Free Rad. Res. Comms., Vol. 7, No. 1, pp. 55–62
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NON-CAERULOPLASMIN COPPER AND FERROXIDASE ACTIVITY IN MAMMALIAN SERUM. FERROXIDASE ACTIVITY AND PHENANTHROLINE-DETECTABLE COPPER IN HUMAN SERUM IN WILSON'S DISEASE

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(Received May 10 1989)

It is thought that 5–10% of human serum copper consists of copper ions complexed with histidine, amino acids or albumin. The phenanthroline assay developed by Gutteridge (*Biochem. J.* **218**, 983–985; 1984) is shown to measure all these forms of copper down to a sensitivity of 0.1μmol/dm^3, yet it does not detect any copper ions in freshly-prepared serum or plasma from rats, mice, rabbits or guinea-pigs, or freshly-prepared serum from humans. It is concluded that the "non-caeruloplasmin copper pool" is much smaller than has previously been supposed. No phenanthroline-detectable copper could be measured in serum freshly prepared from four patients with uncomplicated Wilson's disease, but it could be measured in serum from a patient with fulminant hepatic failure. After liver transplantation, concentrations of phenanthroline-detectable copper in this patient fell to zero within two days. Studies on the ferroxidase activity of freshly-prepared serum or plasma samples shows that little ferroxidase II activity is present in samples from healthy adults or from the patient with Wilson's disease and fulminant hepatic failure. In all the patients, ferroxidase I activities are sub-normal. Freshly-prepared plasma or serum samples from several animal species generally show lower ferroxidase I and greater ferroxidase II activities than do human samples, but only in rabbits does ferroxidase II account for a high proportion of total plasma ferroxidase activity. Storage of biological fluids can cause release of copper from caeruloplasmin and a rise in ferroxidase II activity; these events may have confused some earlier studies.

KEY WORDS: Copper, penicillamine, Wilsons disease, ferroxidase, caeruloplasmin.

INTRODUCTION

According to the literature,[1-3] over 90% of copper in human serum is associated with the protein caeruloplasmin, which assists the loading of iron onto transferrin and may also function as an anti-oxidant (reviewed in[2]). The remaining 5 to 10% of serum copper is believed to be chelated to various amino acids or to serum albumin,[1-6] although the size and nature of this "non-caeruloplasmin copper pool" are poorly defined.[1-6] Gutteridge [7] described an assay that measures "available copper" in body fluids: the assay is based on the fact that a phenanthroline-copper ion complex can degrade DNA in the presence of a reducing agent. Thus, if DNA, phenanthroline and reductant are in excess, the extent of DNA degradation depends upon the amount of copper ion that is available for chelation by phenanthroline. In preliminary experiments, it was found that copper ions attached to albumin or to histidine are available

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for chelation by phenanthroline, but copper ions attached to caeruloplasmin are not.[7] When the phenanthroline assay was applied to a small number of freshly-prepared samples of human plasma or serum, no copper was detected.[7,8] However, storage of serum at 4°C caused copper ions to appear, because of degradation of caeruloplasmin.[7,8] Gutteridge[7,8] therefore raised the possibility that the "non-caeruloplasmin copper pool" of human plasma is an artefact caused by release of copper ions from caeruloplasmin during such procedures as dialysis, prolonged storage or column chromatography. For example, the key paper[4] describing this pool referred to human serum "kept at 4°C and used within 3 days, then subjected to dialysis for 48h at 4°C". These would be ideal conditions for release of copper from caeruloplasmin.[8]

Caeruloplasmin is able to oxidize Fe(II) ions, its "ferroxidase" activity. This activity is thought to be involved in facilitating iron loading onto transferrin, and in the antioxidant activity of caeruloplasmin.[2] The ferroxidase activity of caeruloplasmin, often called "ferroxidase I", is inhibited by 1 mM azide.[9] Several other "ferroxidase" activities have been described in biological fluids. One of the most studied is "ferroxidase II", an azide-resistant activity associated with a complex of lipid, protein and Cu(II) ions. Ferroxidase II has been purified from stored human serum,[9] although the great bulk of human serum ferroxidase activity is attributable to caeruloplasmin.[2,8] Greater amounts of ferroxidase II have been reported in sera from several animal species, such as rabbit.[10,11]

