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Analytical variables affecting exchangeable copper determination in blood plasma

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Abstract To resolve discrepancies observed in the determination of plasma exchangeable Cu (also called direct reacting Cu or loosely bound Cu) by several methods, plasma storage techniques and various aspects of a stable isotope dilution procedure for exchangeable Cu were evaluated. Results indicated that the exchangeable Cu fraction of plasma increased with storage at room temperature, at 5°C and when subjected to repeated freeze/thaw cycles. Samples could be safely stored at −65°C. Exchange between added $^{65}Cu^{2+}$ and endogenous plasma Cu rapidly went to completion in the isotope dilution procedure. Analytical results were unaffected by shaking method, sample size or the presence of heparin. A small difference was observed between serum and plasma. The determination of exchangeable Cu did not vary over a period of 4 h when plasma was exposed to 1.6×10^{-4}-M sodium diethyldithiocarbamate (used in the isotope dilution method) but steadily increased when exposed to 1.1×10^{-2}-M sodium diethyldithiocarbamate, which suggested that tightly bound Cu (probably in ceruloplasmin) was exchanging with isotopic tracer at the higher concentration. Determination of exchangeable Cu was constant from pH 7.2–8.5 but increased substantially at higher pH. Complete recovery of natural Cu added to plasma was obtained. Studies in solution indicated that $^{65}Cu^{2+}$ exchanged readily with albumin- and amino acid-bound Cu. Ultrafiltration of plasma yielded a Cu fraction about half that of the exchangeable Cu fraction. We conclude that the stable isotope dilution procedure for plasma exchangeable Cu yields reliable, physiologically meaningful results.

Keywords Direct reacting copper · Loosely bound copper · Serum · Stable isotope dilution analysis · Ceruloplasmin

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Introduction

Plasma Cu is mainly in the form of ceruloplasmin, a blue plasma cuproprotein that functions in Cu distribution and possibly iron mobilization (Harris 1997). Ceruloplasmin Cu is distinguished from the remaining Cu in blood plasma because it does not exchange isotopically with added free Cu^{2+} (Sternlieb et al. 1961). The plasma Cu fraction that exchanges with added Cu^{2+} is called exchangeable (EX) Cu. It is also called "direct reacting" Cu, because that was the original name given to a plasma Cu fraction (later shown to be isotopically exchangeable) that was first identified by the formation of a coloured complex with diethyldithiocarbamate (DDC) (Gubler et al. 1953). The half-life of the exchangeable (EX) Cu fraction

in vivo is much less than that of ceruloplasmin Cu. The exchangeable fraction includes Cu immediately absorbed from the gut (Gordon et al. 1987; Buckley et al. 1996). Evidence presented earlier indicates that EX Cu determined by isotope dilution analysis is physiologically significant (Buckley et al. 1996). Because of its central role in distribution of absorbed Cu, plasma EX Cu has been included as a compartment in kinetic models of Cu metabolism (Buckley 1991; Turnlund 1998). However, the significance of EX Cu in various physiological or disease states is not well understood. Maryon et al. (2007) discussed the importance of understanding the chemical properties of extracellular Cu with respect to its interaction with the hCTR1 transport protein and, therefore, the entry of Cu into cells. It is not known whether hCTR1 interacts with Cu bound to ceruplasmin, albumin or some other molecule. Knowledge of the properties of EX Cu may contribute to the study of the mechanism of Cu uptake by mammalian cells.

We previously described a method of determining EX Cu based on stable isotope dilution analysis (Buckley et al. 1996). The method was found to be suitable for determining the concentration of EX Cu in plasma as well as the stable isotope ratio of EX Cu obtained from in vivo tracer studies. Although the quantity of EX Cu in plasma varies considerably from species to species, it also varies considerably with the method of determination (Buckley et al. 1996). The values for EX Cu determined with the isotope dilution method (Buckley et al. 1996) agree reasonably well with the original DDC colorimetric method (Gubler et al. 1953), an immunochemistry procedure (Van den Hamer 1988; Louro et al. 2000) and an electrophoresis procedure (Bearn and Kunkel 1954), but are much lower than those determined with a gel filtration procedure (Weiss and Linder 1985; Wirth and Linder 1985). The gel filtration procedure partitions serum Cu into four fractions: transcuprein-bound, ceruloplasmin-bound, albumin-bound and low molecular weight forms. Transcuprein-bound Cu has been shown to exchange readily with albumin-bound Cu in plasma (Weiss and Linder 1985) and the quantity of EX Cu found associated with transcuprein in serum and plasma has been found to vary from about 20% to 100% of that associated with albumin (Wirth and Linder 1985; Barrow and Tanner 1988). In contrast, other researchers have found that albumin is the only plasma protein responsible for the initial transport of Cu after absorption (Gordon et al. 1987).

Linder (1991) argues that ceruloplasmin represents about 60% of plasma Cu as determined by the gel filtration procedure rather than the 90–97% determined by other procedures. Because the concentration of EX Cu for adult humans determined by the stable isotope dilution method (Buckley et al. 1996) is as small as one twelfth of that determined by the gel filtration procedure (Wirth and Linder 1985), we felt it was necessary to conduct detailed evaluations of potential sources of error and various aspects of the isotope dilution procedure with the objective of further establishing the reliability of the technique or otherwise finding a cause for the discrepancy. The results of these investigations are presented here.

