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Estimation of free copper ion concentrations in blood serum using T_1 relaxation rates

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ABSTRACT

The water proton relaxation rate constant $R_1 = 1/T_1$ (at 60 MHz) of blood serum is substantially increased by the presence of free Cu^{2+} ions at concentrations above normal physiological levels. Addition of chelating agents to serum containing paramagnetic Cu^{2+} nulls this effect. This was demonstrated by looking at the effect of adding a chelating agent—D-penicillamine (D-PEN) to $CuSO_4$ and $CuCl_2$ aqueous solutions as well as to rabbit blood serum.

We propose that the measurement of water proton spin–lattice relaxation rate constants before and after chelation may be used as an alternative approach for monitoring the presence of free copper ions in blood serum. This method may be used in the diagnosis of some diseases (leukaemia, liver diseases and particularly Wilson's disease) because, in contrast to conventional methods like spectrophotometry which records the total number of both bound and free ions, the proton relaxation technique is sensitive solely to free paramagnetic ions dissolved in blood serum.

The change in R_1 upon chelation was found to be less than 0.06 s^{-1} for serum from healthy subjects but greater than 0.06 s^{-1} for serum from untreated Wilson's patients.

1. Introduction

Copper is one of trace elements required for proper functionality of living organisms. In blood serum, copper ions are mostly associated with proteins and enzymes, but may also be present in the form of free ions. The mechanisms for regulation of total copper and its metabolism are not yet well understood. Several new techniques, like the use of stable isotope tracers or compartmental modelling, have introduced new approaches to learn about copper metabolism [1].

In this paper, we propose to introduce NMR as an additional technique for monitoring copper ions in blood serum. The simplest approach would be to measure the copper NMR signal directly, but both copper isotopes (^{63}Cu and ^{65}Cu) give very low signals. Indirect methods of measuring the presence of Cu^{2+} are more suitable, for example: proton NMR, which is widely used due to its higher sensitivity. Copper is strongly paramagnetic and both theoretical and experimental studies of its influence on the relaxation times of proteins solutions have been done [2,3]. The NMR signal from water protons near paramagnetic ions are hyperfine shifted and relaxation processes undergo enhancements which depend on the sixth power of the paramagnetic ion–proton distance. In copper solutions, fast exchange between water molecules in the Cu^{2+} coordination sphere and bulk water signal leads to a significant shortening of relaxation times. Hence, the measurement of proton NMR relaxation time constants gives an opportunity to monitor free copper ion levels in body fluids.

In the human body, most of the intake of copper comes from dietary sources and it is transported by blood to the liver. Physiological concentrations of copper in human blood serum are 70–140 μg/ml for healthy men and 76–152 μg/ml for healthy women, corresponding to 1.1–2.2 and 1.2–2.4 $\times 10^{-5}$ mol/l, respectively [4,5]. It is known from the literature [6] that, in aqueous solutions of $CuSO_4$ and $CuCl_2$, the water proton T_1 is sensitive to copper when it is present at concentrations above 2.5×10^{-5} mol/l. In blood serum, which is an aqueous protein solution (with concentration of proteins about 60–80 g/l) [7], $R_1 = 1/T_1$ is increased in solutions of Cu^{2+} at concentrations greater than 2.5×10^{-5} mol/l.

D-penicillamine (D-PEN) is a chelation agent which behaves selectively for Cu^{2+}, forming very stable, water soluble, Cu^{2+} complexes [8–10]. These Cu^{2+} complexes are much less effective than free Cu^{2+} ions in enhancing R_1 [11]. Therefore, in samples containing Cu^{2+}, the extent of R_1 change which occurs upon addition of D-PEN should be indication of a free Cu^{2+} concentration [11].

Wilson's disease is an inborn error of copper metabolism as the result of serum deficiency of ceruloplasmin. Progression of Wilson's disease leads to a significant increase of free copper ions

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Cu^{2+} circulating in the blood stream. Subsequently, it results in an accumulation of copper ions in organs such as the brain, liver and cornea (Kayser–Fleischer rings) [12,13]. The initial presentation of this disease is variable and is usually manifested in the form of liver failure and also neurological or psychiatric symptoms. An early and accurate diagnosis of Wilson's disease is very important because treatment using chelation therapy, mainly with D-penicillamine (D-PEN), is essential to prevent death. Diagnostic tests include: serum ceruloplasmin, 24-h urinary copper, a careful ophthalmologic exam and liver biopsy, but none of these are decisive. MRI of the brain showing the "face of the giant panda", which was first noted by Hitoshi et al. [14], can also be used to document neurological involvement in Wilson's disease.