Gutteridge *et al.*[8] found that storage of human serum, even at −20°C, eventually leads to degradation of caeruloplasmin. The released copper ions can sometimes, depending on the precise conditions, facilitate the peroxidation of plasma lipids, and ferroxidase II activity develops. This raises the possibility that reports of substantial ferroxidase II activity in animal sera might also be artefacts, since published papers rarely make clear how long biological fluids have been stored or how they have been handled. Thus ferroxidase II was purified from "purchased frozen rabbit serum".[10]

Wilson's disease is a rare inherited disorder of copper metabolism in which copper is deposited in several tissues, leading to hepatic and neurological dysfunction.[2,12] Serum caeruloplasmin levels are diminished, apparently due to a decrease in transcription of the gene encoding this protein.[12] However, raised ferroxidase II has been reported in serum from patients with Wilson's disease[9] and it has been speculated that increased low-molecular-mass copper complexes in plasma may contribute to tissue damage in this disease.[8,13] Wilson's disease is often treated by the administration of copper-chelating agents, such as penicillamine or trientine*.[13,18] Since Wilson's disease is rare, however,[8,13] it is likely that many earlier studies on plasma ferroxidase II and non-caeruloplasmin copper have used accumulated stored samples, raising the possibility of artefacts.[7,8]

In this paper, we report the results of a detailed re-evaluation of the non-caeruloplasmin copper content and ferroxidase activity of serum from healthy humans, from patients with Wilson's disease and from healthy specimens of other animal species. Our approach was to obtain blood samples, prepare serum or plasma at once and do the assays immediately, to avoid any question of artefacts due to storage of samples.

MATERIALS AND METHODS

Reagents

Human apotransferrin, human albumin, calf-thymus DNA (type I), 1,10-phenanthroline and the tripeptide glyhislys were obtained from Sigma. Chelex-100 was from

*Trientine N,N′-Bis(2-aminoethyl)1,2-ethanediamine.

Bio-Rad Laboratories, Watford, Herts. All other chemicals were of the highest quality available from BDH Chemicals. Micropartition devices were from Amicon and had a cut-off Mr of 1000.

Body fluids

Patients with an established diagnosis of Wilson's disease by the criteria set by Trey and Davidson,[19] or healthy control subjects, provided blood samples. The study had been approved by the Ethical Committee of King's College Hospital. Full details of the subjects used are given in the legend to Table 3. Blood was withdrawn from the antecubital vein, samples were allowed to clot, and serum removed and assayed immediately. Serum samples were prepared from animals in a comparable way. Other animal blood samples were drawn into heparinized tubes and centrifuged at 1000g for 10 min to pellet cells; plasma was pipetted off and analysed immediately.

Assays

The phenanthroline assay was performed exactly as in,[7] using a Perkin-Elmer fluorimeter. Total ferroxidase activity was measured at 37°C as in.[14] Ferroxidase II was measured by repeating the assay in the presence of 1 mM NaN_3. Feroxidase I was calculated by subtraction. Caeruloplasmin protein was measured imunologically using Nor-Partigen plates.

Micropartition of serum

Samples of serum (usually 1 ml) were centrifuged at 1000g for 1h at room temperature in an Amicon micropartition device, cut-off Mr 1000.

RESULTS

Calibration of the phenanthroline assay

It has been claimed that the non-caeruloplasmin-copper pool of human plasma consists of copper ions attached to albumin, to amino acids or to peptides such as glycylhistidyllysine.[1-6] Table 1 shows that none of these products, at concentrations up to and above physiological, decreased the reactivity of Cu(II) ions in the phenanthroline assay, nor did the iron-binding protein transferrin. Calibration curves showed that the assay was usually sensitive down to 0.1 μmoles Cu(II)/dm^3, although variations in the sensitivity of the assay were sometimes observed, for reasons that we have not been able to ascertain. Experiments on each day were accompanied by a full calibration curve, and the results discarded if the assay did not show its usual sensitivity.