Materials and methods

Determination of exchangeable copper

Exchangeable copper was determined with the stable isotope dilution method described previously (Buckley et al. 1996). Briefly, $^{65}Cu^{2+}$, a stable isotope, (1.5×10^{-9} mol in 0.5 ml 2×10^{-4}-M HNO_3 with 6×10^{-4}-M HCl) was added to plasma samples (5.0 ml) followed by gentle mixing. Sodium diethyldithiocarbamate (1.0×10^{-6} mol in 1.0 ml water) and 8.0 ml mineral oil were added. The Na DDC concentration in the aqueous phase was 1.6×10^{-4} M. The mixture was gently shaken on a blood rocker or horizontal shaker for 30 min, and then the phases were allowed to separate. The aqueous phase was drawn off and the mineral oil was washed three times with 10–15 ml water in order to remove interfering substances. The washing procedure was particularly effective in removing Na, which otherwise formed a polyatomic ion, $NaAr^+$, of the same nominal mass as Cu^{63}, the most abundant stable isotope of Cu. Copper was then back-extracted into 12 ml of 0.63-M HNO_3 by shaking for 3 h. Finally, the back extract was analyzed for Cu stable isotopes by inductively coupled plasma mass spectrometry (ICPMS, Elan Model 250, Sciex). Precautions were taken throughout to avoid Cu contamination from equipment and reagents. The concentration of EX Cu was determined from the stable isotope ratio. This procedure is referred to as the "routine method" and was used in the present study

unless otherwise stated. Analytical precision and the effect of Na DDC concentration on the determination of EX Cu were reported previously (Buckley et al. 1996).

In the present work, reliability of, and sources of error in the EX Cu determinations were studied further. Studies included (1) evaluation of the stability of EX Cu during storage of plasma samples prior to analysis, (2) comparison of six extraction solvents, (3) the effect of duration of equilibration time for isotopic exchange prior to extraction, (4) the effect of duration of extraction time, (5) evaluation of the shaking method used for extraction, (6) the effect of plasma sample size, (7) comparison of EX Cu determination in serum versus plasma, (8) the effect of heparin on EX Cu determination, (9) effect of length of time for back-extraction into acid, (10) the effect of plasma pH on determination of EX Cu, (11) the recovery of natural Cu^{2+} added to plasma, (12) EX Cu determination in solutions of Cu with albumin and amino acids, and (13) a comparison of the routine method with an ultrafiltration procedure.

Statistical comparisons were performed by analysis of variance, Student–Neuman–Keuls multiple comparison of means test, Students *t*-test (SigmaStat Statistical Software, Jandel Scientific Software) and by comparison of slopes of simple linear regression equations (Zar 1984).

Human study protocols were consistent with the Declaration of Helsinki. Blood from cows was obtained by procedures approved by the Animal Care Committee at the Pacific Agricultural Research Centre, Agriculture and Agri-Food Canada.

Results

Sample storage

To test the stability of EX Cu in stored plasma, venous blood samples were taken from six lactating Holstein cows and from two human volunteers. The samples from the humans were taken at 7:00 AM after a 9-h fast. The plasma was separated from cells by centrifugation and analyzed immediately or stored for various times at different temperatures. Some samples were subdivided prior to storage so that the subsamples would be subjected to only one freeze/thaw or warming cycle while others were subjected to multiple freeze/thaw or warming cycles before analysis. Exchangeable Cu in bovine plasma showed relatively minor variation when stored up to 205 d at −65 to −75°C and subjected to only one freeze/thaw cycle (Fig. 1). Linear regression analysis showed that the slope of the response line, 0.035 μg l^{-1} d^{-1}, was slightly greater than 0 ($P \leq 0.05$). Human plasma was found to be stable at room temperature for 8 h after collection, although there was an increase in EX Cu when tested again at 24 h (Fig. 2). When human plasma was stored in a household-type chest freezer

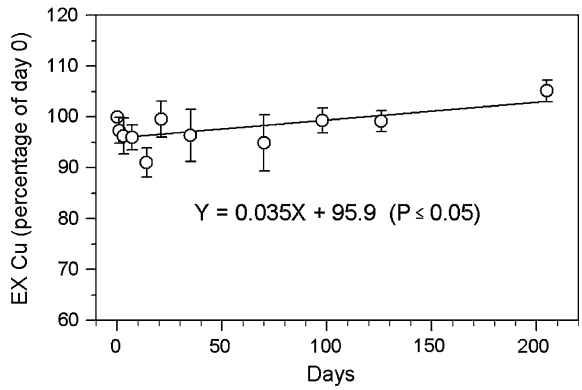

Fig. 1 Stability of exchangeable (EX) Cu in bovine plasma stored at −65 to −75°C. The plasma was divided into subsamples prior to storage so that each subsample was subjected to only one freeze/thaw cycle. Mean ± SE ($n = 5$–6) of EX Cu determined by the routine method (see text) in plasma samples from five or six cows collected at several times are shown. Results are expressed as percentages of zero time values so that SE do not include cow-to-cow variation

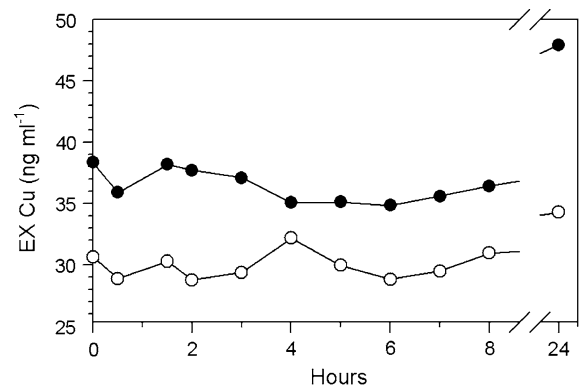

Fig. 2 Stability of exchangeable (EX) Cu in human plasma at room temperature. Repeat analyses of single samples by the routine method (see text) from each of two subjects are shown

(−10°C) there was little change in EX Cu over a period of 10–15 d provided samples were subjected to only one freeze/thaw cycle, whereas a sample subjected to multiple freeze/thaw cycles showed a substantial increase in EX Cu over a period of 35 d ($P \leq 0.05$) (Fig. 3). Also, we observed the sporadic appearance of unusually high concentrations of EX Cu in plasma stored at −18°C, especially after repeated freeze/thaw cycles. One of two samples stored in a refrigerator (2–3°C) for 10–15 days and subjected to only one warming cycle showed an increase in EX Cu ($P \leq 0.05$) (Fig. 4). A sample stored in a refrigerator and warmed prior to every analysis showed a substantial increase in EX Cu over 36 days ($P \leq 0.05$) (Fig. 4).

