3.5.2 Ceruloplasmin and SOD analysis

Serum ceruloplasmin activity was determined in serum by its ability to oxidise *o*-dianisidine. Erythrocyte SOD activity was measured in red blood cell lysates from which the haemoglobin had been precipitated with chloroform and ethanol. SOD activity was determined by its ability to inhibit the auto-oxidation of pyrogallol.

3.5.3 Statistical analysis

Statistical analysis was performed with the personal computer version of the Statistical Analytical System (SAS). Multivariate repeatedmeasures ANOVA was used to compare MPs. Univariate repeatedmeasures ANOVA was performed on data comparing MP and subject. Univariate comparisons were based on all values in each MP.
Univariate analysis was also based on the last day and on the final two measurements of each MP, because these values reflect the longest time on each dietary copper amount. Average plasma copper, ceruloplasmin, and SOD of the 11 subjects were plotted using SAS, with error bars representing the SEM. Averages for the beginning of the study, the end of each MP, and the midpoint of MP2 were determined. Measurements were made for all 11 subjects on those days. Longitudinal data were plotted individually for subjects 7-12, the six subjects in whom copper status indices were measured at frequent intervals.

13 RESULTS AND DISCUSSION

Describe findings. If appropriate, include table. Sample tables are given below.

4.1 Results

Table A6.2(01)-1 shows average copper status data (plasma Cu, ceruloplasmin and erythrocyte SOD) for each MP, based on all data points in each MP for the 11 subjects who completed the study.

Figure A6.2(01)-1 shows average plasma copper, ceruloplasmin, and SOD for all 11 subjects at the beginning of the study, at the end of each MP, and at the midpoint of MP 2.

Figure A6.2(01)-2 shows plots of individual data for subjects 7-12. Plasma copper, ceruloplasmin and SOD differed significantly among individuals. However, on the basis of the indices measured in this study, copper status was not affected by the amount of dietary copper, either when all data points were used, or when only the last day or final two measurements of the MP were used for statistical analysis. Plasma copper and ceruloplasmin were significantly higher on day 1 than during the study by univariate but not by multivariate repeated-measures ANOVA. They did not differ between MPs by either method of data analysis.

Table A6.2(01)-2 shows average urinary copper by MP for 11 subjects and average salivary copper by MP for 10 subjects (it was not possible to connect the saliva collection apparatus to the parotid salivary duct of subject 11). Neither urinary nor salivary copper differed by dietary copper level.

Table A6.2(01)-3 shows averages of plasma, urinary and salivary copper , ceruloplasmin, and erythrocyte SOD for each subject. All of these indices differed significantly among subjects, but in no subject were all indices consistently higher or lower than in other subjects.

Sweat copper from the arm and axillary area ranged from 0.008 to 0.09 µmol/d, with the exception of one collection from one individual, which was 0.2 µmol/d (this was considered to be a result of contamination). There was no evidence that the amount of dietary copper influences

losses of copper in sweat, although the sample size was not large enough or the collections adequately reliable to report mean values or to make statistical comparisons.

4.2 Discussion

Copper status: Copper status, as assessed by plasma copper, ceruloplasmin, and SOD, was not affected by the amount of dietary copper. This suggests that the dietary intake of 0.785 mg/d was sufficient to meet the needs of this group of subjects.

According to univariate repeated-measures ANOVA, ceruloplasmin and plasma copper were higher on day 1 than with either the low-copper or high-copper diet. A possible explanation for this is that the higher initial ceruloplasmin was associated with the stress of beginning the study or was caused by drug, medication, alcohol use or smoking before the study.

The reason for the highly significant differences in indices of copper status are not clear, but **Figure A6.2(01)-2** indicates that they are independent of dietary copper intake. During the different levels of dietary copper intake, plasma copper and ceruloplasmin remained within relatively narrow ranges for each individual, but the ranges differed among individuals. Plasma copper remained consistently high in two individuals and ceruloplasmin remained consistently low in one individual.

Urinary, salivary and sweat copper: Very little copper is normally excreted in urine, and the amount present did not differ significantly as a function of dietary copper. Similarly, the copper content of saliva was low and did not reflect dietary copper, although there were significant differences among subjects. In this study, sweat was collected for an area representing ~10% of the surface area of the body. Assuming that the sweat loss from this area is representative of other areas, total body sweat losses would usually be ~0.08-0.9 µmol/d. This suggests that losses of copper in the sweat do not contribute significantly to copper balance of normal, healthy men.

4.3 Toxic effects, clinical signs

No effects / describe significant effects referring to data in results table

No effects.

4.4 Recovery of labelled compound

state percentage

Not stated.

14 APPLICANT'S SUMMARY AND CONCLUSION

14.1 Materials and methods

Give concise description of method; give test guidelines no. and discuss relevant deviations from test guidelines

Eleven young men were confined to a metabolic research unit for 90 d to determine the effect of the amount of dietary copper on copper nutriture. The study was divided into three metabolic periods (MP): 1) with an adequate-copper diet (1.68 mg/d) for 24 d, 2) with a low-copper diet (0.79 mg/d) for 42 d, and 3) with a high-copper diet (7.53 mg/d) for 24 d. Three indices of copper status (plasma copper; ceruloplasmin; SOD), urinary copper, and salivary copper were determined at intervals throughout the study.

14.2 Results and discussion	*Summarize relevant results; discuss dose-response relationship.* Neither copper status, urinary copper, nor salivary copper differed among MPs. Sweat collections from three subjects suggested that losses of copper through sweat were very low and would not contribute significantly to copper balance. These results suggest that an amount of dietary copper slightly < 0.8 mg/d is adequate to maintain copper status for ≥ 42 d in normal, healthy men and that neither urinary nor salivary copper is affected by the amount of Cu in the diet.
14.3 Conclusion	The results of this study demonstrate the existence of a metabolic mechanism to regulate levels of copper in the body, with no significant changes seen in commonly used indices of copper status following administration of a diet containing total copper in the range 0.8 to 7.5 mg/d.
14.3.1 Reliability	*Based on the assessment of materials and methods include appropriate reliability indicator 0, 1, 2, 3, or 4* 2
14.3.2 Deficiencies	Yes This study was not conducted and/or reported in strict compliance with the principles of GLP. However, this does not compromise the validity of the data generated, or the author's interpretation of that data, given that the study was not carried out for regulatory purposes. Furthermore, the research was published in a peer-reviewed journal, and has therefore been subject to the prior scrutiny of experts in the field. In addition this report has been included in a number of expert reviews of copper toxicokinetics. No internationally accepted guidelines are available that specifically address the objective of the research presented in this summary. Overall, this is a well-reported study, and its findings are considered to make a valuable contribution to the 'weight of evidence' approach that has been adopted for the purposes of the current review of copper toxicokinetics. A reliability indicator of 2 has been assigned on this basis. *(If yes, discuss the impact of deficiencies and implications on results. If relevant, justify acceptability of study.)*

Evaluation by Competent Authorities

Section A6.2	**Metabolism in mammals**
Annex Point IIA6.2	*Specify section no., heading and species as appropriate*
IUCLID: 5.0/01	**A6.2(01), Homeostasis of copper**

	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	[redacted]
Materials and Methods	[redacted]
Results and discussion	[redacted]
Conclusion	[redacted]
Reliability	[redacted]
Acceptability	[redacted]
Remarks	

	COMMENTS FROM ...
Date	*Give date of comments submitted*

Table A6.2(01)-1

Indices of copper status at the beginning of the study and averaged for each dietary copper period.

	Plasma Copper	Ceruloplasmin	Erythrocyte Superoxide dismutase
	µmol/L	*mg*/L	*U/g* Hb
Day 1 (n=11)	15.5*	440*	2250
MP1 †	14.7	400	2055
MP2	14.2	380	2623
MP3	14.6	390	2515
S x‡	0.33	13	200
Effect of copper level	p<0.05	p<0.05	NS
Effect of subject	p<0.01	p<0.01	p<0.01

* Significantly higher than MP 1, 2, or 3 by univariate, but not multi-variate, repeated-measures analysis of variance (ANOVA). There were no significant differences between three levels of dietary copper for any of these indices of copper status.

† Average of all data points in each metabolic period (MP) for 11 subjects.

‡ Pooled SEM.

Table A6.2(01)-2

Urinary and salivary copper

	Urinary copper*	Parotid saliva copper†
	µmol/d	*µmol/L*
MP 1	0.340	1.34
MP 2	0.335	1.30
MP 3 S x ‡	0.326	1.20
Effect of copper level Effect of subject	0.016 NS p<0.001	0.11 NS p<0.001

* n = 11.
† n = 10.
‡ Pooled SEM

Table A6.2(01)-3

Indices of copper status and urinary and parotid salivary copper, averaged for the entire study, by subject*

Subject	Plasma copper	Superoxide dismutase	Ceruloplasmin	Urinary copper	Salivary copper
	µmol/L	*U/g HB*	*mg/L*	*µmol/d*	*µmol/L*
2	13.7	2351	380	0.39	1.73
3	13.9	2009	460	0.33	1.02
4	12.6	3181	360	0.31	0.74
5	15.1	2500	430	0.24	1.95†
6	15.0	1394	460	0.41	1.12
7	12.7	2462	520	0.39	0.76
8	20.9‡	2597	370	0.38	1.54
9	14.2	2800	370	0.30	1.78
10	18.1‡	3370	480	0.39	0.69
11	12.9	2062	190§	0.16#	-
12	12.9	1244	420	0.36	1.13
S_x¶	0.63	331	20	0.03	0.20

* There were significant differences among subjects for each of the variables ($p<0.05$).
† Significantly higher than all but subjects 2 and 9.
‡ Significantly higher and different from each other.
§ Significantly lower than all other subjects.
# Significantly lower than all but subject 5.
¶ Pooled SEM.