In this study, we measured the changes in the water proton R_1 constant which occurred when D-PEN was added to aqueous solutions and in blood serum containing Cu^{2+} ions. It was anticipated that blood serum samples with higher concentrations of free Cu^{2+}, namely those from untreated Wilson's patients, would undergo larger changes in the relaxation rate constant upon chelation.

2. Materials and methods

NMR relaxation studies were performed using a Bruker Minispec NMR spectrometer working at a proton resonance frequency of 60 MHz at a controlled (within ±1 °C) temperature of 22 or 30 °C. Magnetic field stabilisation was achieved using an external probe containing fluorine (^{19}F). The Inversion-Recovery (IR) pulse sequence was applied for measurement of spin–lattice relaxation times T_1. The 90-degree and 180-degree pulses were 1.6 and 3.2 μs long, respectively. Twenty four values of TI varying logarithmetically from 0.2 to 15 s were used for the pure serum samples and 24 TI times varying logarithmetically from 0.001 to 0.2 s were used for measurements on samples with high copper concentration. Nonlinear least square fitting (NLLSQ) of the experimental data allowed evaluation of the spin–lattice time with accuracy better than 1% for the longer TI measurements and 2–3% for the measurements with shorter TI's. All T_1 measurements were repeated at least 6 times. Averages and their errors were calculated from the multiple experiments.

Solutions of $CuSO_4$ and $CuCl_2$ with Cu^{2+} concentrations ranging from 2.5×10^{-5} to 1 mol/l and solutions of $ZnSO_4$ with Zn^{2+} concentrations of 0.2×10^{-5} to 10 mol/l, were prepared by dissolution of salt crystals in twice distilled water. The rabbit serum, produced as a lyophilised powder (Wytwórnia Surowic i Szczepionek Biomed, Kraków, Poland), was dissolved at a concentration of 0.05 g/ml in water. Rabbit serum solutions were mixed with copper and zinc solutions in a volume proportion 1:1.

Two solutions of D-penicillamine (D-PEN) of concentration 20 and 40 mg/ml (0.13 and 0.26 mol/l) were prepared from tablets of the drug Cuprenil (Polfa, Kutno, Poland). The D-PEN solution was added to serum and water solutions of copper and zinc in a volume proportion 1:10. Additional water (1:10 by volume) was then added to the solutions in order to maintain the original protein concentration.

Samples of human blood serum from patients were taken, as part of a routine check-up, at the Neurology Department and Department of Internal Medicine of Collegium Medicum, Jagellonian University, Kraków. For 20 of these blood serum samples, the copper concentrations were estimated by spectrophotometry (Roche Diagnostic, bathocuproin with deproteinisation).

3. Results

Figs. 1 and 2 show the relaxation rate constants ($R_1 = 1/T_1$) as a function of Cu^{2+} concentration in aqueous solutions of $CuSO_4$ and $CuCl_2$ with and without D-PEN. Data are also shown for Cu^{2+} solutions containing rabbit serum. For copper concentrations below 5×10^{-4} mol/l, R_1 was independent of copper concentration. At higher concentrations, R_1 increased with increasing Cu^{2+}. Addition of D-PEN was found to shift the onset of the increase in R_1 to much higher Cu^{2+} concentrations. The extent of this shift increased markedly when the D-PEN concentration was doubled. Addition of D-PEN to solutions containing Zn^{2+} had practically no effect on the dependence of R_1 on Zn^{2+} concentration (data not shown).