Application of the phenanthroline assay to human serum

Serum samples were obtained from ten healthy adult volunteers (8 males, 2 females, age range 25–45 years). No sample showed any copper ions when assayed by the phenanthroline method. When Cu(II) ions were added to the serum as $CuCl_2$ or as complexes of Cu(II) ions with amino acids or the tripeptide glycylhistidyllysine, they

TABLE I

Detection of copper complexes by the phenanthroline assay. The assay was carried out as described by Gutteridge.[7] Amino acids (1 mM), mixtures of amino acids (all at 1 mM) and the peptide glyhislys (1 mM) were preincubated with $CuCl_2$ (10 μM) at pH 6.5 for 30 mins at room temperature to allow complex formation, before addition to the reaction mixture. Controls consisted of the amino acids, mixtures or peptide added to the assay mixture alone. Results presented are the means of duplicate determinatins differing by < 10%. Normal plasma albumin is 40–60 mg/ml, transferrin is ca. 3 mg/ml.

Addition to reaction mixture	Copper concentration measured by the assay (μmol/dm^3)
Amino acids	
His	10.3
Glu	10.6
Val	11.0
Asn	10.6
Cys	11.3
Thr	8.9
Amino acid mixtures	
His, Asn	10.4
His, Glu	10.3
His, Val	10.9
His, Thr	11.5
His, Asn, Glu, Thr	10.6
His, Ser, Val, Ala	11.1
His, Ser, Val, Ala, Glu	10.6
His, Cys	12.0
Other materials	
N,N′-bis(2-aminoethyl)1,2-ethane diamine (Trientine)	10.0
gly-his-lys	10.2
D,L-penicillamine	12.0
Apotransferrin (3 mg/ml)	10.0
*Albumin (10 mg/ml)	10.3
(40 mg/ml)	10.4
(80 mg/ml)	11.8

* The albumin itself contained only trace amounts of copper contamination, which has been corrected for in the data presented.

were measurable on subsequent assay. However, we did note that only about 70–80% of the added copper could be measured: Table 2 shows a typical experimental result (reproducible in five experiments). In confirmation of Gutteridge,[7,8] we found that keeping serum at 4°C for several days, or dialysing it at 4°C, caused phenanthroline-detectable copper to appear.

During the period of this study, five patients with Wilson's disease were admitted to the Liver Unit at King's College Hospital. One was not being treated with a chelator when samples were taken (A). Four others were undergoing chelation treatment, three with penicillamine (C,D,F) and one with trientine (B). Freshly-prepared serum samples from these patients contained no phenanthroline-detectable copper, although added copper ions could be measured in experiments identical to those reported in Table 2 (recovery 70–95% in five experiments). It should be noted that neither trientine nor penicillamine affects the ability of copper ions to react in the phenanthroline assay (Tables 1 and 2), so that any complexes of copper ions with these chelating agents present in the plasma should be measurable in the phenanthroline assay.

Fresh serum samples were also prepared from a patient with Wilson's disease

TABLE II

Detection by the phenanthroline assay of copper complexes added to freshly-prepared serum. Substances were preincubated with $CuCl_2$ at pH 6.5 and 25°C for 30 mins to allow complex formation. An equal volume of serum was then added to this reaction mixture and then an aliquot tested in the phenanthroline assay. The final concentrations in the mixture added to the phenanthroline assay were 1 mM for ligands of Cu(II) and 10 μM for Cu(II) itself. For serum alone an equal volume of 20 μM $CuCl_2$ was added to the serum.

Substance(s) added to the assay with 10 μM $CuCl_2$	Copper concentration measured by assay μmol/dm^3
None	10.0
Serum	8.8
Serum + His	6.6
Serum + His + Thr	7.3
Serum + His + Thr + Asn + Glu	7.0
Serum + His + Val + Ala + Glu + Ser	7.3
Serum + D,L-penicillamine	6.9
Serum + trientine	6.0
Serum + Gly-his-lys	8.8

complicated by fulminant hepatic failure (patient E). Her serum *did* contain phenanthroline-detectable copper (Table 3). One day after patient E received a liver transplant, her phenanthroline-detectable copper fell to 0.7 μmol/dm^3. By two days after transplantation, no phenanthroline-detectable copper was present in her serum. No such copper was detected in samples taken 40 days after transplantation.

