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Determination, stable isotope enrichment and kinetics of direct-reacting copper in blood plasma

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Direct-reacting Cu is defined as Cu^{2+} and other forms of Cu that readily exchange with Cu^{2+} in blood plasma. An analytical method was developed for 1) quantification of direct-reacting Cu by stable isotope dilution and 2) determination of ^{65}Cu enrichment of direct-reacting Cu in plasma samples from in vivo tracer studies. The method involved addition of enriched ^{65}Cu to plasma, extraction with sodium diethyldithiocarbamate in mineral oil, and analysis by inductively coupled plasma mass spectrometry. Optimum sodium diethyldithiocarbamate concentration for the extraction was 0.16 mM. Direct-reacting Cu (means ± SD) varied from 3.4 ± 0.5% of whole plasma Cu in dairy cows (n = 7) and 3.4 ± 0.3% in healthy men (n = 10) to 16.6 ± 3.7% in dogs (n = 3). After intravenous infusion of enriched ^{65}Cu into two healthy men, biological half-lives of 8.7 and 12.3 min were determined for direct-reacting Cu. (J. Nutr. Biochem. 7:488–494, 1996.)

Keywords: direct reacting copper; exchangeable copper; non-ceruloplasmin Cu; stable isotope dilution analysis; human; bovine

Introduction

The existence of a small percentage of plasma Cu that reacts directly with sodium diethyldithiocarbamate (NaDDC) without prior acidification was initially recognized by Gubler et al.[1] They observed that hypercupremia after intravenous administration of Cu in dogs and humans was due to an increase in "direct-reacting" (DR) Cu. Because it had been demonstrated that Cu in ceruloplasmin did not react directly with NaDDC at physiological pH,[2] DR Cu was recognized as non-ceruloplasmin Cu.

The original method of determining DR Cu involved the addition of 2.8×10^{-4} M NaDDC to diluted plasma followed by the measurement of color development at 440 nm.[1] This method, however, has been found to lack sensitivity.[3,4] There have been relatively few publications on DR Cu since its discovery, which may be due, in part, to the lack of a satisfactory method for its determination.

DR Cu is the initial transport form of Cu absorbed from the gut,[5] and is assumed to be the form of Cu released from extrahepatic tissues in kinetic models of Cu metabolism.[6,7,8] Since it has been shown that ceruloplasmin-bound Cu does not exchange with Cu^{2+},[9] whereas albumin-bound,[10] amino acid-bound[11] and transcuprein-bound[12] Cu do exchange readily with Cu^{2+} or with each other, DR Cu may be defined as that fraction of plasma Cu consisting of Cu^{2+} and other species of Cu that exchange readily with Cu^{2+}. This definition describes a physiologically meaningful plasma Cu fraction, which is consistent with the definition of DR Cu in recent compartmental models of Cu metabolism in humans and animals.[6,7,8] The significance of determining DR Cu in improving the reliability of compartmental models of Cu metabolism was discussed by Buckley.[13] Such studies require the use of tracers and, frequently, a stable isotope tracer is preferred. Consequently, the objectives of the present study were 1) to develop methodology for the determination of DR Cu in blood plasma, 2) to develop a meth-

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odology for the determination of stable isotope enrichment of DR Cu and 3) to demonstrate the effectiveness of the methodology in both non-tracer and tracer studies by determining the DR Cu concentrations in several species of animals and by determining the half-life of DR Cu in humans.

Although NaDDC is still utilized as a chelator in this procedure, the principle of analysis is different from that of the original method of Gubler et al.[1] The quantity of exchangeable Cu was determined by stable isotope dilution, not by colorimetry. A measure of plasma Cu that was exchangeable with $^{65}Cu^{2+}$ was obtained, and neither quantitative extraction nor quantitative reaction of DR Cu with NaDDC were required.

Methods and materials

Reagents and equipment

Deionized or quartz sub-boiling distilled water was used throughout the analytical procedures. Enriched ^{65}Cu (Oak Ridge National Laboratory, Oak Ridge, TN, USA) was greater than 99.5% ^{65}Cu (molar). Nitric and hydrochloric acids used for reagent preparation were of high purity (J.T. Baker Ultrex grade, or equivalent). Light mineral oil (Fisher Scientific, Ottawa, Canada), which was low in Cu, was used as the extracting solvent.