In the next step, Cu^{2+} ions were added progressively to a sample of fresh human serum and the difference between relaxation rate constants between fresh serum and the serum after adding of solution of D-PEN (0.13 mol/l), ΔR_1, was measured, where:

$$\Delta R_1 = R_{1(\text{serum})} - R_{1(\text{serum+D-PEN})}.$$

The dependence of ΔR_1 on Cu^{2+} concentration in the human serum samples with added Cu^{2+} is shown on Fig. 3. A large increase in ΔR_1 occurred as the Cu^{2+} concentration was increased from 5×10^{-5} to 0.01 mol/l. The uncertainties in the R_1 and concentration measurements were negligible compared to the observed increases in the relaxation rate constant with Cu^{2+} concentration, as demonstrated by the errors bars on Figs. 1–3. The error in ΔR_1 was estimated by calculating the square root of the sum of squares of the errors in $R_{1(\text{serum})}$ and $R_{1(\text{serum+D-PEN})}$.

More than 80 blood serum samples were obtained from subjects from the Internal Medicine Department and Neurology Departments of Collegium Medicum in Cracow. Thirty six of these samples were from subjects whose state of health was known, including: 11 healthy volunteers, 3 untreated, 2 treated and 2 suspected Wilson's disease patients, 15 heart disease patients and 3 leukaemia patients. For some patients two separate samples, taken over a period of a few days, were measured. Values of R_1 in these serum samples ranged from 0.48 to 0.64 s^{-1} for healthy and Wilson's disease patients (treated, untreated and suspected) [Fig. 4]. Fig. 5 shows a histogram of ΔR_1 for the same samples. The untreated and suspected Wilson's disease patients are clearly distinguishable from the healthy and treated subjects. The value $\Delta R_1 = 0.06 \pm 0.01$ s^{-1} was chosen as an upper limit for healthy subjects (11 volunteers) [see Fig. 5]. Table 1 compares ΔR_1 and the spectrophotometric copper levels for selected samples.

4. Discussion

Due to variations in the composition of serum, room temperature values of R_1 of serum, from a large number of subjects, both

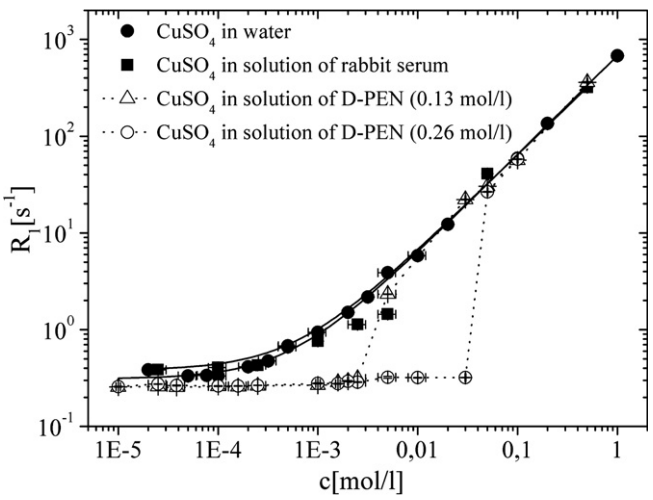

Fig. 1. Dependence of R_1 on copper ions concentration in $CuSO_4$ solutions in water, rabbit serum and aqueous D-PEN solutions (0.13 and 0.26 mol/l) at 30 °C. The solid lines represent fits of the function $R_1(c) = a + b \cdot c$ to the data where c is the Cu^{2+} concentration and a, b are constants.

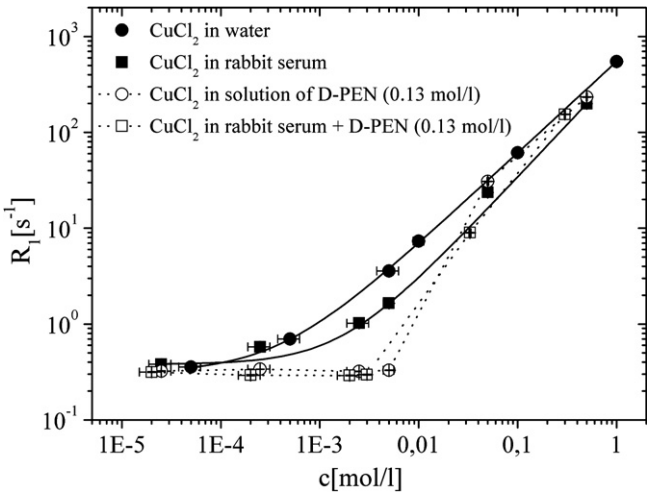

Fig. 2. Dependence of R_1 on copper ions concentration in $CuCl_2$ solutions in water, rabbit serum and aqueous D-PEN solutions (0.13 mol/l) at 30 °C. The solid lines represent fits of the function $R_1(c) = a + b \cdot c$ to the data where c is the Cu^{2+} concentration and a, b are constants.