Figure A6.2(01)-1

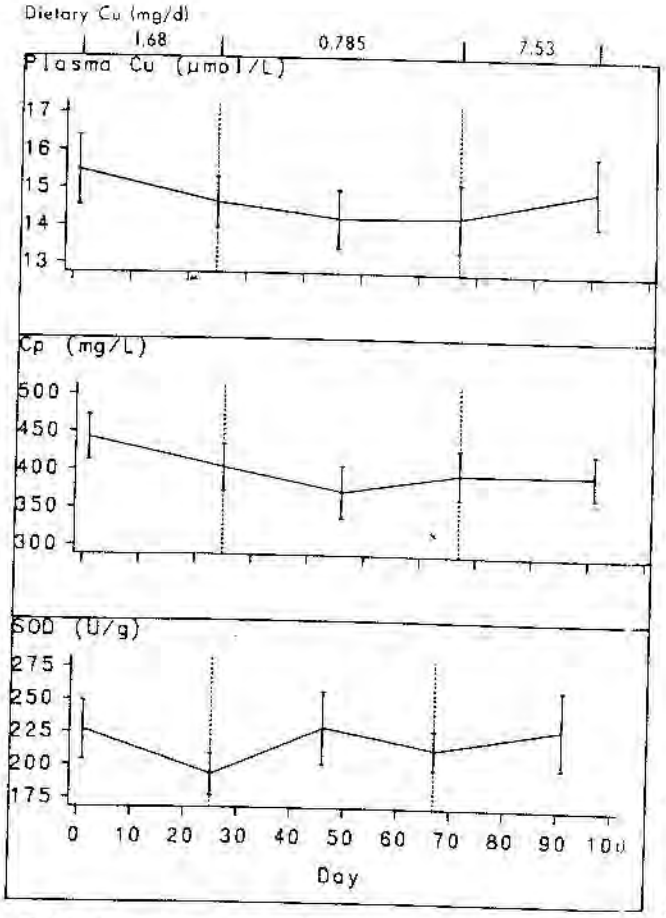


FIG 1. Plasma copper, ceruloplasmin (Cp), and whole-blood superoxide dismutase (SOD) ($\bar{x} \pm$ SEM) at three levels of dietary copper (1.68 mg/d, 0.785 mg/d, and 7.53 mg/d) for 11 subjects. The dashed lines represent a change in the level of dietary copper.

Figure A6.2(01)-2

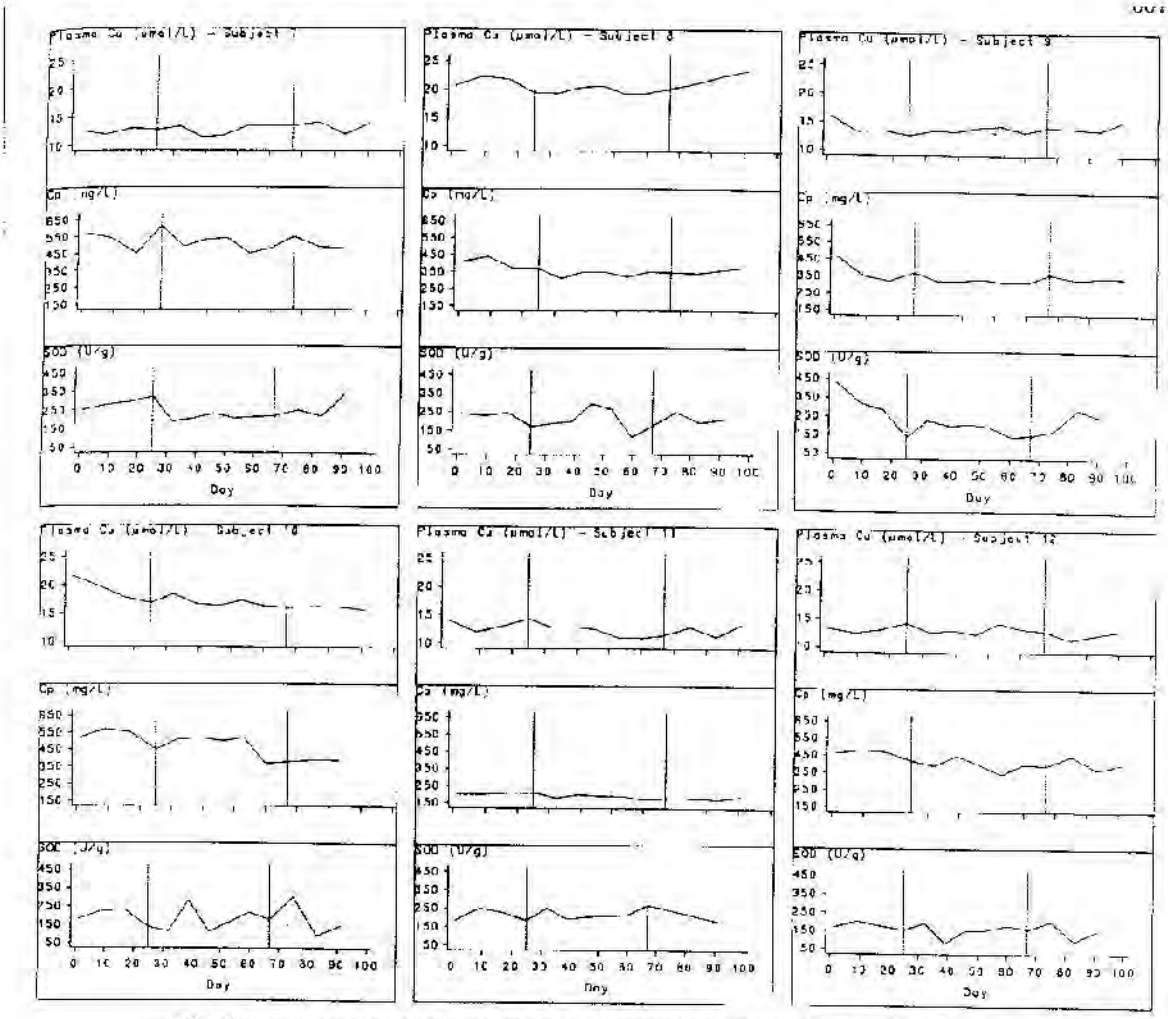


FIG 2. Plasma copper, ceruloplasmin, and whole-blood superoxide dismutase (SOD) ($\bar{x}$ ± SEM) at three levels of dietary copper (1.68 mg/d, 0.785 mg/d, and 7.53 mg/d) for subjects 7–12. All values differed significantly among subjects ($p < 0.01$) but the differences were not due to dietary copper level. The dashed lines represent a change in the level of dietary copper.

			Official use only
		15 REFERENCE	
1.1	**Reference**	*Author(s), year, title, laboratory name, laboratory report number, report date (if published, list journal name, volume: pages) If necessary, copy field and enter other reference(s).* Scott, K.C. & Turnlund, J.R., (1994). Compartment Model of Copper Metabolism in Adult Men. J. Nutr. Biochem. **5**: 342-350. (published).	
1.2	**Data protection**	No *(indicate if data protection is claimed)*	
1.2.1	Data owner	*Give name of company* Public domain	
1.2.2			
1.2.3	Criteria for data protection	Choose one of the following criteria (see also TNsG on Product Evaluation) and delete the others: No data protection claimed	
		16 GUIDELINES AND QUALITY ASSURANCE	
16.1	**Guideline study**	No. This was a non-regulatory study carried out in human volunteers. The experimental protocol was reviewed and approved by the Committee for Protection of Human Subjects, University of California, Berkeley, and by the US Department of Agriculture Human Studies Committee. The purpose of this research was to develop a compartmental model of copper metabolism in humans, based on data from plasma samples, and to elucidate the effect of dietary copper level on copper metabolism in adult men. *(If yes, give guidelines; if no, give justification, e.g. "no guidelines available" or "methods used comparable to guidelines xy")*	
16.2	**GLP**	No. This was a non-regulatory study carried out in human volunteers. *(If no, give justification, e.g. state that GLP was not compulsory at the time the study was performed)*	
16.3	**Deviations**	Yes. Refer to section 5.3.2 for a general discussion of deviations and deficiencies. *(If yes, describe deviations from test guidelines or refer to respective field numbers where these are described, e.g. "see 3.x.y")*	
		17 MATERIALS AND METHODS *In some fields the values indicated in the EC or OECD test guidelines are given as default values. Adopt, change or delete these default values as appropriate.*	
17.1	**Test material**	Cu^{2+} Cupric oxide	X
17.1.1	Lot/Batch number	Not available	
17.1.2	Specification	Deviating from specification given in section 2 as follows *(describe specification under separate subheadings, such as the following; additional subheadings may be appropriate):*	

17.1.2.1 Description	*If appropriate, give e.g. colour, physical form (e.g. powder, grain size, particle size/distribution)* Aqueous solution
17.1.2.2 Purity	*Give purity in % of active substance* [redacted] X
17.1.2.3 Stability	*Describe stability of test material* Not available
17.1.2.4 Radiolabelling	*give structural location of radio labelling, give reason if not labelled* ^{65}Cu
17.2 Test Animals	Non-entry field
17.2.1 Species	Human volunteers
17.2.2 Strain	Not applicable
17.2.3 Source	Not applicable
17.2.4 Sex	Male
17.2.5 Age/weight/height at study initiation	*Young adults recommended* Age: 22 to 33 years. Weight: 71 to 77 kg. Height: 172 to 190 cm.
17.2.6 Number of volunteers	*Give number* *Specify, if there are differences for example for treatment and recovery groups* 5 volunteers were involved in this study.
17.2.7 Controls	No
17.3 Administration/ Exposure	*(fill in respective route in the following, delete other routes)* Oral and intravenous administration of cupric oxide.
17.3.1 Duration of treatment	The total duration of treatment was 90 days. The study was divided into three metabolic periods (MP). Each volunteer received: 1) an adequate-copper diet (1.68 mg/day) for 24 days, followed by 2) a low-copper diet (0.79 mg/day) for 42 days, and then 3) a high-copper diet (7.53 mg/day) for 24 days.
17.3.2 Exposure scenarios	The diet used throughout the study contained low-copper food items, a liquid formula calorie supplement with added minerals and fiber, and a multivitamin tablet. The food and formula in the diet contained ~0.4 mg Cu before copper was added. A solution containing $CuSO_4$ was added to the liquid formula at each meal to achieve the desired copper content of the total diet. The subjects were given an i.v. infusion of ^{65}Cu over a 2-minute period once during each MP, and received the same stable isotope orally once during MP1 and MP3 and twice during MP2. The stable isotope replaced the copper added to the diet during all three metabolic periods, but increased the dietary copper slightly during M2 (low copper period) on the isotope feeding days. The isotope was added to all meals of the feeding days. A summary of the i.v. and oral isotope administration schedule is shown in **Table A6.2(02)-1**.