Sodium diethyldithiocarbamate (Analar grade; British Drug Houses, Poole, UK) was recrystallized to minimize Cu contamination and to remove decomposition products. Five grams NaDDC were dissolved, with warming, in 25 mL redistilled ethanol and filtered through acid-washed glass filter paper. Addition of 25 mL redistilled amyl acetate to the filtrate caused crystals to form, which were collected by suction filtration, rinsed with ice-cold amyl acetate, and allowed to dry.

Stock reagent solutions were: 0.63 M HNO_3; 0.22 M HNO_3 plus 0.55 M HCl; 2.3–3.1 mM enriched ^{65}Cu in 0.22 M HNO_3 plus 0.55 M HCl; 0.1 M NaDDC. Reagent solutions prepared daily were: spike solution, a 1,000-fold dilution of enriched ^{65}Cu stock solution; dilute acid solution, 2.2×10^{-4} M HNO_3 plus 5.5×10^{-4} M HCl; and 1.02×10^{-3} M NaDDC.

With the exception of disposable plastic syringes and vials, which were found to contribute insignificant Cu contamination, labware was submerged for a minimum of 1 hr in 1.6-M nitric acid and rinsed with deionized water before using.

Two inductively coupled plasma mass spectrometers were used for determination of Cu stable isotope ratios: an Elan Model 250 (Sciex, Thornhill, Ontario, Canada) and a PlasmaQuad 2+/Turbo (VG Elemental, Winsford, Cheshire, UK). Analysis by inductively coupled plasma mass spectrometry (ICPMS) used routine instrument procedures with sample introduction by nebulization.

Recommended procedure for sample preparation

Duplicate plasma samples were required for the procedure if plasma was already labeled with enriched ^{65}Cu from an in vivo tracer experiment, otherwise a single sample was adequate. We used either 5.0-mL or 2.5-mL sample sizes. A smaller ratio of Cu contamination (from glassware, reagents, etc.) to DR Cu was achieved with larger samples, although both sizes yielded acceptable results in our laboratories. The following procedure is described for 5.0-mL sample sizes. Disposable, 25-mL all-plastic blood collection syringes were an integral part of the procedure (Safety-Monovette®, W. Sarstedt, Inc., Princeton, NJ USA). For 2.5-mL sample sizes, 10-mL Safety-Monovettes were used and reagent volumes were adjusted accordingly.

Safety-Monovettes were prepared for the extraction procedure by retracting the plunger and unscrewing the plunger rod. The cap was removed and 5.0 mL plasma was added without producing foam. Reagent blanks were prepared by substituting 5.0 mL water for plasma. One half milliliter of spike solution (approximately 100 ng enriched ^{65}Cu) was added to natural abundance samples and one of each pair of labeled samples. One half milliliter of dilute acid solution was added to the reagent blanks and the remaining half of the labeled samples. The tube contents were thoroughly mixed without forming surface bubbles. One milliliter of 1.02×10^{-3} M NaDDC reagent and 8.0 mL of mineral oil were added and the Safety-Monovettes capped. The concentration of NaDDC in the extraction mixture (plasma + spike solution + NaDDC reagent) was 1.6×10^{-4} M. The mineral oil volume was equivalent to the void volume remaining in a 25-mL Safety-Monovette with the plunger fully depressed. For a 10-mL Safety-Monovette this volume was 4.0 mL. The Safety-Monovettes were gently shaken on a tube rocker for mixing blood or a horizontal shaker for 30 min to convert DR Cu into copper diethylthiocarbamate and extract the complex into the mineral oil phase. During shaking, the mineral oil broke up into globules of various sizes, but shaking was not so vigorous as to form an emulsion.

After shaking, the air was expelled and the phases were allowed to separate with the Luer fitting down. The plasma layer was discarded by fully depressing the plunger and the remaining mineral oil was washed three times with 10- to 15-mL volumes of water. The washing steps removed Na, which interferes with the determination of Cu stable isotope ratio by ICPMS. Washing was efficiently performed by injecting water via a bottle top dispenser through a Luer connection into the Monovette, and then briefly and gently mixing the Monovette before allowing phases to separate and expelling the water.

After washing, 12 mL of 0.63 M HNO_3 was injected into the Monovettes, and the plunger was retracted. The Monovettes were shaken for 3 hr to extract the Cu into the acid layer, using the same shaking procedure as previously described. After shaking, the air was expelled, phases were allowed to separate, and the acid layer was dispensed into 20-mL polystyrene vials (W. Sarstedt, Inc., Princeton, NJ USA). Since there was usually a drop or two of mineral oil on the acid solution, the vials were capped and allowed to stand for several hours. While standing, the mineral oil collected on the sides of the vials, after which oil-free, 9-mL subsamples were taken for ICPMS analysis.