Extraction solvents

Six potential solvents for the extraction of EX Cu from serum or plasma were evaluated. The solvents were amyl acetate, heavy mineral oil, light mineral oil, octanol, toluene and xylene. The use of amyl acetate for extraction of EX Cu from serum was described previously by Suttle and Field (1968). Octanol, toluene and xylene tended to form stable emulsions with serum, even with gentle shaking, and consequently were found to be unsuitable for the method. Amyl acetate and heavy and light mineral oils were more promising but amyl acetate required centrifugation for phase separation to occur and also produced a light-colored interfacial layer of unknown composition. In addition, solubility of amyl acetate in water is about 2%, which is undesirable for routine operation of an inductively coupled plasma. Of the solvents tested, light mineral oil was the most suitable with respect to ease of phase separation, absence of interfacial layers or cloudiness and viscosity. There were no visible effects of either heavy or light mineral oil on plasma.

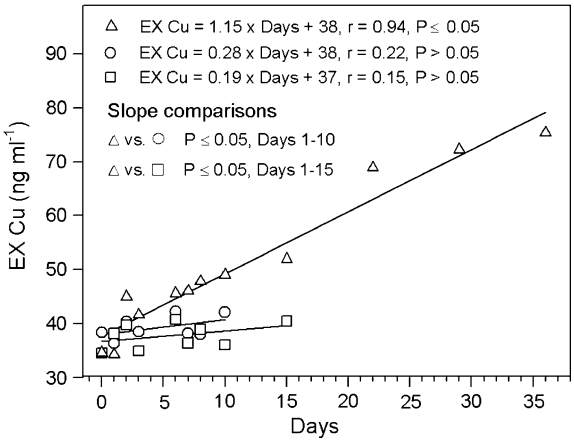

Fig. 3 Stability of exchangeable (EX) Cu in human plasma stored at −10°C. Repeat analyses of single samples by the routine method (see text) from two subjects are shown. Circles (subject A) and squares (subject B): samples were subdivided prior to freezing so that each subsample was subjected to only one freeze/thaw cycle. Triangles (subject B): the entire sample was thawed and refrozen each time a subsample was removed for analysis

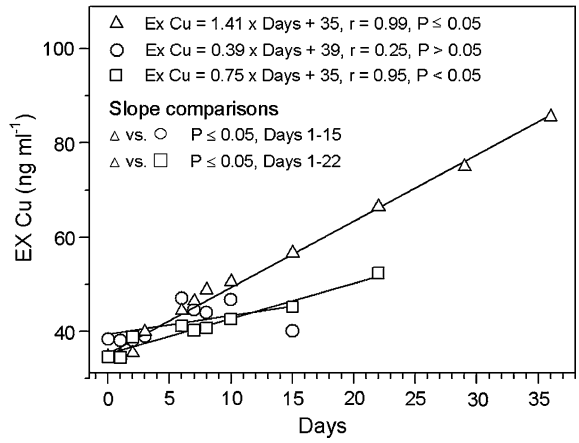

Fig. 4 Stability of exchangeable (EX) Cu in human plasma stored at 2–3°C in a refrigerator. Repeat analyses of single samples by the routine method (see text) from two subjects are shown. Circles (subject A) and squares (subject B): the samples were subdivided prior to transferring to the refrigerator so that each subsample was warmed to room temperature only once. Triangles (subject B): the entire sample was warmed to room temperature then returned to the refrigerator each time a subsample was removed for analysis

Time required for exchange between stable isotope tracer and plasma Cu

The time required for equilibration of added $^{65}Cu^{2+}$ with the EX Cu fraction in plasma was investigated with human plasma. In the routine method, the time available for equilibration of added $^{65}Cu^{2+}$ with EX Cu varied from 0.5 to 5 min after mixing the tracer with the plasma and before adding the Na DDC reagent. To determine the amount of time required for equilibration, subsamples were mixed gently for various times from <1 min to 5.0 h in 0.5-h increments after addition of tracer and prior to addition of Na DDC. No effect of varying the equilibration time on

the determination of EX Cu was observed (data not shown).

Extraction time at two sodium diethyldithiocarbamate concentrations

The effect of extraction time in bovine plasma was evaluated at the concentration of Na DDC selected for the routine method, 1.6×10^{-4} M, and at the highest concentration reported in the literature for Na DDC extraction of direct reacting Cu from plasma, 1.1×10^{-2} M (Suttle and Field 1968). Isotopically exchangeable Cu was determined by isotope dilution analysis while extractable Cu was determined by the addition of external calibration standards. Extraction times for bovine samples varied from 10 to 240 min. At 1.6×10^{-4}-M Na DDC, EX Cu determination by isotope dilution in bovine plasma appeared to be fairly constant when extraction time was increased from 20 min to 4 h, while the first measurement at 10 min seemed low (Fig. 5a, solid lines). Extraction of bovine plasma with 1.1×10^{-2}-M Na DDC yielded substantially more EX Cu than extraction with 1.6×10^{-4}-M Na DDC. In addition, with 1.1×10^{-2}-M Na DDC, the estimated concentration of EX Cu continued to increase throughout the 4-h extraction (Fig. 5a, dashed lines).

In human plasma, the effect of extraction time was evaluated with 1.6×10^{-4}-M Na DDC. There was no effect of varying extraction time from 10 to 120 min on concentration of EX Cu in human plasma (Fig. 6, compare with Fig. 5a, solid lines).