TABLE III

Serum parameters in five patients with Wilson's disease

Patient	Phenanthroline-detectable copper μmol/dm^3	Caeruloplasmin protein g/dm^3	Ferroxidase activity units/ml Ferroxidase I	Ferroxidase II
Wilson's disease				
A	0	< 0.05	0.007	0.021
B	0	< 0.05	0.080	0.040
C	0	0.01	0.022	0.024
D	0	0.14	0.560	0.037
F	0	0.19	0.540	0.035
Wilson's disease plus fulminant hepatic failure				
E	5.1*	0.15	0.480	0.056
Normal controls (n = 10) mean ± SD	0	0.259 ± 0.079	1.12 ± 0.16	0.06 ± 0.02

Patient A Male, born 1964, diagnosed 1977 in Ireland. Took penicillamine 750 mg/day until 1983, then stopped, seen at King's, March 1988, not taking any chelator.
Patient B Male, born 1959, diagnosed 1986. Allergic to penicillamine. On trientine (1g/day) since 1986.
Patient C Male, born 1961, diagnosed 1983. On penicillamine 750 mg/day.
Patient D Female, born 1957, diagnosed 1976. On penicillamine 250 mg daily.
Patient E Female, born 1964, diagnosed 1975. On penicillamine 750 mg/day. presented at King's July 1988 with fulminant hepatic failure. Transplanted August 1988.
Patient, F Male, born 1934, diagnosed 1947. On penicillamine 1.5 g/day.
All samples were taken in the time period August-October 1988 and were analyzed immediately for the above parameters.*Mean of quadruplicate determinations differing by ⩽ 10%. Sample taken 24 h before liver transplantation.

Application of the phenanthroline assay to animal serum or plasma

Serum or plasma samples were prepared from blood freshly drawn from mice, rats, guinea-pigs and rabbits (see Table 4 for details of species used). In at least four separate plasma or serum samples from each species, no phenanthroline-detectable copper was observed. Cu(II) ions added to the plasma or serum samples could readily be detected (recovery 70–95% in four experiments). If rabbit sera were kept at 4°C for several days, phenanthroline-detectable copper appeared.

Ferroxidase activity of human sera

Table 3 shown that normal human serum contains substantial ferroxidase activity. As expected,[2,3,8] almost all of this activity is inhibited by 1 mM sodium azide and thus can be attributed to ferroxidase I, the ferroxidase activity of caeruoplasmin. Raising the azide concentration (tested up to 10 mM) did not increase the inhibition obtained. In three of the patients with Wilson's disease (A,B,C), ferroxidase I activities and amounts of immunologically-detectable caeruloplasmin were very low, consistent with a defect of synthesis of caeruloplasmin.[2,3,12,13] In the fourth and fifth patients (D and F), ferroxidase I was approximately 50% of normal. Caeruloplasmin protein, as measured immunologically, was also present in sub-normal amounts. In none of the Wilson's patients were ferroxidase II activities elevated: indeed, they were lower than normal in four of the patients (A,C,D,F) and at the lower end of the normal range in the fifth (B). The patient with fulminant hepatic failure (E) showed ferroxidase I activities of about 40–50% of normal, but levels of ferroxidase II were not elevated.

Ferroxidase activity of animal serum or plasma

Table 4 shows the results of ferroxidase assays performed upon freshly-prepared serum or plasma samples from a minimum of four animals of each species. Mice, rats and rabbits showed lower ferroxidase I activities than humans, but guinea pigs did not. In rabbits and mice, ferroxidase II activities were generally higher than in