Calculation of tracer and DR Cu mass

Output from ICPMS analysis was expressed as ion intensities, which were corrected for blanks and used to calculate tracer[a]/tracee mass ratio (TTMR) as the unit of isotopic enrichment:

$$TTMR = \frac{mass\ of\ tracer}{mass\ of\ tracee} \quad (1)$$

$$TTMR = \frac{M_{(65)}A_{63(n)}}{M_{(n)}A_{63(65)}} \times \frac{R_{(n)}(1 - \Theta_{(m)}/\Theta_{(n)})}{R_{(n)}\Theta_{(m)}/\Theta_{(n)} - R_{(65)}} \quad (2)$$

where M = atomic mass; A = molar abundance; R = molar ratio, $^{65}Cu/^{63}Cu$; Θ = observed ion intensity ration for mass/charge = 65 over mass/charge = 63; 63 = ^{63}Cu; (65) = enriched ^{65}Cu tracer; (n) = natural abundance Cu; (m) = a mixture of tracee and

[a] Definitions: natural abundance sample—a plasma sample that has not been enriched with ^{65}Cu; labeled sample—a plasma sample that has been enriched with ^{65}Cu as part of an in vivo tracer experiment; spiked sample—a natural abundance or labeled sample to which ^{65}Cu has been added in vitro as part of stable isotope dilution analysis; tracee—naturally occurring Cu; tracer—an enriched preparation of ^{65}Cu.

tracer. Thus, for example, $A_{63(n)}$ = molar abundance of ^{63}Cu in natural abundance Cu; and $R_{(65)}$ = molar ratio, $^{65}Cu/^{63}Cu$, in enriched ^{65}Cu tracer. Values of *M*, *A*, and *R* were obtained from reference texts and from specifications supplied by Oak Ridge National Laboratory for enriched ^{65}Cu. A correction for instrumental bias is included in equation 2 and consists of multiplying observed ion intensity ratios by $R_{(n)}/\Theta_{(n)}$. Multiple determinations of $\Theta_{(n)}$ were performed with each batch of samples and the average for the batch was used for instrumental bias correction. Equation 2 (without the instrumental bias correction) can be obtained by algebraic manipulation of an equation originally proposed by Hintenberger.[14]

The mass of tracer (*Tr*) and natural abundance DR Cu ($DRCu_{nat}$) in labeled samples was obtained from:

$$Tr = \frac{S \times TTMRL}{TTMRLS - TTMRL} \quad (3)$$

and

$$DRCu_{nat} = \frac{S}{TTMRLS - TTMRL} \quad (4)$$

where *S* = mass of spike, *TTMRL* = *TTMR* of a labeled sample, *TTMRLS* = *TTMR* of a labeled, spiked sample. The total DR Cu (enriched and natural abundance) in a labeled sample was the sum of Tr and $DRCu_{nat}$.

The mass of DR Cu in natural abundance samples was obtained from:

$$DRCu_{nat} = \frac{S}{TTMRS} \quad (5)$$

where TTMRS = TTMR of a spike sample.

Optimization of NaDDC concentration and method evaluation

The effect of NaDDC concentration was evaluated with bovine and human plasma samples. Sodium diethyldithiocarbamate concentration was varied from 1.6×10^{-8} M to 1.6×10^{-2} M in the extraction mixture (plasma + spike solution + NaDDC reagent). Otherwise the sample preparation was identical to that as previously described. Analysis of the final extract from each sample was performed both by stable isotope dilution and by the method of external calibration standards. Both methods were performed simultaneously by ICPMS. The former method determined isotopically exchangeable Cu in plasma, whereas the latter method determined the amount of Cu that could be extracted with NaDDC and mineral oil. Finally, analytical precision and sensitivity were determined by multiple analysis of samples of human and bovine plasma selected for low DR Cu concentration.

Animal and human studies

The method was applied in two studies. First, a survey of DR Cu concentration in humans and animals was conducted. Blood samples from healthy human males, lactating Holstein dairy cows, brown Leghorn roosters, beagle dogs, and Sprague Dawley rats were transported immediately to the laboratory, separated into plasma and cells, and the plasma was frozen at −65 to −70°C until analysis. The samples were collected with all-plastic blood collection syringes (Sarstedt, Inc., St. Laurent, PQ) which contributed insignificant Cu contamination. DR Cu was determined by the method described herein and whole plasma Cu was determined by isotope dilution-ICPMS after addition of enriched ^{65}Cu internal standard and decomposition with hot nitric acid.