Shaking method and sample size

Exchangeable Cu was determined in 5-ml subsamples of a pooled plasma while using two shaking methods: a clinical blood tube rocker (rocking frequency: 0.3 Hz) and a horizontal, reciprocating shaker in which tubes were held at an angle of 5° vertically from the axis of motion and which was operated with an amplitude of 2.5 cm and frequency of 2.5 Hz. In addition, 2.5-ml and 5.0-ml subsamples of the same pooled plasma were analyzed with the horizontal shaker to compare the effect of sample size.

A slightly lower value for EX Cu was obtained with the horizontal shaker compared to the rocker (5-ml samples) ($P \leq 0.05$), but no differences between 2.5- and 5-ml sample sizes (horizontal shaker) were

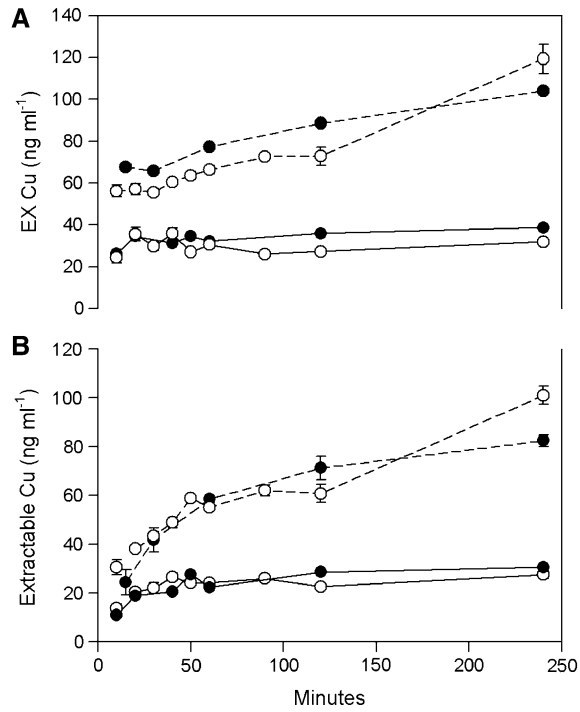

Fig. 5 Effect of extraction time and Na diethyldithiocarbamate (DDC) concentration on determination of exchangeable (EX) and extractable Cu in bovine plasma (mean ± SE, $n = 3$ or 4 cows). Broken lines show extraction with 1.1×10^{-2}-M Na DDC; solid lines show extraction with 1.6×10^{-4}-M Na DDC. Pairs of lines (same line style, different symbols) represent repeat experiments. (**a**) Stable isotope dilution method (EX Cu). (**b**) Calibration standard method (extractable Cu)

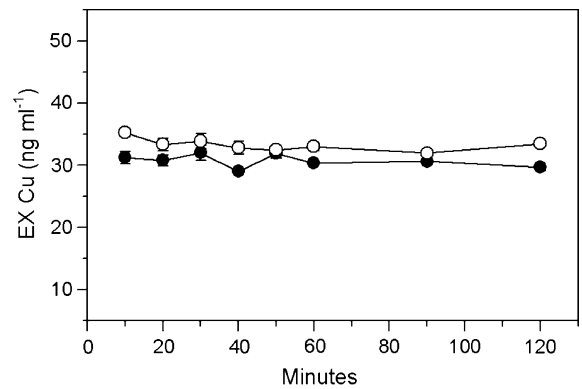

Fig. 6 Effect of extraction time on determination of exchangeable (EX) Cu in human plasma (mean ± SE, $n = 4$). Analyses were by the routine procedure (see text) except for the variations in extraction time. Multiple subsamples of a single plasma sample were analyzed immediately (solid circles) or after 2 d storage at −65 to −70°C (open circles)

observed ($P > 0.05$). Mean (±SE, $n = 8$) results were: rocker with 5-ml samples, 37.5 ± 0.99; horizontal shaker with 5-ml samples, 33.0 ± 1.1 and horizontal shaker with 2.5-ml samples, 35.5 ± 0.53 μg l^{-1}. Although statistically significant, the difference between shaking methods was considered small and not investigated further. Both shaking methods caused the mineral oil to break into small globules without forming an emulsion. The small globules readily coalesced without centrifugation after completion of shaking.

Serum versus plasma and the effect of heparin

To determine if there was a difference in EX Cu concentration between serum and plasma, EX Cu was measured in bovine and human plasma (with heparin as the anticoagulant) and in serum prepared from the same blood samples. To isolate the effect of heparin itself, heparin (60 units/ml) was added to subsamples of human serum prior to EX Cu determination.

Small but consistent differences were found between serum and plasma for both the bovine and human blood. Means (±SE) for bovine blood from three lactating Holstein cows are 27.9 ± 1.3 μg l^{-1} ($n = 3$) for plasma and 32.0 ± 0.87 μg l^{-1} ($n = 3$) for serum ($P > 0.05$). Results for multiple determinations on a single human blood sample were 33.6 ± 0.85 μg l^{-1} ($n = 4$) for plasma, 37.2 ± 0.53 μg l^{-1} ($n = 7$) for serum and 43.3 ± 3.8 μg l^{-1} ($n = 4$) for serum with 60 units heparin ml^{-1} added after the serum was prepared.

Back-extraction time

In the routine method, back-extraction of Cu from mineral oil to 0.63-M HNO_3 was performed for 3 h in order to maximize recovery of analyte. In the present work, the need for a lengthy back-extraction time was re-evaluated by varying back-extraction time from 15 min to 4 h. Recovery of natural and tracer ^{65}Cu from labeled plasma increased with back-extraction time for at least 2 h. On the other hand, the determination of EX Cu (calculated from the isotope ratio of the extracted Cu) stabilized after about 30 min (Table 1).