3.3.1.1 Intravenous administration	A solution containing 392 µg of ^{65}Cu was infused on days 7, 49 and 73 of the study. The solution was prepared by weighing ^{65}CuO, dissolving it in a minimum of ultrapure HCl, and diluting with distilled, deionised water.
3.3.1.2 Oral administration	The isotope was given orally over a 1-day interval for MP1 and MP3, and over 2-day intervals for MP2, on days 13, 31/32, 55/56 and 79 of the study. The amounts of isotope fed were 1600, 1020, 1020 and 7660 µg for the four feedings. Two-day intervals were necessary during MP2 to obtain sufficient ^{65}Cu enrichment while maintaining dietary copper as close as possible to the level used on other days of the low copper diet. The enriched isotope was added to the liquid supplement.
17.4 Examinations	Non-entry field
17.4.1 Body weight	*yes/no (give time periods for determinations).* Yes. Body weight was monitored over the course of the study.
17.4.2 Blood collections following intravenous dosing.	*yes/no (give time periods for determinations).* Blood was sampled at 0, 5, 15, 30, 60, 120, 240, 360, 480, 600, 960, 1,440, and 2,880 minutes post intravenous infusion.
17.4.3 Blood collections following oral dosing.	Blood was sampled the morning after feedings 1 and 4 (days 14 and 80, respectively), and the morning after the first day of feedings 2 and 3 (days 32 and 56, respectively). Blood was also drawn 11 days after the initial draws for feedings 1, 3, and 4, and 14 days after the initial draw for feeding 2.
3.5 Sample processing and analysis	Non-entry field.
3.5.1 Copper analysis	Blood samples taken at the intervals outlined in section 3.4.2 were collected in heparinised trace element-free polypropylene tubes, centrifuged, and the plasma removed and stored frozen until needed. Thawed plasma samples were evaporated, ashed, then dissolved in 6 mol/l ultrapure HCl. Minerals were separated on ion exchange columns with decreasing concentrations of ultrapure HCl. The copper fraction was collected and evaporated to a concentration of approximately 1g/l. The isotopic ratios were determined by magnetic sector, thermal ionisation mass spectrometry, and enrichment was calculated. The ^{65}Cu to ^{63}Cu ratio and plasma copper concentration , determined by atomic absorption spectrophotometry, were used to calculate the concentration of enriched ^{65}Cu in the samples. The mass of enriched ^{65}Cu in plasma was calculated from plasma enriched ^{65}Cu concentration, blood volume, and haematocrit. Blood volume was calculated based on a nomogram using body mass (kg) and age (yr).

3.6 Modelling of data

The isotopic masses were modelled using the CONSAM (version 30.1) computer modelling program. The model is based on the known physiology of copper metabolism, and is based on the assumption that the subjects were in, or quickly obtained, steady state conditions. Model-predicted enriched ^{65}Cu masses were converted to total copper masses as follows:

Total ^{65}Cu = predicted mass of enriched ^{65}Cu / ^{65}Cu enrichment. Total Cu = Total ^{65}Cu / 0.3083 ^{65}Cu in total Cu.

The model was developed initially using plasma data averaged over all subjects and the model was then applied to each subject separately. The rate constants were allowed to vary (through hand-fitting and iteration) to obtain a best fit for each individual. The model utilised pathways known to be physiological, although not all physiologically possible pathways were used. The size of the data set limited the number of pathways in the model.

The i.v. and oral inputs were modelled in series as they occurred (**Table A6.2(02)-1**) to produce one continuous model covering the entire study.

First, the rate constants, L(I,J) (representing transfer from compartment J to compartment I in units of time^{-1}), were fitted to the data from the first i.v. input, while the remaining data were unweighted. Each L(I,J) may be used only once and was used to fit the first input. Parameters were varied until the desired fit was obtained.

Then the parameters, P(I), representing L(I,J) of the next input, were fitted to the data from the first oral input. P(I) are variable parameters used to represent each L(I,J) for all inputs subsequent to the first use. All subsequent data were unweighted during the fitting process. Parameters were varied until a best fit was obtained, then the next set of P(I) values were fitted to the next input (**Table A6.2(02)-1**), until the entire data set was fitted. During each stage in the fitting process, all subsequent data were unweighted to give a more accurate fit to each data set of interest. The final data set was fitted by the model with all points equally weighted.

The intravenous inputs were treated as "boluses" because they occurred over a short time (2 minutes) relative to the length of the study. The oral inputs were treated as "inputs over time" to account for the slower transfer of copper through the gastrointestinal tract and into the blood. "Input over time" refers to an input occurring over an extended period (4 hours in this study) and requiring the use of time interrupts within the CONSAM input file to start and stop the flow into the system. Because oral inputs 2 and 3 each occurred over consecutive days, each input was treated as two separate events; one for each day of feeding. All inputs went directly into the plasma, as the size of the data set did not permit inclusion of a gastrointestinal tract compartment. The oral input into the model was the amount of ^{65}Cu absorbed (**Table A6.2(02)-2**). Absorption was determined at each level of dietary copper as described in study summary **A.6.2(04)**. The percentage of copper absorbed decreased as dietary copper increased, but the absolute amount absorbed increased. Total urinary copper was measured (study summary **A6.2(01)**). Urinary copper was too low to measure enrichment, so urinary copper enrichment data could not be included in the model.

18 RESULTS AND DISCUSSION

Describe findings. If appropriate, include table. Sample tables are given below.

18.1 Results

No effects / describe significant effects referring to data in results table

The model consists of five compartments, two delay components, and two routes of excretion. These were interpreted as representing two plasma, two liver, one 'other tissues' compartments, and urinary and faecal excretion, as shown in **Figure A6.2(02)-1**. Plasma compartment 6 is considered to represent nonceruloplasmin-bound copper, while compartment 8 contains ceruloplasmin-bound copper. The delay components simulate the passage of copper through the system, and may contain any combination of body tissue and storage and transport forms of copper. The delay in the faecal excretion pathway is 72 hours, while that in the other-tissues pathway is 4 to 7 hours. The duration of the faecal delay component was estimated based on an observed delay in the passage of a faecal marker given to the subjects.

A total of six sets of parameters (**Table A6.2(02)-3**) were used to fit the plasma data , with sets of parameters corresponding to the different inputs into the system; 1st i.v., 1st oral input, 2nd and 3rd oral input, 2nd i.v., 3rd i.v. and 4th oral input.

Figures A6.2(02)-2a to e illustrate the fit of the total model for the 90-day study to data for each of the five subjects, while **Figures A6.2(02)-3a to e** show a time scale enlargement of the first i.v. period for each of the five subjects. The i.v. tracer was expected to produce detailed information on the metabolism of copper, whereas the oral dose was expected to produce absorption data.

Of the eight rate constants that were allowed to vary, two were affected by dietary copper level and one differed with route of input.

L(8,4), the rate constant representing transfer from compartment 4 (liver-4) to compartment 8 (plasma-8), was lower during MP2 (0.8 mg Cu/d) by approximately 30% than it was during the other two metabolic periods (1.68 mg Cu/d and 7.53 mg/d). However, there was no difference between MP1 and MP3 for this parameter. L(9,8), representing transfer from compartment 8 (plasma-8) to compartment 9 (other tissues), varied with the level of dietary copper. The value was lowest during MP2, the low copper period, and highest during MP3, the highest copper period. The range for L(9,8) was from 0.39 hr^{-1} during MP2 through 0.78 hr^{-1} during MP3 (**Table A6.2(02)-3**). Rate constants [L(I,J)] for all subjects are shown in **Table A6.2(02)-3**. An influence of route of administration (oral versus i.v.) was seen on the rate constant L(5,6), from compartment 6 (plasma–6) to compartment 5 (liver-5). In all subjects, L(5,6) was larger following oral doses of tracer than it was after intravenous doses.

The parameters DT(10), L(10,9) and L(1,6), representing a delay between compartments 9 and 10, transfer from compartment 9 to delay component 10, and the urinary excretion route, respectively, could each be represented by a single value for all inputs. Urinary output [L(1,6)] was predicted to be approximately 4 orders of magnitude smaller than faecal output. The 3 parameters exerting most influence on the shape of the curves following intravenous doses were L(5,6), L(8,4) and L(9,8).

Total copper masses for all subjects at the times of the 2nd i.v. and 3rd oral doses of tracer are listed in **Table A6.2(02)-4** and **Table A6.2(02)-**

5, respectively. These times were chosen because they were during the low copper period (MP2), and because the length of MP2 allowed the longest stabilisation period prior to the administration of these particular tracer doses. However, little or no difference was seen in predicted masses at the times of the two oral doses of tracer in MP2 (7 and 31 days into MP2, respectively). The mass in plasma-6 increased following i.v. doses in comparison with oral doses of tracer. In contrast, the mass in plasma-8 (ceruloplasmin-bound copper) was higher after oral tracer administration. However, no influence of the level of dietary copper was noted on this compartment. Liver-5 and the 'other-tissues' compartment 9 also showed higher mass with oral doses, while the mass of Cu in liver-4 was higher with i.v. doses. Both liver compartments and the 'other-tissues' compartment displayed an increase in mass as the study progressed except during the low copper period (MP2), when the Cu in these compartments was predicted to decrease between the two oral doses of tracer. The delay component 10 showed a pattern similar to that seen with liver-4, though there was no increase in mass over the course of the study.

Major storage sites include the second liver compartment (compartment 4), and delay components 10 and 12. The model predicted 72.2 ± 9.3% and 62.1 ± 7.9% (mean ± SEM) of the plasma copper was bound to ceruloplasmin (compartment 8) following intravenous and oral doses, respectively (**Table A6.2(02)-4** and **Table A6.2(02)-5**), and that approximately 3.1 ± 0.2% and 3.4 ± 0.7% (mean ± SEM) (**Table A6.2(02)-3**) of total body copper circulated in the plasma following i.v. and oral tracer administration, respectively. Predicted masses followed similar patterns for all subjects following each dose of tracer.