In the second study, the half-life of plasma DR Cu was determined in humans. Two apparently healthy human males (subject 1:37 y, 79.4 kg, and 165 cm; and subject 2: 44 y, 82.6 kg, and 180 cm) fasted from 10:00 p.m. until 12:00 noon the following day. Beginning 12 hr after the start of the fast (10:00 a.m.) they were infused with 12 to 16 μg enriched $^{65}Cu^{2+}$ in 100 mL physiological saline into an arm vein over a period of 34 to 39 min. Serial blood samples were taken from the opposite arm vein immediately before starting the infusion and at intervals for about 120 min thereafter. Direct reacting Cu as well as ^{65}Cu enrichment in the DR Cu fraction were determined.

The tracer study data was analyzed as a compartmental model (*Figure 1*) utilizing the SAAM and CONSAM computer programs.[15,16]

Human study protocols were approved by the Institutional Review Board of the University of North Dakota and the Human Studies Committee, USDA, ARS, and were consistent with the Declaration of Helsinki. Blood from cows and chickens was obtained by procedures approved by the Animal Care Committee at the Pacific Agricultural Research Centre for routine health monitoring of livestock. Blood sampling protocols for other animals were approved by the Animal Care and Use Committee of the Human Nutrition Research Center following guidelines set forth by the U.S. Animal Welfare Act, the National Institute of Health's Guide for the Care and Use of Laboratory Animals and, for rats, the American Veterinary Medical Association Panel on Euthanasia.

Results

Analytical sensitivity

Eight determinations of DR Cu on a single human plasma sample with unusually low DR Cu yielded a mean (±SD) of 9.93 ± 0.48 ng/mL. Nine determinations of DR Cu on a single bovine plasma sample yielded a mean of 30.7 ± 1.1 ng/mL. Detection limits, calculated as 3 × SD, were 1.4 ng/mL for human plasma and 3.3 ng/mL for bovine plasma.

Effect of sodium diethyldithiocarbamate concentration

DR Cu determined by stable isotope dilution analysis in bovine plasma was relatively constant with 1.6×10^{-6} to 1.6×10^{-4} M NaDDC in the reaction mixture (plasma + spike solution + NaDDC reagent) but increased from 1.6×10^{-4} to 1.6×10^{-2} M (*Figure 2*). On the other hand, the same

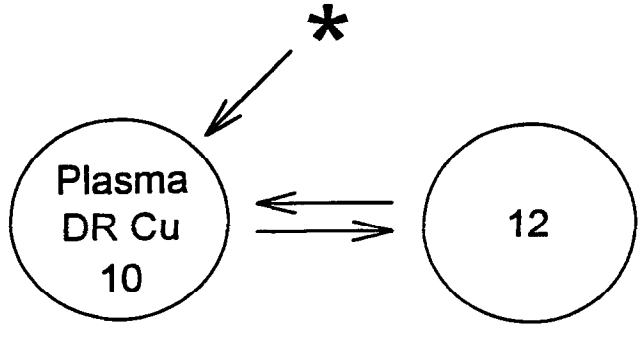

Figure 1 Two-compartment model of DR Cu metabolism. Circles are compartments with arbitrary numbers. Arrows between compartments represent fractional transfer coefficients (units: min^{-1}). The arrow with an asterisk represents tracer input. Compartment 12 is not within plasma.

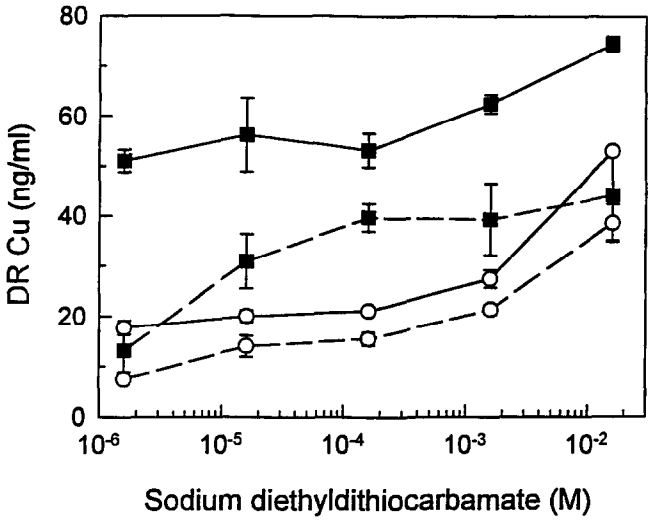

Figure 2 Effect of sodium diethyldithiocarbamate (NaDDC) concentration on determination of DR Cu in bovine plasma (mean ± SD, $n = 4$). Different symbols represent different plasma samples. Solid lines were obtained by stable isotope dilution analysis and indicate isotopically exchangeable Cu (DR Cu). Broken lines were obtained by external calibration standard techniques and indicate extracted Cu.