Table 1 Effect of back-extraction time on analyte recovery and plasma exchangeable (EX) Cu determination ($n = 4$)

Back-extraction time (min)	ICPMS[a] ion intensity at *m/z* 65		Plasma EX Cu	
	Mean (counts s^{-1})	Relative SD (%)	Mean (μg l^{-1})	Relative SD (%)
15	4,070	98	81	89
30	2,443	36	36	4.5
60	5,606	31	33	5.0
120	15,105	6.5	33	2.4
240	16,894	6.5	33	2.5

Copper that had been extracted from plasma into light mineral oil was back-extracted for varying lengths of time into 0.63 M HNO_3

[a] Inductively coupled plasma mass spectrometry

pH

The effect of pH on determination of EX Cu was investigated with bovine and human plasma. Because dropwise addition of acid or base to plasma would cause local variations in pH that might release ceruloplasmin Cu, plasma pH was adjusted by the following procedure. Tris(hydroxymethyl)aminomethane (TRIS), 0.2 M, was shaken with Chelex 100 in order to remove Cu contamination. Buffers in the range of pH 7.0–10 were prepared by addition of nitric acid to 0.02-M TRIS (for human plasma) or 0.1 M-TRIS (for bovine plasma). Equal volumes of buffer and plasma were mixed and the diluted plasma was then analyzed for EX Cu. To minimize contamination, measurements of pH were performed on plasma samples only after extraction of EX Cu. Exchangeable Cu concentration in human and bovine plasma was relatively constant from pH 7.2 (the lowest pH measured) to 8.5 (Fig. 7). Above pH 8.5, the concentration of EX Cu increased. The range of pH of unbuffered plasma samples (after EX Cu extraction) was within the stable pH range for EX Cu determinations.

Recovery of natural Cu added to plasma

Recovery of natural Cu added to human plasma was studied over a broad range of additions from 0 to 507 μg added Cu l^{-1} of plasma. Copper was added to plasma as $Cu(NO_3)_2$. The added Cu was mixed well with the plasma prior to adding the $^{65}Cu^{2+}$ tracer, and then determination of EX Cu was performed by the routine procedure. Ninety-seven to one hundered and seven percent of natural Cu added to human plasma was found within the EX Cu fraction determined by isotope dilution analysis (Table 2).

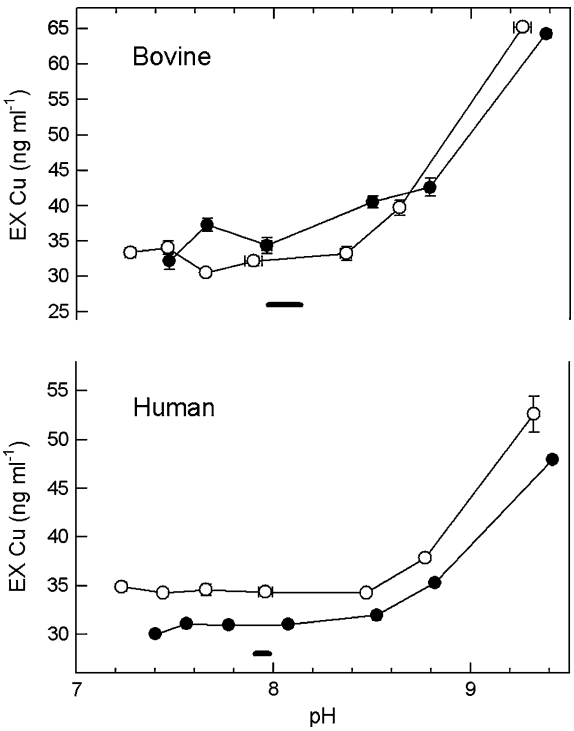

Fig. 7 Effect of extraction pH on determination of exchangeable (EX) Cu in buffered bovine and human plasma. Analyses were by the routine procedure (see text), except for pH adjustments, details of which are described in the text. Solid and open symbols indicate different plasma samples. Means ± SE of repeat determinations for EX Cu (vertical bars) and pH (horizontal bars) are presented ($n = 3$ or 4). Heavy horizontal bars indicate the range of pH observed in unbuffered plasma samples. All pH determinations were performed with plasma samples after extraction of EX Cu

Table 2 Recovery of added Cu from human plasma (means, $n = 4$)

Cu added (μg l^{-1})	Exchangeable Cu		Recovery of added Cu	
	μg l^{-1}	SE	%	SE
0	33.9	0.8	–	–
9.38	43.0	1.0	96.7	11.1
28.2	62.4	0.5	101	1.7
84.5	119	1.0	101	1.2
253	304	1.3	107	0.5
507	556	3.1	103	0.6

Natural Cu was added to plasma as $Cu(NO_3)_2$ followed by gentle mixing. Then exchangeable Cu was determined by stable isotope dilution analysis with $^{65}Cu^{2+}$

Table 3 Determination of total and exchangeable (EX) Cu in solutions with albumin and amino acids (means, $n = 4$)

Cu added to solution (μg l^{-1})	EX Cu (μg l^{-1})		Total Cu (μg l^{-1})		Ex Cu as a percentage of total Cu	
	Mean	SE	Mean	SE	Mean	SE
0.0	–	–	53.7	0.4	–	–
0.0	–	–	47.5	1	–	–
10.0	52.2	0.3	61.7	1	84.6	5.5
30.0	76.9	0.7	79.2	1	97.1	1.5
90.0	133	2	149	4	89.3	2.7
270	316	2	316	3	100	1.1
540	557	16	582	2	95.7	2.8

Natural Cu was added to an isotonic, buffered (pH 7.5) solution of human albumin and 22 amino acids. The albumin and amino acids were at concentrations found in human plasma