healthy and suffering from a wide range of diseases, range from 0.5 to 0.7 s^{-1} [[5], this work]. Proteins and paramagnetic ions in aqueous solution affect the relaxation rate constant of water protons according to formula (valid at low concentrations) [15,5]:

$$R_1 = (1 - f_1 - f_2) \cdot R_{1w} + f_1 \cdot R_{1\text{protein}} + f_2 \cdot R_{1\text{para}}, \quad (1)$$

where f_1 is the fraction of protons associated with proteins, $R_{1\text{protein}}$—the effectiveness of protein as a proton relaxation agent (serum blood contains proteins in the concentration range 60–80 g/l), f_2—the fraction of protons associated with paramagnetic ions and $R_{1\text{para}}$ represents the effectiveness of the paramagnetic ions as a proton relaxation agent. At low concentrations of paramagnetic copper f_2 is proportional to the Cu^{2+} concentration. Figs. 1 and 2 demonstrate that the paramagnetic term in Eq. (1), begins to have an effect for Cu^{2+} concentrations greater than 5×10^{-4} mol/l.

The data for copper solutions in water and rabbit serum were fit to the function:

$$R_1(c) = a + b \cdot c, \quad (2)$$

where a represents the first two terms of Eq. (1) and b is a parameter associated with $R_{1\text{para}}$. Values of parameter a for water and serum solutions of copper were fixed to 0.31 and 0.38 s^{-1}, respectively, as measured at 30 °C (see Figs. 1 and 2). The results for solutions were: $b(CuSO_4) = (680 \pm 2)\ s^{-1}$, $b(CuCl_2) = (550 \pm 2)\ s^{-1}$. For the rabbit serum samples, the best fit was obtained for $b(CuSO_4) = 640 \pm 7\ s^{-1}$ and $b(CuCl_2) = 400 \pm 3\ s^{-1}$. The values for b suggest that $R_{1\text{para}}$ was larger in $CuSO_4$ than in $CuCl_2$ solutions.

Figs. 1 and 2, demonstrate that when D-PEN was added, the onset of R_1 increase with Cu^{2+} concentration was pushed to higher Cu^{2+} concentrations. When the D-PEN concentration was doubled in the $CuSO_4$ solutions, this onset was pushed to a much higher concentration, suggesting that many more Cu^{2+} ions were complexed by the additional D-PEN. In the chelated preparations at higher Cu^{2+} levels, once the chelate was unable to suppress the paramagnetic ion contribution to the relaxation rate, the measured R_1 were similar to those from preparations at the same Cu^{2+} level without D-PEN.

To extract the paramagnetic ion contribution to the relaxation rate constant, we examined the change in R_1 upon addition of D-PEN, ΔR_1, which was found to increase sharply with Cu^{2+} level up to a Cu^{2+} concentration of about 1×10^{-2} mol/l. The fact that the marked increase of ΔR_1 with increased Cu^{2+} concentration (Fig. 3) was observed just above the normal physiological concentration of copper ($1.2 - 2.5 \times 10^{-5}$ mol/l) is the basis for application of a NMR relaxation technique for estimating the increased level of Cu^{2+} ions in blood serum from some patient groups. Table 1 and Fig. 5 show the values of ΔR_1 from human serum samples from healthy volunteers, and patients suffering from Wilson's disease, heart diseases and leukaemia. For healthy volunteers the ΔR_1 values were less than $0.06 \pm 0.01\ s^{-1}$, while for patients with elevated copper level, ΔR_1 values were larger than 0.06 s^{-1}. Hence, based upon these results, one can conclude that the observation of serum ΔR_1 values greater than 0.06 s^{-1} is strongly indicative of elevated levels of copper.