samples analyzed by the external calibration standard method yielded DR Cu concentrations that increased at the low as well as at the high range of NaDDC concentrations (*Figure 2*). The results by isotope dilution analysis were consistently greater than the results obtained by the external calibration standard method. In human plasma, DR Cu determined by stable isotope dilution analysis also was relatively constant up to 1.6×10^{-4} M NaDDC (*Figure 3*). At higher concentrations, the determination of DR Cu increased, but to a much greater extent than with bovine plasma. Results by isotope dilution analysis were consistently greater than results by the external calibration standard method, which agreed with bovine plasma results, except that the difference between isotope dilution and external calibration standard determinations was much greater for human compared to bovine plasma. Isotope dilution analysis measured isotopically exchangeable Cu, whereas the external calibration standard method measured the fraction of Cu that could be extracted from plasma with NaDDC and mineral oil.

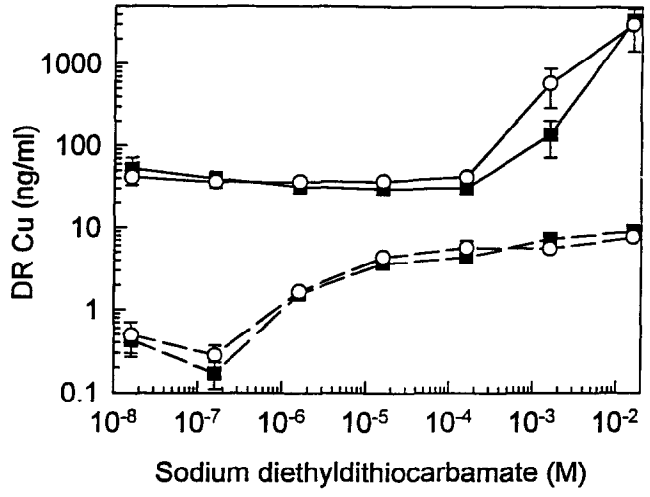

Figure 3 Effect of sodium diethyldithiocarbamate (NaDDC) concentration on determination of direct reacting DR Cu in human plasma (mean ± SD, $n = 3$). Plasma from a single blood collection was analyzed fresh (open circles) and again after storage at −65 to −70 C (solid squares). Solid lines were obtained by stable isotope dilution analysis and indicate isotopically exchangeable Cu (DR Cu). Broken lines were obtained by external calibration standard techniques and indicate extracted Cu.

Species Survey

DR Cu varied from a low of 28.4 ng/mL in lactating Holstein dairy cows to 95.0 ng/mL in male beagle dogs (*Table 1*). When expressed as a percentage of whole plasma Cu, DR Cu varied from a low of 3.4% in dairy cows and humans to 18.5% in a female beagle. The relative order for percentage DR Cu was human = cattle < rat < guinea pig < dog. Unfortunately, whole plasma Cu was not determined for chickens.

Plasma kinetics of DR Cu in humans

The concentration of enriched ^{65}Cu in the DR Cu fraction rose rapidly during infusion and dropped rapidly thereafter, but did not return to zero (*Figure 4*). The pattern indicated recycling of tracer from another pool or other pools. Because of the recycling, a simple single-pool model could not adequately describe the data and a second compartment was required (*Figure 1*). The model was solved for three parameters (adjustable parameters): the fractional transfer coefficient to compartment 12 (recycling sink) from compartment 10 (plasma DR Cu), the volume of distribution of tracer, and the initial mass of compartment 12. Other parameters were derived from the adjustable parameters and the assumption of steady state. The model solutions for parameters are given in *Table 2*. Correlation coefficients among adjustable parameters did not exceed 0.8, indicating satisfactory independence. Fractional SD of adjustable parameters did not exceed 0.1, indicating good precision for the estimates of those parameters. Half-lives of DR Cu (calculated from the fractional transfer coefficients to compartment 12 from compartment 10) for subjects 1 and 2 were 8.7 and 12.3 min.