Exchange with albumin and amino acids

To study the exchange of free Cu with albumin-bound and amino acid-bound Cu, solutions were prepared in a TRIS/HCl buffer, pH 7.5. The solutions included human albumin, 0.0500 g ml^{-1} and 22 amino acids at concentrations found in human plasma. The amino acids were (concentrations, mg l^{-1}, in parentheses): l-alanine (60), arginine (19), l-asparagine (6.6), l-aspartic acid (2.2), l-cysteine (7.0), l-cystine (7.0), l-glutamic acid (16), glutamine (96), glycine (39), l-histidine (21), l-isoleucine (13), l-leucine (23), l-lysine (30), l-methionine (6.0), l-phenylalanine (18), l-proline (33), trans-4-hydroxyl-l-proline (4.1), l-serine (26), l-threonine (40), l-tryptophan (14), l-tyrosine (13) and l-valine (37). Precautions were taken to minimize Cu contamination from reagents and glassware. Osmolality was adjusted to 264–289 mOsmol kg^{-1} with NaCl to be consistent with human plasma. Copper was added to the solution at 10–540 μg l^{-1}. Total Cu in the solution was determined by isotope dilution ICPMS and EX Cu was determined by the routine procedure. Exchangeable Cu as a percentage of total Cu determined in solution with albumin and amino acids varied from 85 to 100% (Table 3).

Ultrafiltration

Exchangeable Cu determined by the routine procedure was compared with the Cu fraction that could be obtained from plasma by ultrafiltration. Subsamples of pooled human plasma were filtered by

centrifugation (2,550*g*, 2 h, 4°C) through a membrane designed to retain substances of 10 kDa or greater (Amicon). Other subsamples of the same pooled plasma were analyzed for EX Cu by the routine procedure. Ultrafiltration yielded a Cu fraction a little more than half that of EX Cu. Ultrafiltrate of pooled human plasma passing a 10 kDa membrane contained 18.2 ± 0.8 (mean ± SE, $n = 6$) μg Cu l^{-1}. Analysis of the same sample for EX Cu by the routine method yielded 32.8 ± 0.1 μg Cu l^{-1}. Analysis for total plasma Cu by isotope dilution ICPMS yielded 942 ± 23 μg Cu l^{-1}.

Discussion

The procedure for determination of plasma EX Cu used in this study is based on stable isotope dilution analysis. The stable isotope tracer used was $^{65}Cu^{2+}$, which is chemically identical to free Cu^{2+} in plasma. The tracer was mixed with free plasma Cu^{2+} in an environment that, as much as possible, allowed the isotopically enriched plasma free Cu^{2+} to exchange with ligands in the same manner as natural Cu^{2+}. Then the concentration of EX Cu was calculated from the isotopic ratio of enriched free Cu^{2+} extracted from plasma. It was not necessary to completely extract the EX Cu fraction from plasma to accurately measure its isotopic ratio. Only a quantity of EX Cu large enough for isotope ratio analysis by ICPMS was required.

Exchangeable Cu is present in low concentrations in plasma. Ceruloplasmin-bound Cu, on the other hand, is present in much higher concentrations. Thus, there is a risk of contaminating the EX Cu fraction with ceruloplasmin Cu during analysis. Stable isotope dilution analysis is particularly suited for the determination of EX Cu because it permits the use of gentle techniques that matched the conditions in natural plasma as closely as possible. Since it was necessary only to extract a portion of the total EX Cu for ICPMS analysis, mild conditions that greatly reduced the potential of simultaneously extracting Cu from ceruloplasmin could be used. Probably the most important factors that minimized the risk of contaminating EX Cu with ceruloplasmin Cu were a low concentration of Na DDC and the use of a light mineral oil for the solvent extraction step.

Results of storage studies indicated that samples should be stored in an ultracold freezer (≤−65°C) as soon as possible after collection. Storage for a short time (<2 weeks) in a household-type chest freezer (−10°C) may be acceptable. Samples should never be subjected to more than one freeze/thaw cycle, nor should they be stored in a refrigerator or at room temperature for more than a few hours. Gutteridge et al. (1985) found that phenanthroline-detectable Cu appeared in human serum upon storage at 4°C for more than 2 weeks. Storage at −20 or −70°C slowed but did not eliminate the appearance of the phenanthroline-detectable Cu fraction. They recommended that analysis of low-molecular-mass Cu^{2+} complexes in body fluids, including serum, be performed on fresh samples (Gutteridge et al. 1985). Musci et al. (1992) observed the transfer of ceruloplasmin Cu to albumin in plasma and serum upon standing at room temperature for three or more hours. However, the transfer was not consistent in all samples. Sometimes several days or repeated freeze/thaw cycles were required. It is likely that the increase in the EX Cu fraction which we observed under a variety of storage conditions also was due to release of Cu from ceruloplasmin.

Equilibration between plasma EX Cu and added $^{65}Cu^{2+}$ apparently occurs rapidly. Thus, there was no need to include a delay step following ^{65}Cu addition to plasma samples prior to addition of Na DDC and mineral oil. It seems likely that equilibration also occurs during the 30 min period of shaking for extraction of the Cu DDC complex into light mineral oil, which may explain why we were unable to observe incomplete exchange when 1 min or less was allowed before proceeding to the extraction step.

In previous work it was shown that the measured amount of EX Cu remained constant as Na DDC concentration was raised four orders of magnitude from 1.6×10^{-8} to 1.6×10^{-4} M (Buckley et al. 1996). The broad range of Na DDC concentrations, over which the measured value of EX Cu did not vary, indicated that EX Cu is a distinct Cu fraction. The Na DDC concentration at the upper end of this range, 1.6×10^{-4} M, was selected for the routine method. Above this concentration, the measured amount of EX Cu increased, apparently because higher concentrations of Na DDC caused exchange of $^{65}Cu^{2+}$ with forms of plasma Cu that normally do not exchange with ionic Cu. The additional Cu may have come from ceruloplasmin, which is the most abundant form of Cu in plasma. Sodium DDC is a strong

Cu chelator and one would expect that eventually, as the Na DDC concentration increased, Cu would exchange with one or more of the Cu sites on ceruloplasmin.