TABLE IV
Serum parameters in animals

Animal studied (no of blood samples taken from different animals)	Phenanthroline-detectable copper μmol/dm^3	Ferroxidase activity units/ml mean ± SD		
		Ferroxidase I	Ferroxidase II	Ferroxidase II as a % of total ferroxidase
Adult male mice, T.O strain (6)	0	0.480 ± 0.130	0.097 ± 0.017	17.2 ± 1.5
Adult male rats, Wistar strain (5)	0	0.647 ± 0.050	0.057 ± 0.009	8.0 ± 0.8
Adult male guinea pigs, Dunkin Hartley Albino strain (12)		0.980 ± 0.300	0.045 ± 0.010	4.4 ± 0.6
Adult male rabbits, New Zealand White (NZW) strain (9).	0	0.490 ± 0.210	0.280 ± 0.140	38.9 ± 13.2
Adult female rabbits, Dutch X NZW crosses (4)	0	0.567 ± 0.065	0.142 ± 0.035	19.8 ± 2.2

humans, but they were of about the same magnitude in rats and guinea pigs. Indeed, in NZW male rabbits, ferroxidase II represented over 30% of total plasma ferroxidase activity (Table 4, last vertical column). Raising azide concentrations (tested up to 10 mM) did not decrease the ferroxidase II activity measured. When rabbit sera were centrifuged in Amicon micropartition devices (cut-off Mr 1000), the bulk of ferroxidase activity was recoved in the concentrate and not in the filtrate, suggest that the ferroxidase II activity has Mr > 1000.[9] When a human serum sample was allowed to "age" at 4°C for several days, so that ferroxidase II activity had risen,[8] the bulk of this activity also remained in the concentrate upon centrifugation in a micropartition device.

DISCUSSION

Gutteridge[7,8] raised the possibility that reports of non-caeruloplasmin copper and ferroxidase II activity in animal and human serum could be artefacts due to the use of stored samples. We therefore decided to examine this problem by direct assay of freshly-prepared material. We have confirmed previous claims that the ferroxidase I activity of most animal sera is usually less than that of human sera and that ferroxidase II is more prominent, expecially in rabbit and guinea pig sra.[2,9,10,11] The ferroxidase II in rabbit appears to be of Mr > 1000, consistent with its being the copper-protein complex isolated by Topham *et al.*[10] rather than some low-molecular-mass "pseudo-ferroxidase" activity. On the other hand, we have been *unable* to confirm reports of elevated ferroxidase II in the serum from patients with Wilson's disease: in the five cases studied, ferroxidase II activity was actually lower than normal or at the low end of the normal range. Since these studies were performed upon freshly-prepared samples,and it is known that ferroxidase II activity develops upon storage of human serum,[8] it may be that previous reports of elevated ferroxidase II were artefacts.[8] Even in a patient with fulminant hepatic failure, ferroxidase II activity was not elevated (Table 3).

In agreement with,[7,8] no phenanthroline-detectable copper was present in freshly-prepared human serum, or even in serum from the patients with Wilson's disease. We have shown that the same is true of serum or plasma from other animal species. Table 1 shows that Cu(II) bound to amino acids, peptides or proteins of the type thought to chelate the "non-caeruloplasmin copper",[3–6] can readily be detected in the assay. Copper ions added to animal or human sera are also readily detected, although not with 100% recovery (eg. Table 2). The assay is sensitive down to 0.1 μmol/dm^3 of copper ion, and it has detected copper ion in "aged" serum from both animals and humans, and in serum from a patient with fulminant hepatic failure, whose liver was necrosing. Since total serum copper in male humans has been quoted[2] at 17 ± 3 μmol/dm^3, it follows that the "non-caeruloplasmin copper pool" can be no greater than about 0.6% of total serum copper, rather than the 5–10% normally quoted.[1–3] This raises the possibility that current models of human copper metabolism are in error. Indeed, the suggestion [reviewed in [2,3]] that a copper-albumin complex serves a transport role for copper has already been questioned.[15,16]

It is often assumed that the role of penicillamine in the therapy of Wilson's disease is to bind copper from the liver and promote its excretion from the body, although this view has been challenged.[17] Our failure to find plasma "low molecular mass" copper in serum from patients on penicillamine therapy suggests that penicillamine-copper complexes were not present at detectable concentrations in the blood samples studied.

Acknowledgements

We are very grateful to the Wellcome Trust for financial support and to Dr. J.M.C. Gutteridge for his most helpful comments and advice about the phenanthroline assay.

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Accepted by Prof. J.V. Bannister