18.2 Discussion

Dietary copper level influenced the rate of transfer for two pathways in this model. L(8,4), transfer from the second liver compartment to the second plasma compartment, was lower by approximately 30% in MP2, the lowest dietary copper level, than it was in MP1 and MP3 (**Table A6.2(02)-3**). This would be consistent with the slower turnover during the lowest level of dietary copper. L(9,8), representing transfer from compartment 8 (plasma-8) to compartment 9 (other tissues) followed dietary copper level, ranging from a low of 0.39 during MP2 (0.78 mg Cu/day) to a high of 0.78 during MP3 (7.53 mg Cu/day). This corresponds to a half-life range of approximately 4 to 7 days; shorter during the high dietary copper period, longer during the low copper period. This explains why it was possible for the subjects to maintain relatively constant ceruloplasmin and plasma copper levels despite widely different levels of dietary copper.

One parameter, L(5,6), was influenced by route of administration of the stable isotope, but did not appear to be influenced by the dietary copper level. The parameter values (**Table A6.2(02)-4**) illustrate that L(5,6) (flow between plasma-6 and liver-5) is higher with oral inputs than with i.v. inputs, suggesting that i.v. and oral copper may be handled differently. Following an i.v. dose of tracer, L(5,6) is approximately 80% of the value following an oral dose of tracer. This may be due, in part, to a difference in the form of the infused copper as opposed to that found in plasma following oral administration. Another possibility for this difference may be a first-pass hepatic extraction of infused copper from the portal blood, with increased retention in liver compartment 4. This would explain the higher mass of infused copper in liver compartment 4.

Some rate constants appear to have more influence than others in the regulation of copper metabolism. Small changes in the parameters L(5,6), L(8,4) and L(9,8) have a major effect on the shape of the curve. This suggests that movement of nonceruloplasmin copper into the liver, incorporation of copper into ceruloplasmin within the liver, and subsequent release into plasma and transport to other tissues are points of regulation of copper metabolism. The parameters with the least influence on the rate of copper metabolism, are those from the other-tissue compartment 9 to the delay component 10 [L(10,9)] and the urinary excretion route [L(1,6)]. This suggests that release of copper from the tissues and urinary excretion are relatively constant.

The pathways used in this model were adequate to fit the data and allow recirculation of copper through the system. Liver is the major tissue involved in metabolism of copper, and plasma is the major fluid through which copper is transported. Two liver compartments were required (**Figure A6.2(02)-1**) to adequately fit the data. Incorporation of copper into ceruloplasmin; storage of copper bound to metallothionein; activity of the copper-containing enzyme, superoxide dismutase; transfer of Cu into cytochrome oxidase in mitochondria; and excretion of Cu into bile are hypothesised to occur within these liver compartments. Two plasma compartments appear to be required because they represent the different properties of ceruloplasmin-bound and nonceruloplasmin-bound copper.

The model predicts approximately 62 – 72% ceruloplasmin-bound copper in plasma (**Table A6.2(02)-4** and **Table A6.2(02)-5**), with ranges of 66.3% to 87.2% (following i.v. tracer) and 52.5% to 73.9% (following oral tracer). The increase in ^{65}Cu enrichment of plasma seen between 5 and 15 hours postinfusion (**Figure A6.2(02)-3**) is considered to be due to ^{65}Cu turnover in the tissues and the subsequent release back into the plasma (delay component 10 between the two plasma compartments).

The movement of copper between connecting compartments in the model is all in a single direction, resulting in a recycling model (**Figure A6.2(02)-1**). Recycling refers to the repeated movement of copper through the liver, blood, and tissues, and back through the liver.

The pathways incorporated within the model are consistent with what is known to occur in copper metabolism, although not all known pathways could be included in this model due to the limited size of the data set. The model-predicted total body copper ranged from 1.18 to 1.54 mmol.

19 APPLICANT'S SUMMARY AND CONCLUSION

19.1 Materials and methods

Give concise description of method; give test guidelines no. and discuss relevant deviations from test guidelines

A compartmental model of copper metabolism in adult men was developed from stable isotope data obtained in a 90-day study during which three levels of dietary copper (1.68, 0.785, 7.52 mg/d) were fed. The tracer ^{65}Cu was administered intravenously (i.v) three times and orally four times during the study period. The model contains five compartments, two delay components, and two excretion pathways interpreted as two plasma compartments, two liver compartments, an other-tissue compartment, and faecal and urinary excretion routes.

19.2 Results and discussion

Summarize relevant results; discuss dose-response relationship.

Dietary copper level was found to influence the flow from the second

	liver compartment to the second plasma compartment and from the second plasma compartment to the other-tissues compartment. The model suggested that the tissue uptake of oral and i.v. copper was different, with flow from plasma to the first liver compartment varying with route of isotopic administration. The model-predicted masses of copper within the compartments and delay components followed a pattern of expected masses within the body. The major storage sites predicted were the second liver compartment, the delay after the othertissue compartment, and the delay in the faecal excretion pathway. The model predicted that approximately 65% of plasma copper was bound to ceruloplasmin. Approximately 4.1 ± 0.8% of total body copper was predicted to be in plasma.
19.3 Conclusion	The results of this study demonstrate the existence of a metabolic mechanism to regulate levels of copper in the body, with no significant changes seen in commonly used indices of copper status following administration of a diet containing total copper in the range 0.8 to 7.5 mg/d.
19.3.1 Reliability	*Based on the assessment of materials and methods include appropriate reliability indicator 0, 1, 2, 3, or 4* 2
19.3.2 Deficiencies	Yes This study was not conducted and/or reported in strict compliance with the principles of GLP. However, this does not compromise the validity of the data generated, or the author's interpretation of that data, given that the study was not carried out for regulatory purposes. Furthermore, the research was published in a peer-reviewed journal, and has therefore been subject to the prior scrutiny of experts in the field. In addition this report has been included in a number of expert reviews of copper toxicokinetics. No internationally accepted guidelines are available that address the objective of the research presented in this summary. Overall, this is a well-reported study, and its findings are considered to make a valuable contribution to the 'weight of evidence' approach that has been adopted for the purposes of the current review of copper toxicokinetics. A reliability indicator of 2 has been assigned on this basis. *(If yes, discuss the impact of deficiencies and implications on results. If relevant, justify acceptability of study.)*

Evaluation by Competent Authorities

Section A6.2	**Metabolism in mammals**
Annex Point IIA6.2	*Specify section no., heading and species as appropriate*
IUCLID: 5.0/02	**A6.2(02), Homeostasis of copper**

	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	[redacted]
Materials and Methods	[redacted]
Results and discussion	[redacted]
Conclusion	[redacted]
Reliability	[redacted]
Acceptability	[redacted]
Remarks	

	COMMENTS FROM ...
Date	*Give date of comments submitted*

Table A6.2(02)-1

STUDY DESIGN

MP[a]	1	2	3
Number of days	24	42	24
Days of study	1-24	25-66	67-90
Cu level (mg/d)	1.68	0.79	7.53
Intravenous ^{65}Cu (d)	7	49	73
Oral ^{65} Cu (d)	13	31/32[b], 55/56[b]	79

[a]Metabolic period.
[b]Oral ^{65}Cu administered over a 2-day period during MP2, the low Cu period, to maintain a low Cu level in the diet.

Table A6.2(02)-2

^{65}Cu absorption at three levels[a] of dietary copper[b]

Subject	MP1[c]	MP2	MP3
		%	
2	42.1	50.6	12.6
3	34.2	57.5	10.4
4	40.1	54.3	7.4
5	29.5	61.0	14.0
6	34.8	57.7	11.1
Mean ±SEM	36.1 ±5.0	56.2 ±3.9	11.1 ±2.5

a 1.68mg Cu/d, 0.79 mg Cu/d, 7.53 mg Cu/d.
b Taken from Turnlund er al. 10
c MP = Metabolic period, refers to the three levels of dietary copper.