In the present study, the stability of the EX Cu fraction during extended exposure of plasma to 1.6×10^{-4} M Na DDC was compared with 1.1×10^{-2}-M Na DDC. The measurement of EX Cu remained relatively stable for up to 4 h at 1.6×10^{-4} M Na DDC. On the other hand, Ex Cu increased continuously for 4 h when bovine plasma was extracted with 1.1×10^{-2}-M Na DDC (Fig. 5a, dashed lines), which indicated that other forms of Cu were exposed to exchange over the 4 h. The stability of the EX Cu measurement at the lower Na DDC concentration supported the choice of 1.6×10^{-4} M Na DDC for the routine method. The biological half-life of EX Cu in humans is only about 10 min (Buckley et al. 1996). It seems unlikely that any forms of Cu that do not exchange with $^{65}Cu^{2+}$ within 4 h (at room temperature) could be a component of the rapidly turning over EX Cu pool.

The concentration of EX Cu determined by isotope dilution was consistently greater than extractable Cu determined by the calibration standard procedure (Fig. 5a compared with 5b). This result demonstrated that the isotope dilution method for EX Cu measured a fraction of plasma Cu which was greater than that which was extracted under the same conditions. Thus, the concentration of Na DDC utilized was not enough to completely extract the EX Cu fraction. However, a higher concentration of Na DDC, as discussed above, was unacceptable because other Cu fractions would began to exchange with free Cu^{2+}. Fortunately, incomplete extraction of the EX Cu fraction is not a disadvantage, because it is not necessary to extract the entire EX Cu fraction in order to perform isotope dilution analysis.

The difference between plasma and serum EX Cu was investigated because whole plasma Cu concentration is reported to be greater than that for serum Cu, which may be the result of trapping of some ceruloplasmin in the fibrin clot (Kincaid et al. 1986). It was reasoned that a protein such as transcuprein, which may be a ligand for EX Cu, might react in a similar fashion. However, slightly lower values of EX Cu were found in plasma, which is inconsistent with the loss of high-molecular weight ligands of EX Cu in the clot.

Suitable analytical precision to measure EX Cu concentration in plasma can be achieved with a 30-min back-extraction time (Table 1). On the other hand, higher precision can be achieved with higher analyte recovery resulting from longer back-extraction times. Although not required for determination of EX Cu concentration, higher precision is available, if desired, for other studies, such as $^{65}Cu^{2+}$ tracer investigations. We chose a 3 h back-extraction for the routine method so we could use the same procedure for EX Cu analysis as well as for stable isotope tracer studies.

Results showed that there was no need for pH adjustment of plasma samples for determination of EX Cu by the routine method. There was adequate natural buffering to maintain plasma samples within a safe pH range for the analysis. This is an advantage because adjustment of plasma pH could be a significant source of Cu contamination. An increase in the concentration of EX Cu observed above pH 8.5 (Fig. 7) may have been caused by a release of Cu from ceruloplasmin. Marriott and Perkins (1966) investigated the removal of Cu from purified ceruloplasmin by 5×10^{-3}-M DDC. They found that Cu was stable at pH 5.8, but could be partially removed at pH 5.2. Thus, although we didn't investigate isotopic exchange below pH 7.2, the determination of EX Cu would be expected to increase below pH 5.8.

Recovery of natural Cu added to plasma demonstrated that the EX Cu method has adequate linear range for even unusually high EX Cu concentrations, such as might be found in cases of Wilson's disease. Because both the natural Cu and stable isotope tracer were added to plasma in the same chemical form, although at different times, complete equilibration between tracer and tracee was expected.

Studies in water solution were conducted to investigate the role of albumin and amino acids in the determination of EX Cu. Copper associates with albumin with a molar ratio of 1:1 until the first binding site becomes saturated (Sarkar and Wigfield 1968). The molar ratio of albumin:Cu in the solution with no added Cu was 875:1 indicating that albumin and presumably the amino acids were not saturated with respect to Cu. The highest addition of Cu to the solutions represented an albumin:Cu ratio of 80:1, also unsaturated with respect to albumin. In general, there was good agreement between the total Cu and EX Cu determinations in solution, which indicated

that Cu complexed by albumin and amino acids exchanged readily with the Cu stable isotope tracer under the analysis conditions, and, therefore, Cu bound to these ligands could be components of the EX Cu fraction.

One would not expect to obtain the same measurement by ultrafiltration as by isotope dilution because EX Cu bound to albumin and other proteins greater than 10 kDa would not be determined. Noubah and Al-Awqati (1990) found 16 ± 3.9 (SD) μg Cu l^{-1} in the ultrafilterable components (<10 kDa) of serum of normal nonpregnant women, which agrees well with our result from a healthy man. Thus, it appears that low-molecular-weight components (<10 kDa) may form a significant portion of EX Cu.

Comparison of isotope dilution results with those of other methods for plasma exchangeable copper

Earlier workers (Gubler et al. 1953; Cartwright and Wintrobe 1964; Evans and Wiederanders 1967), using the method of Gubler et al. (1953), found direct reacting Cu in human plasma and serum to be about 4–6.2% of total Cu. In the study of Cartwright and Wintrobe (1964), 30 male and female subjects, 17–45 yr old, were found to have a mean direct reacting Cu concentration in serum of 70 μg l^{-1} with 95% confidence limits of 0–200 μg l^{-1}. Buckley et al. (1996) reported that ten human males, 27–36 yr, had a mean EX Cu concentration of 31 μg l^{-1}, with 95% confidence limits of 28–33 μg l^{-1}. Although there likely would be more variation in the direct reacting Cu concentrations in the subjects of Cartwright and Wintrobe (1964) than in the EX Cu of the subjects of Buckley et al. (1996), the variation in the former is very large and may indicate analytical difficulties. Others have concluded that the method of Gubler et al. (1953) lacks analytical sensitivity (Suttle and Field 1968; Wirth and Linder 1985). Inadequate sensitivity typically leads to large variability, which, because the variance is also a function of the mean, leads to elevated mean values. That apparently was the case with the results of Cartwright and Wintrobe (1964) and may explain why the results of Buckley et al. are nearly half of those of Cartwright and Wintrobe (1964). Furthermore, earlier workers did not describe plasma or serum storage conditions or time periods between sampling and analysis. Thus, it is also possible that elevated direct reacting Cu determinations in early studies may have resulted from improper sample storage.