Means (± SD) of DR Cu determinations on plasma samples collected over the 2 hr period of the tracer study were 35.5 ± 2.3 ($n = 10$) ng/mL for subject 1 and 33.9 ± 1.0 ($n = 10$) ng/mL for subject 2.

Discussion

The original method of DR Cu determination by Gubler et al.[1] was criticized for lack of sensitivity.[3,4] Attempts to use modifications of the original method in our laboratory, including a modification similar to that proposed by Suttle and Field,[3] were not successful. Thus, a method based on stable isotope dilution analysis was developed. Ideally, the detection limit for a new method should be determined at an analyte concentration at or near the detection limit, itself. However, the present method is very sensitive and we were

Table 1 DR Cu determined by isotope dilution analysis in plasma of humans and animals

Source[a]	Age	Sex	n	Plasma Cu		DR Cu			
								Percentage of plasma Cu	
				ng/mL	SD	ng/mL	SD	%	SD
Cattle	3–4 y	F	7	860	133	28.4	2.0	3.4	0.5
Chicken	9 mo	M	4	—	—	47.7	11.1	—	—
Dog	4, 11 y	M	2	605	12	95.0	30.7	15.7	4.7
Dog	16 mo	F	1	437	—	80.9	—	18.5	—
Guinea pig	4 mo	M	2	580	291	52.8	0.8	10.4	5.7
Guinea pig	6 mo	F	4	507	91	38.4	3.0	7.7	0.8
Human	27–36 y	M	10	920	96	30.7	3.6	3.4	0.3
Rat	3 mo	M	5	883	62	62.2	8.4	7.0	0.5

[a]Lactating Holstein dairy cows, brown Leghorn roosters, beagle dogs, Sprague Dawley rats.

unable to find samples of DR Cu concentration low enough to allow an ideal measure of detection limit. Thus, the detection limit was estimated using samples with the lowest available analyte concentration. Detection limits were 1.4 ng/mL for human plasma and 3.3 ng/mL for bovine plasma. Detection limits determined under ideal conditions would be equivalent or lower. Because the measured detection limits are much lower than the lowest DR Cu concentrations found in the species surveyed (*Table 1*), it is concluded that the method is sufficiently sensitive for determination of DR Cu in human and bovine plasma.

The method of analysis of DR Cu is based on the exchange of $^{65}Cu^{2+}$ with loosely bound forms of Cu in plasma. These forms include albumin,[10] peptides,[17] amino acids,[11,18] transcuprein,[12] and possibly an exchangeable site on ceruloplasmin,[19] but not with the tightly bound Cu atoms on ceruloplasmin.[9,20] Several minor copper-binding components in the molecular weight range of 13 to 30 kda have also been identified in human serum,[4] but it is unknown whether or not the Cu on these components is readily exchangeable. The proportion of Cu associated with transcuprein in plasma varies from as much as 43% of nonceruloplasmin Cu[4] to nondetectable amounts[5] in different studies for reasons that are not clear. Other than ceruloplasmin, albumin was found to be the only protein to bind appreciable amounts of Cu in the portal blood of rats.[5] A copper radioisotope, ^{67}Cu, was shown to exchange readily between albumin and transcuprein in vivo and in vitro.[12] Because of this exchange, determinations of direct reacting Cu by the present method would include transcuprein-bound copper if it is present.

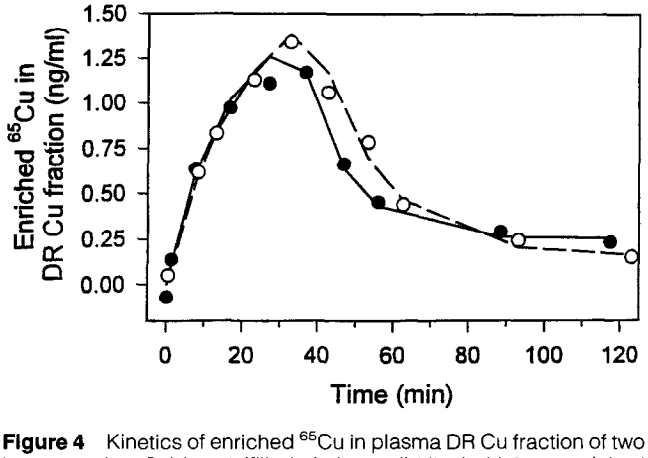

Figure 4 Kinetics of enriched ^{65}Cu in plasma DR Cu fraction of two human males. Subject 1 (filled circles, solid line): 11.8 μg enriched ^{65}Cu was infused during 34.4 min. Subject 2 (open circles, dashed line): 15.9 μg enriched ^{65}Cu was infused during 38.7 min. Infusions began at 0 min. Lines are calculated model solutions obtained with SAAM and CONSAM computer programs, symbols are observations.