Table A6.2(02)-3
Rate Constants for all subjects and for all inputs

	MP1[a]		MP2		MP3	
	1st	1st	2nd	2nd & 3rd	3rd	4th
	i.v.[b]	Oral[c]	i.v.	Oral	i.v.	Oral
			hours^{-1}			
Subject 2						
L(12,5)[d]	0.86 (0.023)[e]	0.81 (0.007)	0.75 (0.009)	0.78 (0.006)	1.7 (0.013)	2.0 (0.011)
L(5,6)	1.2 (0.001)	1.5 (0.016)	1.1 (0.005)	1.5 (0.009)	1.4 (0.006)	1.6 (0.009)
L(4,5)	8.3 (0.013)	7.0 (0.002)	7.2 (0.006)	7.3 (0.007)	8.1 (0.010)	7.5(0.003)
L(8,4)	0.060(0.001)	0.071 (0.005)	0.039 (0.001)	0.039 (0.005)	0.072 (0.002)	0.081 (0.004)
L(1,6)	1.9E-06 (0.009)[f]					
L(9,8)	0.56 (0.003)	0.56 (0.002)	0.42 (0.010)	0.48 (0.007)	0.61 (0.005)	0.62 (0.005)
L(10,9)	2.6 (0.021)					
DT(10)[g]	6.9 (0.006)					
Subject 3						
L(12,5)	0.95 (0.018)	0.95 (0.011)	0.91 (0.010)	0.92 (0.004)	2.0 (0.003)	2.1 (0.025)
L(5,6)	1.1 (0.004)	1.4 (0.013)	1.1 (0.059)	1.4 (0.031)	1.1 (0.014)	1.4 (0.014)
L(4,5)	9.2 (0.048)	9.0 (0.072)	9.5 (0.023)	9.3 (0.017)	9.0 (0.024)	9.2 (0.019)
L(8,4)	0.037(0.001)	0.038 (0.018)	0.025 (0.040)	0.027 (0.025)	0.036 (0.021)	0.034 (0.032)
L(1,6)	4.0E-06 (0.026)					
L(9,8)	0.67 (0.092)	0.65 (0.022)	0.47 (0.071)	0.41 (0.025)	0.78 (0.035)	0.78 (0.032)
L(10,9)	1.9 (0.044)					
DT(10)	4.9 (0.005)					
Subject 4						
L(12,5)	1.1 (0.013)	1.3 (0.055)	0.95 (0.055)	1.0 (0.035)	2.3 (0.011)	2.5 (0.022)
L(5,6)	0.95 (0.022)	2.1 (0.059)	0.93 (0.032)	1.8 (0.021)	1.1 (0.044)	2.5 (0.001)
L(4,5)	9.5 (0.023)	9.4 (0.012)	9.0 (0.013)	9.1 (0.025)	9.3 (0.024)	9.7 (0.027)
L(8,4)	0.021 (0.014)	0.020 (0.008)	0.010 (0.053)	0.009 (0.051)	0.023 (0.047)	0.025 (0.052)
L(1,6)	7.1E-06 (0.032)					
L(9,8)	0.61 (0.015)	0.58 (0.021)	0.41 (0.025)	0.41 (0.028)	0.69 (0.036)	0.71 (0.023)
L(10,9)	1.6 (0.025)					
DT(10)	5.8 (0.006)					
Subject 5						
L(12,5)	0.63 (0.031)	0.61 (0.023)	0.57 (0.035)	0.62 (0.052)	1.0 (0.014)	1.3 (0.025)
L(5,6)	0.96 (0.025)	1.7 (0.027)	1.0 (0.033)	1.7 (0.017)	0.92 (0.023)	1.9 (0.011)
L(4,5)	7.4 (0.023)	7.1 (0.024)	7.3 (0.071)	7.0 (0.016)	7.5 (0.012)	7.3 (0.024)
L(8,4)	0.035 (0.007)	0.0036 (0.017)	0.021 (0.014)	0.020 (0.023)	0.034 (0.026)	0.034 (0.029)
L(1,6)	8.5E-06 (0.042)					
L(9,8)	0.59 (0.027)	0.60 (0.036)	0.39 (0.032)	0.42 (0.024)	0.72 (0.013)	0.68 (0.036)
L(10,9)	2.2 (0.029)					
DT(10)	5.7 (0.002)					
Subject 6						
L(12,5)	0.45 (0.012)	0.50 (0.037)	0.53 (0.015)	0.50 (0.027)	0.95 (0.034)	1.0 (0.031)
L(5,6)	0.95 (0.012)	0.96 (0.011)	0.90 (0.004)	0.99 (0.014)	0.91 (0.007)	1.1 (0.024)
L(4,5)	7.4 (0.028)	7.6 (0.036)	7.5 (0.015)	7.9 (0.029)	8.0 (0.029)	7.3 (0.056)
L(8,4)	0.073 (0.009)	0.070 (0.045)	0.061 (0.043)	0.059 (0.018)	0.069 (0.031)	0.075 (0.024)
L(1,6)	8.1E-06 (0.042)					
L(9,8)	0.59 (0.039)	0.61 (0.025)	0.49 (0.027)	0.51 (0.025)	0.68 (0.025)	0.71 (0.021)
L(10,9)	2.5 (0.032)					
DT(10)	5.5 (0.009)					

[a]MP = Metabolic period, corresponds to different levels of dietary copper.
[b]i.v. = ^{65}Cu intravenous input.
[c]oral = ^{65}Cu oral input.
[d]L(I,J) represents the fractional rate of transfer from compartment J to compartment I. Columns without entries used the most recent value listed to the left of the empty space.
[e]Values in parentheses are fractional standard deviations (FSD). Rate constants without FSD values were held constant to the values determined for the first input.
[f]The "E" designates an exponential. For example, 1E - 05 = 1 x 10^{-5}.
[g]DT(I) represents the delay component I. Units are in hours. Columns without entries used the most value listed to the left of the empty space.

Table A6.2(02)-4

Model-predicted masses [M(1)] of copper at the time of the second infusion (metabolic period 2) (0.78 mg Cu/d) listed by compartment for each subject.

	Subject				
	2	3	4	5	6
		mg total copper			
M(3) [a]	3.50	3.13	4.00	2.81	2.76
M(4)	52.33	80.12	89.13	58.46	73.94
M(5)	0.17	0.11	0.15	0.17	0.07
M(6)	1.17	1.06	1.22	1.01	0.94
M[illegible]	2.33	2.07	2.86	1.81	2.39
M[illegible]	0.79	0.42	0.67	0.44	0.86
M[illegible]	46.69	12.18	18.10	25.61	14.04
M(12)	8.16	5.56	7.02	4.63	7.59
Total mass	111.64	101.52	119.15	92.13	99.86
% Cu in plasma [b]	3.1	3.1	3.4	3.1	2.7
% plasma Cu in Cp [c,d]	66.6	66.3	71.5	64.4	87.2

[a] See *figure 1* for identification of compartment numbers.
[b] Mean ± SEM = 3.1 ± 0.2%.
[c] CP = ceruloplasmin, see text for calculation.
[d] Mean ± SEM = 71.2 ± 9.3%.

Table A6.2(02)-5

Model-predicted masses [M(1)] of copper at the time of the third oral dose (metabolic period 2) (0.78 mg Cu/d) listed by compartment for each subject.

	Subject				
	2	3	4	5	6
		mg total copper			
M(3) [a]	4.91	3.97	4.84	4.65	2.64
M(4)	67.06	96.85	102.65	80.83	95.56
M(5)	0.34	0.37	0.40	0.41	0.09
M(6)	2.01	1.59	1.68	2.21	0.69
M[illegible]	2.91	2.38	3.15	2.44	1.95
M[illegible]	0.98	0.48	0.73	0.91	0.24
M[illegible]	46.68	12.06	17.47	28.86	18.78
M(12)	4.99	5.92	4.86	4.90	2.88
Total mass	123.97	119.64	130.95	120.55	120.19
% Cu in plasma [b]	4.0	3.3	3.7	3.9	2.2
% plasma Cu in Cp [c,d]	58.9	59.9	65.1	52.5	73.9

[a] See *Figure 1* for identification of compartment numbers.

[b] Mean ± SEM = 3.4 ± 0.7%.
[c] CP = ceruloplasmin, see text for calculation.
[d] Mean ± SEM = 62.1 ± 7.9%.

Figure A6.2(02)-1

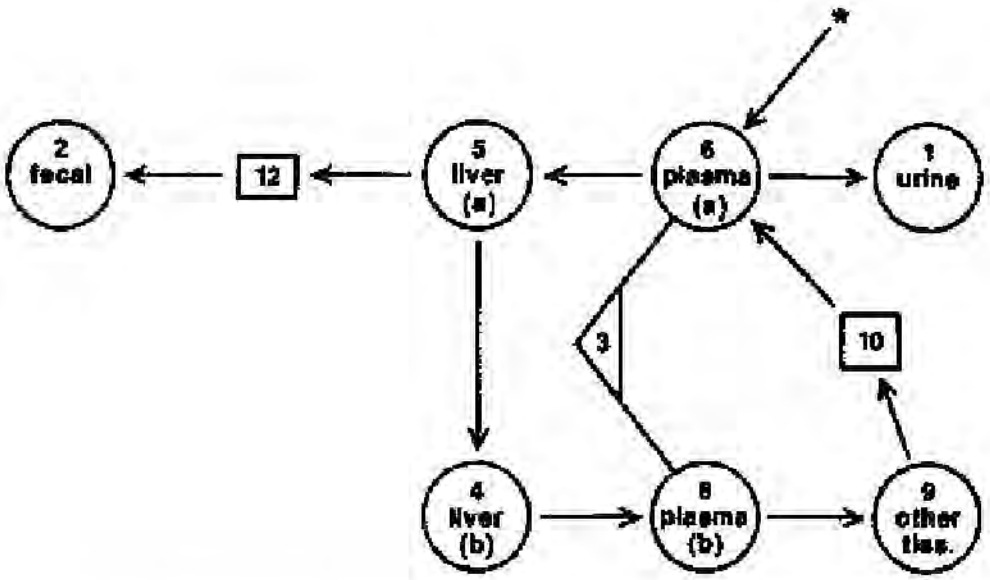


Figure 1 Schematic representation of compartmental model of copper metabolism in adult men. Circles represent compartments, rectangles are delay elements, triangle is the sum of the indicated compartments, * is the site of ^{65}Cu tracer input. Plasma samples were taken from compartment 3. Compartment 8 is hypothesized to be ceruloplasmin Cu, while compartment 6 is noncaeruloplasmin Cu. Delay 10 is 4 to 7 hours, delay 12 is 72 hours. Compartment numbers were chosen arbitrarily.