Wirth and Linder (1985) reported that transcuprein- and albumin-bound Cu separated from normal adult human serum by Sephadex G-150 chromatography were of approximately equal concentrations and totaled 270 μg l^{-1}. An additional 90 μg Cu l^{-1} was found associated with low molecular weight components and 600 μg l^{-1} with ceruloplasmin (Wirth and Linder 1985). Non-ceruloplasmin Cu forms totaled 360 μg l^{-1}, or 38% of serum Cu, which is about 12 times the concentration of EX Cu found by the isotope dilution method. Gordon et al. (1987), also using Sephadex G-150 chromatography, were unable to identify a copper-binding protein other than albumin in serum and concluded that albumin was the only protein that binds an appreciable amount of the copper absorbed directly from the gastrointestinal tract. The results of Gordon et al. (1987) raise uncertainty about the existence of transcuprein and, therefore, the amount of copper bound to it.

One possible explanation for the difference between our previous results (Buckley et al. 1996) and those of Wirth and Linder (1985) was that enriched $^{65}Cu^{2+}$ added in vitro in the isotope dilution method had not exchanged with the entire albumin- and transcuprein-bound Cu fraction. However, present observations and the known Cu binding properties of albumin and transcuprein do not support this hypothesis. Because the capacity of the first binding site of albumin to complex Cu greatly exceeds the quantity of EX Cu, it does not appear that other sites on albumin, with lower affinities for Cu, could be involved. Therefore, because $^{65}Cu^{2+}$ would have interacted with a single homogeneous binding site on albumin and, because albumin-bound Cu readily exchanges with transcuprein-bound Cu (Weiss and Linder 1985), it seems likely that enriched $^{65}Cu^{2+}$ would exchange readily with the entire albumin- and transcuprein-bound Cu fraction. Furthermore, present results indicate that exchange between $^{65}Cu^{2+}$ and the plasma EX Cu fraction rapidly goes to completion under the conditions of the isotope dilution procedure. Thus, the difference between the present results and those of Wirth and Linder (1985) are unlikely to be caused by slow or incomplete exchange of tracer and tracee.

Wirth and Linder (1985) stored samples for up to 6 months at −20°C. They reported that ceruloplasmin

oxidase activity was stable under their storage conditions for up to a year with as many as four freeze/thaw cycles. The results of present studies suggest that more than one freeze-thaw cycle should be avoided. Nevertheless, since the loss of Cu from ceruloplasmin is associated with loss of oxidase activity, it appears that the storage conditions adopted by Wirth and Linder (1985) were adequate.

Gutteridge et al. (1985), using an assay based on the degradation of DNA in the presence of 1,10-phenanthroline, O_2 and a reducing agent, were unable to detect "loosely bound" copper in freshly collected human serum in spite of a claimed capability to detect concentrations as low as 0.1 μ mol l^{-1} (6.4 μg l^{-1}). The authors suggested that reported measurements of low-molecular-mass Cu^{2+} complexes in body fluids may be an artifact of storage and handling due to the release of Cu from ceruloplasmin. However, our storage and handling procedures were at least as conservative as those of Gutteridge et al. (1985).

In contrast to the extreme values of 30% (Wirth and Linder 1985) and 0% (Gutteridge et al. 1985), the results of four other independent methods indicate that non-ceruloplasmin or EX Cu is greater than 0% and less than 10% of total Cu in human plasma. Exchangeable Cu was found to be 3.4% of plasma Cu in healthy males by stable isotope dilution analysis (Buckley et al. 1996). Workers using the Na DDC colorimetric method of Gubler et al. (1953) reported values ranging from 4 to 6.2% (Cartwright and Wintrobe 1964; Evans and Wiederanders 1967; Gubler et al. 1953) for direct reacting Cu in apparently healthy subjects (including pregnant subjects that had higher total plasma Cu as well as higher direct reacting Cu). Determinations of ceruloplasmin-bound Cu by immunochemistry procedures showed that non-ceruloplasmin Cu was 5–8% of total plasma Cu from apparently healthy subjects as well as patients with rheumatoid arthritis (Van den Hamer 1988; Louro et al. 2000). Finally, Bearn and Kunkel (1954) separated the Cu components of normal human plasma by zone electrophoresis and found that more than 90% of plasma Cu was bound to ceruloplasmin. Thus, it appears that non-ceruloplasmin Cu constitutes greater than 0% and less than 10% of plasma Cu in healthy humans.

Although EX Cu represents a small percentage of total plasma Cu, especially in bovine and human plasma (Buckley et al. 1996), the present evaluation of the stable isotope dilution procedure indicates that consistent, reliable measurements of EX Cu can be made. A significant source of error in the determination of EX Cu by isotope dilution was not identified in the present work. Furthermore, the studies presented here and those presented previously (Buckley et al. 1996) indicate that EX Cu represents a physiologically meaningful Cu fraction that is metabolized separately and distinctly from ceruloplasmin Cu. Although EX Cu has been recognized as the absorbed form of Cu (Gordon et al. 1987) and its central role in Cu metabolism has been included in kinetic models of Cu metabolism (Buckley 1991; Turnlund 1998), its metabolism and potential diagnostic attributes are largely unexplored.

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