Sodium diethyldithiocarbamate concentration

Sodium diethyldithiocarbamate was used as the chelating agent because it was reported in early work not to remove Cu from ceruloplasmin.[2] However, Jackson et al.[21] found various chelators to remove Cu from ceruloplasmin with the following order of efficacy: triethylenetetraamine > diethylenetriaminepentaacetic acid > ethylenediaminetetraacetic acid > D-penicillamine ≅ NaDDC ≅ Ascorbic acid. No extraction of Cu from serum was observed with the addition of 2.5×10^{-4} M NaDDC (using ultrafiltration to separate the Cu complex), but a 5% release of Cu from purified ceruloplasmin occurred at pH 7.4 at the same NaDDC concentration.[21] Because of the possibility that NaDDC might remove some Cu from ceruloplasmin, we tested the exchange of $^{65}Cu^{2+}$ with plasma Cu over a range of NaDDC concentrations.

Table 2 Parameters determined by kinetic modeling of direct reacting Cu metabolism

Parameter	Subject 1	Subject 2
L(12,10)[a]	0.079	0.056
L(10,12)[a]	0.0058	0.0027
Half-life of Dr Cu (min)	8.7	12.3
Plasma volume[b] (L)	3.17	4.53
DR Cu mass (mg)	0.11	0.15
Compartment 12 mass (mg)	1.48	3.21

[a]Fractional transfer coefficients. L(i,j) represents fraction of compartment j transferred to compartment i per min. Refer to *Figure 1*.
[b]Volume of distribution of the tracer was assumed to be plasma volume.

The apparent concentration of DR Cu in both human and bovine plasma increased at NaDDC concentrations above 1.6×10^{-4} M (*Figures 2 & 3*). Because the increase in DR Cu was observed by isotope dilution, it was due to an expansion of the exchangeable Cu pool and not due to progressively more complete extraction of an unchanged DR Cu pool. Thus, it appeared that NaDDC concentrations above 1.6×10^{-4} M caused exchange of $^{65}Cu^{2+}$ with forms of plasma Cu which normally do not exchange with ionic Cu. Because most plasma Cu is bound to ceruloplasmin, exchange with one or more sites on ceruloplasmin was likely. Expansion of the exchangeable Cu pool was much greater for human than for bovine plasma as NaDDC concentration increased. Thus, we concluded that NaDDC concentrations above 1.6×10^{-4} M are not suitable for the determination of DR Cu. However, because measurement of small increments in isotope enrichment in tracer experiments is usually limited by analytical precision and because precision of isotope ratio determinations usually is improved with increased sample size (subject to limitations of the mass spectrometer detector) maximum extraction of DR Cu was desirable. Both human and bovine results showed that extraction of DR Cu increased with no change in exchangeable Cu pool size up to a NaDDC concentration of 1.6×10^{-4} M (*Figures 2 & 3*). Thus, a concentration of 1.6×10^{-4} M NaDDC was chosen for the method.

Incomplete extraction of the DR Cu fraction was evident because the results by isotope dilution were consistently greater than the results by use of an external calibration standard, even at higher NaDDC concentrations (*Figures 2 & 3*). Thus, complete extraction of DR Cu without causing release of other plasma Cu forms cannot be achieved, even by careful selection of the NaDDC concentration. It was concluded that the pool of Cu exchanging with enriched ^{65}Cu tracer was larger than the pool of extractable Cu under the extraction conditions tested. Thus, extractable Cu is not a reliable estimator of exchangeable Cu and probably should not be used as a method of determining DR Cu. *Figures 2* and *3* show that the determination of DR Cu by isotope dilution in human and bovine plasma was independent of quantity of DR Cu extracted (at least up to 1.6×10^{-4} M NaDDC). The present method avoids the problems associated with obtaining 100% recovery of analyte during extraction procedures because isotopic dilution analysis is dependent on isotopic ratio not quantity of extracted analyte.