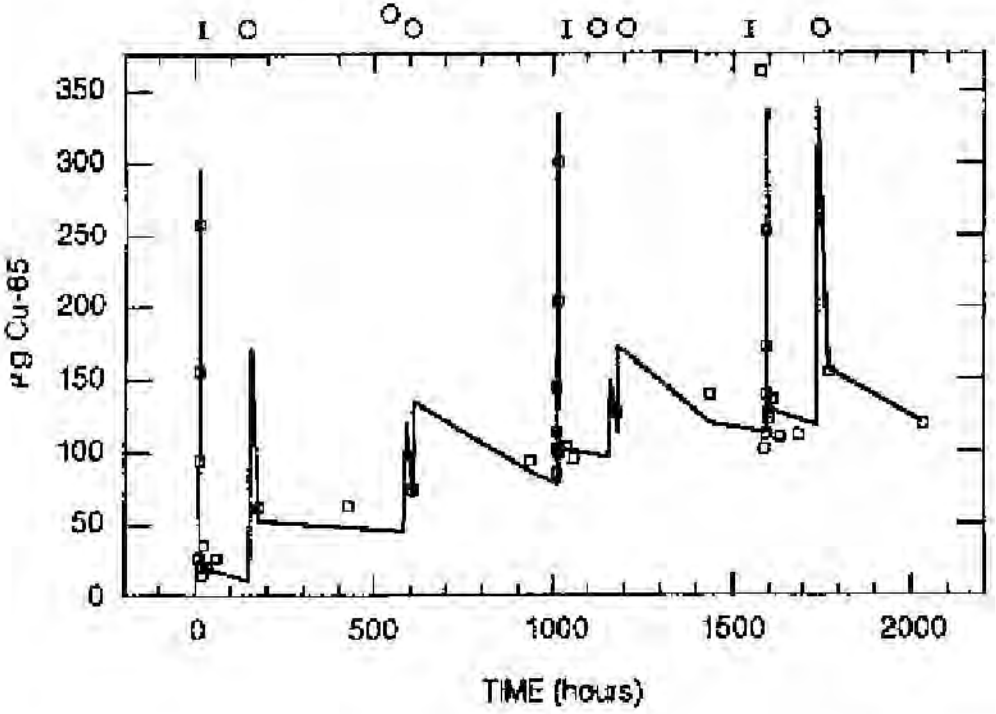
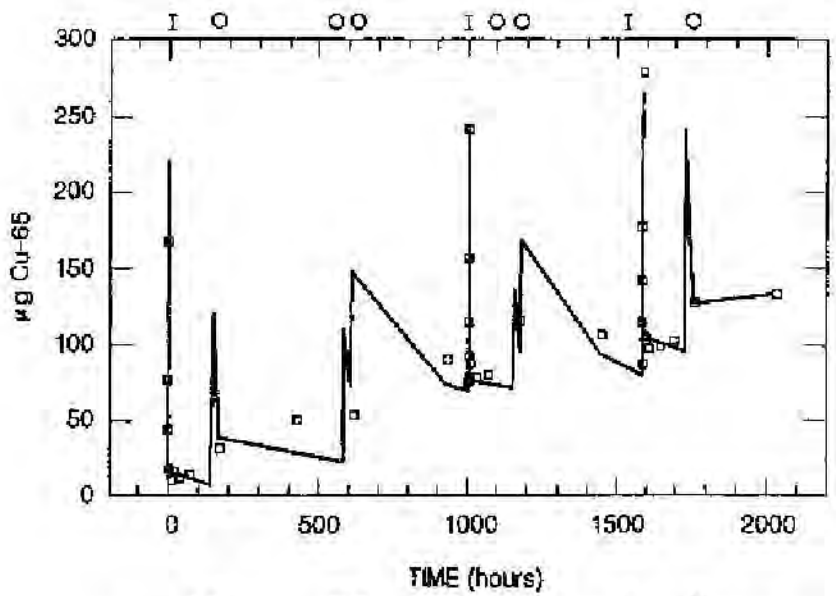
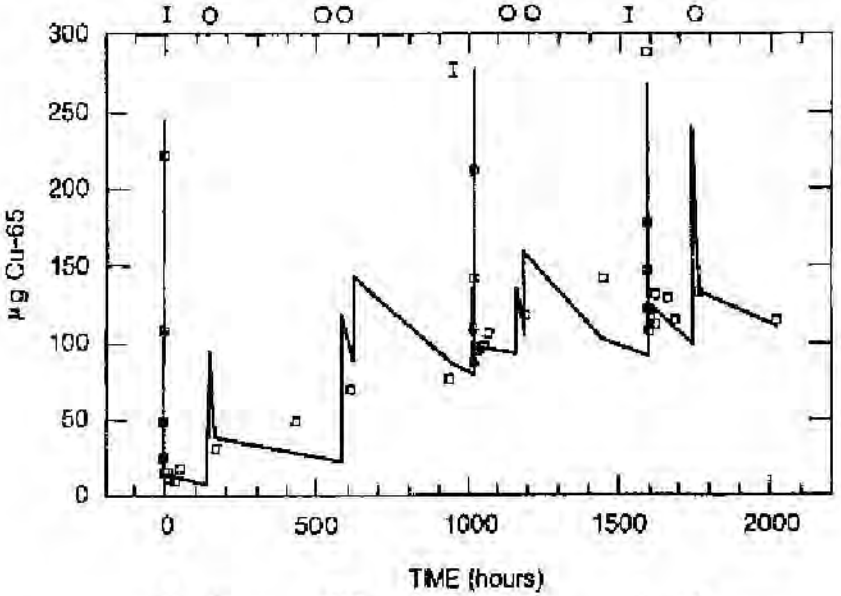
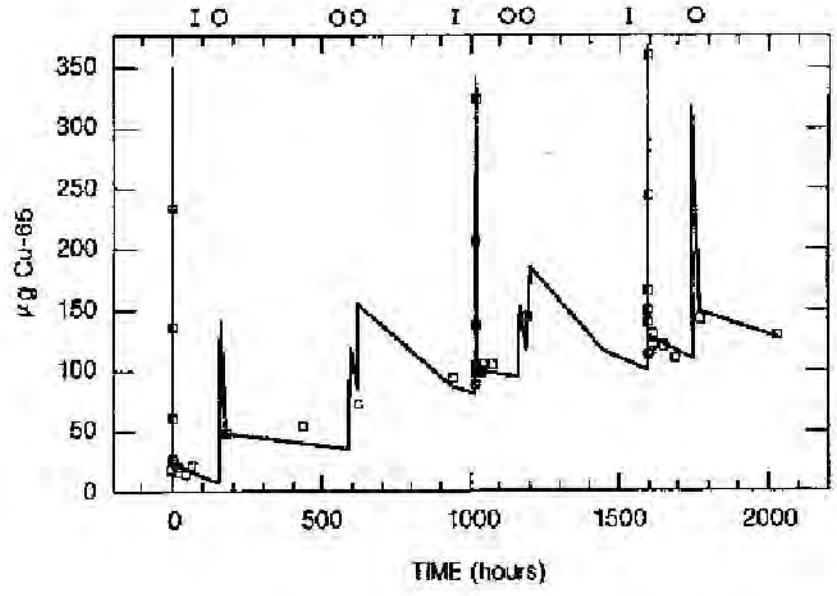


Figure A6.2(2)-2

Figure 2 Copper model fit to plasma-enriched ^{65}Cu tracer data for the three i.v. and four oral tracer inputs for subjects 2 through 5. Line is model simulation, open squares represent observed data. I = i.v. input, O = oral input. (a) Subject 2. (b) Subject 3. (c) Subject 4. (d) Subject 5. (e) Subject 6. Hours are relative to the first ^{65}Cu input.


I O OO I OO I O
300
250
200
150
100
50
0
µg Cu-65
0
500
1000
1500
2000
TIME (hours)


I O OO OO I O
300
250
200
150
100
50
0
µg Cu-65
0
500
1000
1500
2000
TIME (hours)


I O OO I OO I O
350
300
250
200
150
100
50
0
µg Cu-65
0
500
1000
1500
2000
TIME (hours)

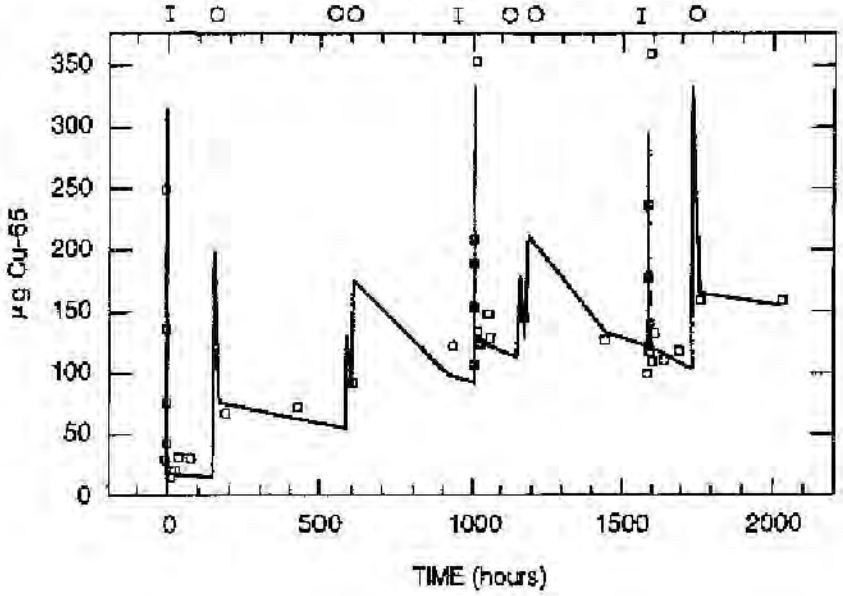

I O OO I OO I O
350
300
250
200
150
100
50
0
µg Cu-65
0
500
1000
1500
2000
TIME (hours)