The maximum of ΔR_1 in Fig. 3, which corresponds to maximum chelation, occurred at an added Cu^{2+} concentration of about 0.01 mol/l. In Fig. 1, the maximum increase in ΔR_1 was observed at a Cu^{2+} of 0.003 mol/l for the same concentration of added D-PEN (0.13 mol/l). The discrepancy between these two concentrations results from the fact Fig. 1 is for water solutions and Fig. 3 is for solutions containing human serum. One might expect that the protein rich serum solutions would cause increased R_1 compared to pure water solutions. However, Figs. 1 and 2 demonstrate, for higher Cu^{2+} concentrations, that R_1 in serum solutions were slightly decreased relative to those in pure Cu^{2+} solutions. It seems likely that serum solutions are themselves capable of binding Cu^{2+}

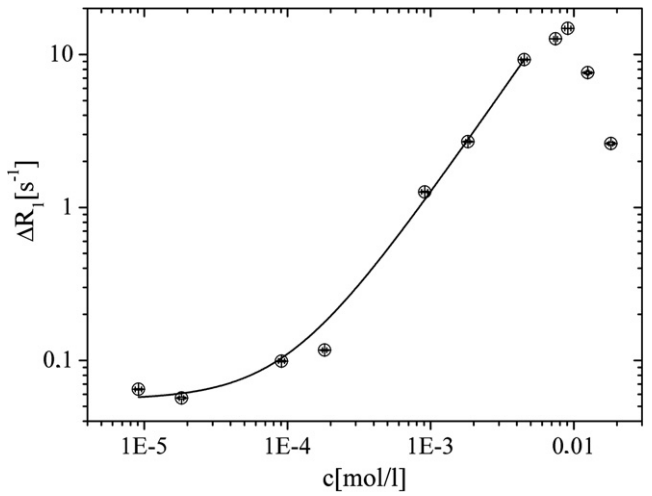

Fig. 3. Dependence of $\Delta R_1 = R_{1(\text{serum})} - R_{1(\text{serum+D-PEN})}$ on copper ion concentration in solutions of human serum with added $CuSO_4$ at 22 °C.

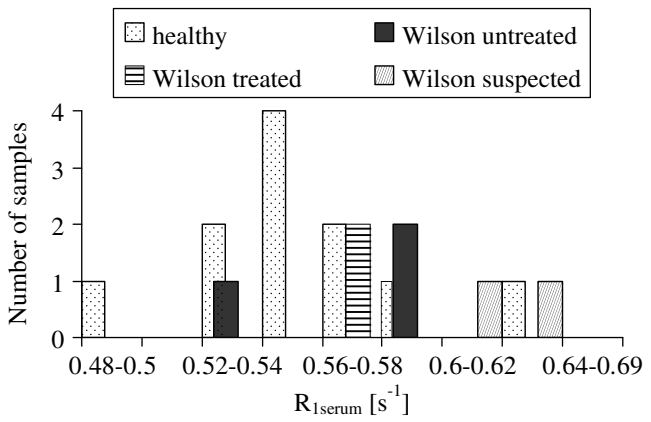

Fig. 4. Histogram of $R_{1(\text{serum})}$ for healthy volunteers and patients suffering from Wilson's disease (treated, untreated and suspected).

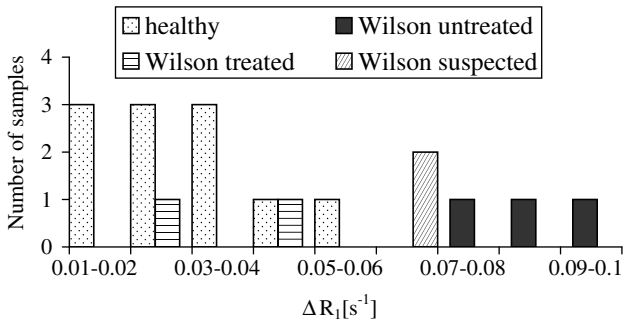

Fig. 5. Histogram of $\Delta R_1 = R_{1(\text{serum})} - R_{1(\text{serum+D-PEN})}$ for healthy volunteers and patients suffering from Wilson's disease, treated, untreated and suspected.

