From FT-IR spectroscopic data as well as from ICP-MS elemental analysis it was found that exposure of patients to lead of various concentrations induced toxic effects leading to epileptic behavior. The characteristic infrared spectral changes were associated to lead concentrations. From the shifts of the absorption bands of amide I and amide II of proteins it was indicated that Pb^{2+} was causing alterations to misfolding of proteins and amyloid protein formation, which have been observed in many neurological diseases, such as Alzheimer and Parkinson. The presence of Pb^{2+} in serum affected also the DNA phosphate-sugar backbone structure and this is most likely the cause for epileptic seizures. Moreover, the characteristic band at 1170 cm^{-1} could be used as a "marker band" for a DNA damaged backbone structure due to lead exposure, present in the environment of DNA. Finally, the Pb^{2+} concentration in serum altered the concentrations of Cu^{2+} and Zn^{2+} cations ([Cu/Zn] is decreasing) affecting thus the pathophysiological conditions of the brain leading to epileptogenesis.

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