Figure A6.2(2)-3

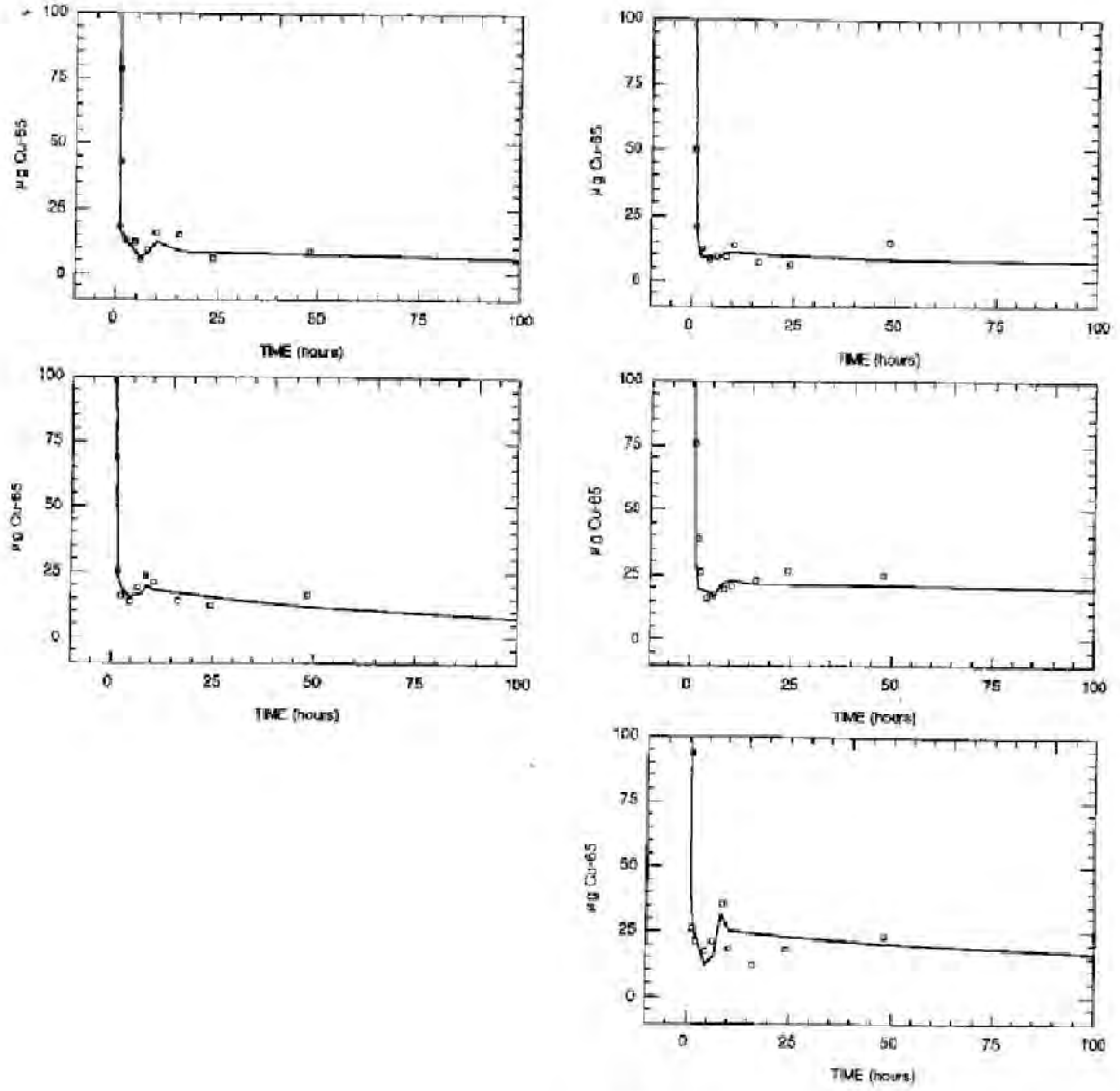


Figure 3 Copper model fit to plasma-enriched ^{65}Cu tracer data for the first intravenous time period for subject 2 through 6. Line is model simulation, open squares represent observed data. (a) Subject 2. (b) Subject 3. (c) Subject 4. (d) Subject 5. (e) Subject 6. Hours are relative to the first ^{65}Cu input.

			Official use only
		20 REFERENCE	
1.1	**Reference**	*Author(s), year, title, laboratory name, laboratory report number, report date (if published, list journal name, volume: pages)* *If necessary, copy field and enter other reference(s).* Walker, R.W. (1982). The Result of a Copper Bracelet Clinical Trial and Subsequent Studies. P 469 – 478. In: J. R. J. Sorenson (ed) Inflammatory Diseases and Copper: The Metabolic and Therapeutic Roles of Copper and Other Essential Metalloelements in Humans; Humana Press; Clifton N.J., USA. (published).	
1.2	**Data protection**	No. *(indicate if data protection is claimed)*	
1.2.1	Data owner	*Give name of company* Public domain.	
1.2.2	Criteria for data protection	Choose one of the following criteria (see also TNsG on Product Evaluation) and delete the others: No data protection claimed	
		21 GUIDELINES AND QUALITY ASSURANCE	
21.1	**Guideline study**	No. This was a non-regulatory clinical trial carried out in human volunteers. The purpose of this study was to subjectively assess the therapeutic value of copper bracelets in the treatment of arthritis and to confirm the ability of copper to dissolve in human sweat and to subsequently penetrate the skin. *(If yes, give guidelines; if no, give justification, e.g. "no guidelines available" or "methods used comparable to guidelines xy")*	
21.2	**GLP**	No. This was a non-regulatory clinical trial carried out in human volunteers. *(If no, give justification, e.g. state that GLP was not compulsory at the time the study was performed)*	
21.3	**Deviations**	Yes. Refer to section 5.3.2 for a general discussion of deviations and deficiencies. *(If yes, describe deviations from test guidelines or refer to respective field numbers where these are described, e.g. "see 3.x.y")*	
		22 MATERIALS AND METHODS *In some fields the values indicated in the EC or OECD test guidelines are given as default values. Adopt, change or delete these default values as appropriate.*	
22.1	**Test material**	Cu^{2+} as Copper metal	
22.1.1	Lot/Batch number	Not available	
22.1.2	Specification	As given in section 2 Deviating from specification given in section 2 as follows *(describe specification under separate subheadings, such as the following; additional subheadings may be appropriate):*	

Section A6.2	**Metabolism in mammals**
Annex Point IIA6.2	*Specify section no., heading and species as appropriate*
IUCLID 5.0/03	**A6.2(03), Absorption of copper**

22.1.2.1 Description	*If appropriate, give e.g. colour, physical form (e.g. powder, grain size, particle size/distribution)* Copper bracelets, each measuring about 15.0 x 1.2 x 0.1 cm.	
22.1.2.2 Purity	*Give purity in % of active substance* [redacted]	X
22.1.2.3 Stability	*Describe stability of test material* Not available	
22.1.2.4 Radiolabelling	*give structural location of radio labelling, give reason if not labelled* The test material was not radiolabelled.	
22.2 Test Animals	Non-entry field	
22.2.1 Species	Human volunteers	
22.2.2 Strain	Not applicable	
22.2.3 Source	Not applicable	
22.2.4 Sex	Male and female	
22.2.5 Age/weight/height at study initiation	*Young adults recommended* Both males and females were involved in this study. The principal age group was between 50 and 70 years old.	
22.2.6 Number of volunteers	*Give number* *Specify, if there are differences for example for treatment and recovery groups* 240 subjects were randomly allocated to one of three treatment groups, each comprising equal numbers of those who had previously worn copper bracelets and those who had not.	
22.2.7 Controls	Yes (refer to section 3.3.2.1).	
22.3 Administration/ Exposure	*(fill in respective route in the following, delete other routes)* Dermal	
22.3.1 Duration of treatment	2 months.	
22.3. 2 Exposure scenario	Non-entry field.	
22.3.2.1 Psychological groups, analysis	240 subjects were randomly allocated to one of three treatment groups, each comprising equal numbers of those who had previously worn copper bracelets and those who had not. Group 1 wore a 14 gram copper bracelet (15.0 x 1.2 x 0.1 cm) for 1 month, followed by 1 month wearing an aluminium placebo. Group 2 wore a placebo bracelet for 1 month, followed by 1 month wearing a copper bracelet. Group 3 did not wear any bracelet. All subjects were asked to maintain normal diet,	

activity, etc. over the course of the study.

22.3.2.2 Chemical analysis The author wore a pair of copper bracelets (22 x 1.3 x 0.1 cm) around his ankles for 50 days and an associate wore a similar bracelet around his wrist. The loss of weight (mg) was recorded with time.

The associate also collected his own sweat after vigorous exercise wrapped in plastic and warm clothing.

22.3.2.3 Dermal penetration The author wore a copper ring on the ring finger of one hand.

22.4 Examinations Non-entry field

22.4.1 Psychological analysis of subjects. A follow-up questionnaire was sent to all subjects at the end of each treatment period. On the second occasion, subjects were also invited to compare the effectiveness of the two bracelets.

22.4.2 Chemical analysis phase. The associate collected his own sweat after vigorous exercise while wrapped in plastic and warm clothing and several samples, each of approximately 25 ml, were obtained for analysis of copper content.

22.4.3 Skin biopsy. A skin biopsy was taken by a surgeon from the finger upon which the study author had worn a copper ring. A control biopsy was also taken from the other hand.

22.5 Sample/data processing and analysis Non-entry field

22.5.1 Psychological analysis of subjects Copper bracelets were weighed after use.

The therapeutic value of copper bracelets was subjectively evaluated by study subjects. Evaluation categories were as follows:

Cu++ copper bracelet much better than aluminium placebo.

Cu+ copper bracelet a little better than placebo.

= no difference between the two bracelets.

Al++ aluminium bracelet much better than copper bracelet.

Al+ aluminium bracelet a little better than copper bracelet.

22.5.2 Chemical analysis phase. Copper bracelets were weighed to determine loss of weight over time.

The copper content of collected sweat was determined before and after shaking for 24 hours with copper turnings. A colorimetric method involving oxalylhydrazide was used and checked by atomic absorption spectroscopy.

22.5.3 Dermal penetration. Skin biopsies were sectioned, stained and examined histologically.

23 RESULTS AND DISCUSSION

Describe findings. If appropriate, include table. Sample tables are given below.

23.1 Results Non-entry field.

23.1.1 Psychological analysis of subjects The perceived effectiveness of the bracelet is summarised in **Table A6.2(03)-1**.

The average weight loss was about 13 mg per bracelet, each of which weighed about 14 g. There were no differences in weight loss between the two evaluation groups ($F < 1$ in both cases).

A dietary survey confirmed that a large majority of subjects did not

	regularly consume foods considered to be high in copper (liver; shellfish; mushrooms and nuts). The consequences for those asked not to wear any bracelet are shown in **Table A6.2(03)-2**.
23.1.2 Chemical analysis phase	The average weight loss per 14 g bracelet was 13 mg/month. The bracelets worn by the author lost 80 mg over 50 days. The analyses of copper in human sweat are summarised in **Table A6.2(03)-3**.
23.1.3 Dermal penetration.	Copper was present in the sample taken from the author's ring finger.
23.2 Discussion	Non-entry field
23.2.1 Psychological analysis of subjects	Of the people invited to take part in this study, 4/10 had a condition described as arthritis, 2/10 had rheumatism or rheumatoid arthritis, and 2/10 had a combination of these and other conditions. These people were given a copper bracelet to wear for a period of one month, and a bracelet of aluminium for a second month, in varying order. Others who normally wore a copper bracelet were asked to leave it off for the same period. All were asked to report the state of their condition after each period. When returned, the bracelets were weighed and were found to weigh less. Results of the experimental study showed that the medical conditions of previous users were significantly worse when not wearing their copper bracelet ($\chi^2 = 8.00$, df = 2, $p<0.02$), and that the bracelets were therefore of some therapeutic value. There was no change with the non-users.
23.2.2 Chemical analysis phase	The concentration of dissolved copper in sweat was found to increase following the introduction of copper turnings (**Table A6.2(03)-3**).
23.2.3 Dermal penetration	There was evidence that copper was present in the sample taken from the author's ring finger, thereby demonstrating that small amounts of complexed dissolved copper are capable of penetrating the skin.
23.3 Toxic effects, clinical signs	*No effects / describe significant effects referring to data in results table* No toxic effects or clinical signs were observed.
23.4 Recovery of labelled compound	*state percentage* Not applicable.