Table 1
ΔR_1 values measured at 60 MHz and 22 °C and copper concentrations from spectrophotometry of blood serum samples from healthy volunteers and patients suffering from heart diseases, leukaemia and Wilson's disease (only for one of seventh subjects the spectrophotometry data were available)

Subject	ΔR_1 [s^{-1}] (±2%)	μmol/l[a]	Diagnosis
1	0.029	11.8	Healthy
2	0.011	9.50	
3	0.028	15.4	
4	0.042	10.7	
5	0.094	26.9	Untreated Wilson's disease with full clinical symptoms (two measurements of T_1)
6	0.059	24.7	Heart diseases
7	0.065	18.8	
8	0.061	20.2	
9	0.072	27	
10	0.064	22.2	
11	0.055	19.7	
12	0.045	21.4	
13	0.047	19.1	
14	0.042	22.5	
15	0.034	25.3	
16	0.067	19.1	
17	0.042	27.2	Leukaemia
18	0.073	24.3	
19	0.031	19.4	
D-PEN water solution	−0.006 ± 0.001		Shortening done by D-PEN added to water

[a] The error in the Cu concentration measured by spectrophotometry was ±15–20%.

ions. While they are not as effective as D-PEN in binding Cu^{2+}, they shift the ΔR_1 vs added Cu^{2+} concentration plot in Fig. 3 to the right.

Ions other than Cu^{2+} can also cause increased R_1 [2,3]. Blood serum also contains: Fe, Co, Cr, Mn and Zn ions [7], which increase both R_1 and R_2. The extent of relaxation rate constant increase depends on the type of ion and is often characterised by broadening of proton NMR lines [3]. The broadening parameter [3] for Cu^{2+} ions is one of the highest (1000–2000 Hz), whereas for Zn is much smaller. The broadening parameter is also large for Fe and Co, but D-penicillamine can chelate only Cu and Zn [8–10,16]. Therefore we can assume ΔR_1 is selectively effective for copper ions only since adding D-PEN to zinc solutions had no effect on R_1 (data not shown).

We used $\Delta R_1 = 0.06 \pm 0.01\ s^{-1}$ as the upper limit for normal free copper level. For Wilson's disease patients, all ΔR_1 values were larger than $0.06\ s^{-1}$ [Fig. 5]. However, values of ΔR_1 for samples from patients suffering from heart diseases and leukaemia, which is also characterised by evaluated level of copper or copper to zinc ratio [17,18], ranged from 0.035 to $0.075\ s^{-1}$, below and above our threshold value (Table 1). The values of ΔR_1 did not correlate well with copper concentrations measured by spectrophotometry. This is likely because copper ions in serum can exist as free copper and as bound copper in ceruloplasmin. D-PEN is not able to remove ions from ceruloplasmin, hence it can not affect their contribution to the water proton relaxation rate constant [19]. Spectrophotometry detects both free and bound copper ions. This is why, for heart disease and leukaemia our relaxation method may not be useful. However, those diseases are easily diagnosed by other methods; this is not always the case for Wilson's disease.

Wilson's disease is a rare illness with incidence 1/30,000; this was a factor in the low number of serum samples in the current study—we investigated samples from only 3 untreated subjects and 2 suspected subjects with this disease. Fig. 5 shows that all untreated and suspected subjects with Wilson's disease gave rise to serum ΔR_1 values greater than $0.06\ s^{-1}$, while all treated Wilson's subjects and healthy volunteers had serum ΔR_1 values less than $0.06\ s^{-1}$.

The advantage of the proposed technique for estimating copper concentration in blood serum is that it is sensitive only to free copper ions (which are substantially more toxic), while spectrophotometry is sensitive to both free and bound Cu^{2+}. The accuracy of ΔR_1 is better (2%) than spectrophotometry (15–20%). The relaxation technique is also fast (about 10 min for single measurement) and inexpensive, as it may be carried out with low-cost, low-field time-domain NMR spectrometers. Sample preparation is simple—in our case it involved collecting 2 ml of serum solution and measuring R_1 before and after adding D-PEN. While the application to heart disease and leukaemia may not be worthwhile, these initial results suggest that this spin–lattice relaxation technique could be very useful as a diagnostic for Wilson's disease. Since Wilson's disease is inherited, a promising application would be to use this technique to screen family members of Wilson's patients.

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