Species survey

DR Cu in several species was determined using the new isotope dilution procedure (*Table 1*). Results by other workers for some of the same species are shown in *Table 3*. Considerable variation among and within species is apparent in the results of others, which were obtained using the methods of Gubler et al.[1] and Weiss and Linder.[12] Although some of the variation within species reported by others (*Table 3*) may be due to differences in breed, age, sex, diet, and other factors, variation also may be attributed to the poor sensitivity reported for the method of Gubler et al.[3,4] Gubler et al.[1] used 2.8×10^{-4} M NaDDC compared with 1.6×10^{-4} M in the present study. However, it seems unlikely that significantly greater Cu would react colorimetrically at 2.8×10^{-4} M NaDDC compared with 1.6×10^{-4} M (*Figures 2 & 3*). The present results with dogs agree with the values reported by Evans and Wiederanders[22] and the present results for humans agree with the original results of Gubler et al.[1] Otherwise, except for some results with rats, our results are lower than previously published data. Because the present method has adequate sensitivity for the determination of DR Cu in blood plasma, whereas the method of Gubler et al. does not,[3,4] the new results (*Table 1*) should be more accurate. Results obtained with the gel filtration method of Weiss and Linder[12] are considerably greater than results obtained with either the method of Gubler et al.[1] or the current method (*Tables 1 & 3*). The reason for this discrepancy is not clear.

Plasma kinetics of DR Cu in humans

Subjects were in a fasting state during the tracer trial, so it was assumed that there was no absorption of Cu from the gut, i.e., input of natural Cu into compartment 10 was fixed at zero in the model solution. Also, the model was assumed to be in steady state except for the accumulation of tracer, so there also was no net loss from the system. While, compartment 10 represented DR Cu in plasma, the physiological

Table 3 Published DR and exchangeable Cu concentrations

Source	Age	Sex	n	DR/exchangeable Cu		Method	Reference
				ng/mL	Percentage of plasma Cu		
Cattle	adult	—[a]	16	220	24.2	Gubler et al.[1]	22
Dog	—	—	10	80	11.9	Gubler et al.[1]	22
Dog	adult	M & F	—	187	58	Weiss & Linder[12]	25
Human	—	—	7	40	4	Gubler et al.[1]	1
Human	—	M & F	30	70	6.1	Gubler et al.[1]	26
Human	—	—	16	80	6.2	Gubler et al.[1]	22
Human	adult	F	—	430	42	Weiss & Linder[12]	4
Rat	adult	M	10	10	1	Gubler et al.[1]	1
Rat	adult	—	20	10	0.8	Gubler et al.[1]	22
Rat	12 wk	—	—	190	13.6	Gubler et al.[1]	27
Rat	adult	F	5	360	30	Weiss & Linder[12]	12
Rat	adult	F	—	332	34	Weiss & Linder[12]	25

[a]Information not provided by authors is left blank.

significance of compartment 12 is unclear and it could represent more than one pool. Amino acid-bound Cu is reported to be about 40% of red cell Cu.[23] However, we measured DR Cu in hemolyzed whole human blood with the present method and found only 26 ng/mL compared with 36 ng/mL for plasma from the same blood sample. Thus, the quantity of exchangeable Cu in red blood cells is too small to be a significant portion of compartment 12. On the other hand, preliminary estimates of DR Cu in bovine liver homogenates (Buckley and Godfrey, unpublished results) indicate that liver Cu is predominantly exchangeable, and could be one of a number of possible identities for compartment 12.

The half-lives of DR Cu, 8.3 and 12.7 min, are consistent with an earlier report.[24] D'Addabbo et al.[24] observed half-lives of 8.5 and 10 min for the initial disappearance of ^{64}Cu in two healthy subjects after intravenous injection. No other direct determinations of the half-life of DR Cu in humans were found in the literature. An indirect measurement of DR Cu in half-life was estimated from a kinetic model to be 32 min in normal humans.[8]

The infusion of 12-16 μg enriched ^{65}Cu over 34 to 39 min had only a minor effect on the concentration of DR Cu and, therefore, was unlikely to have affected DR Cu metabolism. Maximum concentrations of enriched ^{65}Cu achieved near the end of the infusion period were 1.17 and 1.34 ng/mL (*Figure 4*), less than 4% of mean DR Cu concentration for each subject.

Based on the current method parameters, DR Cu may be defined as ionic Cu^{2+} plus all other Cu forms in plasma that exchange readily (within 30 min) with ionic Cu at room temperature. Because the turnover of DR Cu is very rapid in vivo, it is unlikely that any greater exchange between Cu^{2+} and other plasma Cu forms will occur in vivo than occurs during the in vitro measurement of DR Cu. We believe that this functional definition has physiological significance that may be applied to kinetic models of Cu metabolism and to other studies.

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