24 APPLICANT'S SUMMARY AND CONCLUSION

24.1 Materials and methods	*Give concise description of method; give test guidelines no. and discuss relevant deviations from test guidelines* A clinical trial was carried out in which 240 arthritis sufferers were randomly allocated to one of three treatment groups. In two of the groups, subjects wore bracelets of either copper or an aluminium placebo for a period of 1 month, after which a bracelet of the alternate metal was worn for a second month. Subjects in the third group did not wear bracelets. Subjects were asked to evaluate the therapeutic effectiveness of their bracelets in the treatment of arthritis. Bracelets were also weighed.

	In a separate phase of the study, the solubility in human sweat of copper derived from turnings was investigated. In a third phase, a skin biopsy was taken from the finger upon which the subject had worn a copper ring. The degree of dermal penetration by copper was then assessed.
24.2 Results and discussion	*Summarize relevant results; discuss dose-response relationship.* The medical condition of subjects was reported to be significantly worse when copper bracelets were not worn by those who had previously done so. When returned, bracelets were found to weigh less. The concentration of copper in sweat was found to increase following the introduction of copper turnings. There was evidence that copper was present in the sample taken from the author's ring finger, demonstrating that small amounts of complexed dissolved copper are capable of penetrating the skin.
24.3 Conclusion	Small amounts of copper dissolved in human sweat have been shown to absorb through the skin and may have therapeutic value in the treatment of arthritis.
24.3.1 Reliability	*Based on the assessment of materials and methods include appropriate reliability indicator 0, 1, 2, 3, or 4* 2
24.3.2 Deficiencies	Yes. This study was not conducted and/or reported in strict compliance with the principles of GLP. However, this does not compromise the validity of the data generated, or the author's interpretation of that data, given that the study was not carried out for regulatory purposes. No internationally accepted guidelines are available that address the objective of the research presented in this summary. The findings of this study are considered to make a valuable contribution to the 'weight of evidence' approach that has been adopted for the purposes of the current review of copper toxicokinetics. A reliability indicator of 2 has been assigned on this basis. *(If yes, discuss the impact of deficiencies and implications on results. If relevant, justify acceptability of study.)*

Evaluation by Competent Authorities

Section A6.2	**Metabolism in mammals**
Annex Point IIA6.2	*Specify section no., heading and species as appropriate*
IUCLID 5.0/03	**A6.2(03), Absorption of copper**

	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	[redacted]
Materials and Methods	[redacted]
Results and discussion	[redacted]
Conclusion	[redacted]
Reliability	[redacted]
Acceptability	[redacted]
Remarks	

	COMMENTS FROM ...
Date	*Give date of comments submitted*

Table A6.2(03)-1

Copper Bracelet Clinical Trial

Subjective evaluation of Copper (Cu) and placebo (A1) bracelets. Evaluation categories:
Cu^{++} (copper bracelet much better than aluminium placebo).
Cu^{+} (copper bracelet a little better than placebo) = (no difference between two bracelets).
$A1^{+}$, $A1^{++}$ (as for copper but vice versa).

		Cu^{++}	Cu^{+}	=	$A1^{+}$	A^{+++}	Total
Previous users	Cu → A1	12	3	6	2	0	23
	A1 → Cu	8	2	7	3	1	21
Previous non-users	Cu → A1	1	2	5	1	2	11
	A1 → Cu	4	5	12	0	1	22
Total		25	12	30	6	4	77

Table A6.2(03)-2

The evaluation categories of the table are: "better" (much better and little better); "same" and "worse" (little worse and much worse).

	"better"	"same"	"worse"	Total
Previous users	1	7	11	19
Non-users	6	10	6	22
Total	7	17	17	41

Table A6.2(03)-3

Copper analyses of human sweat. Concentration M/l

Sample No.	Concentration before	Concentration after
1	$3.5x10^{-5}$ $(1.7x10^{-5})$	$0.7x10^{-3}$ $(0.2x10^{-3})$
2	$3.0x10^{-5}$	$0.6x10^{-3}$
3	$1.1x10^{-5}$ $(1.8x10^{-5})$	$3.4x10^{-3}$
4	$2.7x10^{-5}$	$0.8x10^{-3}$
5	$1.0x10^{-5}$	$2.0x10^{-3}$

			Official use only
		25 REFERENCE	
1.1	**Reference**	*Author(s), year, title, laboratory name, laboratory report number, report date (if published, list journal name, volume: pages) If necessary, copy field and enter other reference(s).* Pirot, F., Panisset, F., Agache, P. & Humbert, P. (1996). Simultaneous Absorption of Copper and Zinc through Human Skin in vitro. Skin Pharmacol. **9:** 43-52 (published).	
1.2	**Data protection**	No *(indicate if data protection is claimed)*	
1.2.1	Data owner	*Give name of company* Public domain	
1.2.2	Criteria for data protection	Choose one of the following criteria (see also TNsG on Product Evaluation) and delete the others: No data protection claimed	
		26 GUIDELINES AND QUALITY ASSURANCE	
26.1	**Guideline study**	No. This was a non-regulatory study carried out to investigate the simultaneous absorption of copper and zinc sulphates or chlorides through ex vivo sliced human skin. No guidelines are available to address this objective. This summary addresses only those sections of the report that relate to copper salts. *(If yes, give guidelines; if no, give justification, e.g. "no guidelines available" or "methods used comparable to guidelines xy")*	
26.2	**GLP**	No. This was a non-regulatory study. *(If no, give justification, e.g. state that GLP was not compulsory at the time the study was performed)*	
26.3	**Deviations**	Yes. Refer to section 5.3.6 for a general discussion of deviations and deficiencies. *(If yes, describe deviations from test guidelines or refer to respective field numbers where these are described, e.g. "see 3.x.y")*	
		27 MATERIALS AND METHODS *In some fields the values indicated in the EC or OECD test guidelines are given as default values. Adopt, change or delete these default values as appropriate.*	
27.1	**Test material**	Cu^{2+} as copper sulphate ($CuSO_4.5H_2O$) and copper chloride ($CuCl_2.2H_2O$); Zn^{2+} as zinc sulphate ($ZnSO_4.7H_2O$) and zinc chloride ($ZnCl_2$).	
27.1.1	Lot/Batch number	Not available	
27.1.2	Specification	Deviating from specification given in section 2 as follows *(describe specification under separate subheadings, such as the following; additional subheadings may be appropriate):*	

Section A6.2	**Metabolism in mammals**	
Annex Point IIA6.2	*Specify section no., heading and species as appropriate*	
IUCLID 5.0/04	**A6.2(04), Absorption of copper**	
27.1.2.1 Description	*If appropriate, give e.g. colour, physical form (e.g. powder, grain size, particle size/distribution)* Four preparations were tested: (A) 5% (w/w) copper sulphate and 5% (w/w) zinc sulphate in white petrolatum. (B) 5% (w/w) copper sulphate and 5% (w/w) zinc sulphate in carboxypolymethylene gel. (C) 5% (w/w) copper chloride and 5% (w/w) zinc chloride in white petrolatum. (D) 5% (w/w) copper chloride and 5% (w/w) zinc chloride in hydroxypropylmethylcellulose gel. Formulae A and B contained 1.27% Cu and 1.14% Zn. Formulae C and D contained 1.86% Cu and 2.40% Zn.	
27.1.2.2 Purity	*Give purity in % of active substance* [redacted]	X
27.1.2.3 Stability	*Describe stability of test material* Not stated	
27.1.2.4 Radiolabelling	*give structural location of radio labelling, give reason if not labelled* The test material was not radiolabelled.	
27.2 Test Animals	*Non-*entry *field*	
27.2.1 Species	Skin samples obtained from humans (see section 3.2.3).	
27.2.2 Strain	Not applicable	
27.2.3 Source	Skin samples used for the permeation studies was obtained from three women (37, 40 and 45 years old). The samples of skin were obtained from either breast or abdomen during surgery.	
27.2.4 Sex	Female	
27.2.5 Age	Three adults (37, 40 and 45 years old).	
27.3 Procedures	Non-entry field	
27.3.1 Skin permeation studies	Skin samples were sliced with a dermatome to an average thickness of 410 µm as assessed by high frequency (25 MHz) B-scan ultrasound imaging. All skin samples were randomised to avoid source effect. Samples were then mounted in Franz-type static diffusion cells with a 3.14 cm^2 surface area, and 20 mg/cm^2 of a formula was applied to the outer skin surface. The amounts of Cu and Zn deposited within each formula are presented in **Table A6.2(04)-1**. The receptor fluid consisted of isotonic saline containing 5% human albumin, 5000 IU/l penicillin G, 5 mg/l streptomycin, and 1.25 mg/l amphotericin B. During the experiment, it was continuously stirred with a magnetic rod and maintained at 33°C. It was totally withdrawn after 1.5, 3, 6, 12, 24, 48 and 72 hours and kept for analysis. The receptor chamber was immediately refilled with fresh receptor fluid to maintain a constant acceptor volume (9 ml). Five cells were used for each formula. After tenfold dilution, the collected receptor fluids were subjected to flame atomic absorption spectrometry for cu and Zn quantification at 324.7 and 213.9 nm, respectively, with an acetylene-air flame.	

27.3.2 Copper and zinc levels in the skin. At the 72nd hour, the white petrolatum ointment remaining on the skin surface was washed off with toluene. The hydrogels were washed off with distilled water. The whole epidermis was removed from the dermis with forceps, dried for 24 hours at 105°C, weighed, and dissolved in nitric acid (68%) for 69 hours. The mixtures were then passed through a 0.2 µm nitro-cellulose filter before determination of the copper and zinc.

To estimate the metal content of fresh tissue, the dry weight of the stratum corneum and viable epidermis and dermis were estimated at 90 and 28% of fresh weight, respectively; the density of both fresh tissues equal to unity and the volume per cm2 surface area of fresh epidermis and dermis 5 mm3 and 36 mm3, respectively.

27.3.3 PH measurements The pH of aqueous formulations and receptor fluids were measured after verification of the accuracy of the pH measurements (the coefficient of variation of the reproducibility was 0.08%). The pH of Formulae B (sulphates) and D (chlorides) were 1.6 ± 0.01 and 4.43 ± 0.01, respectively.

27.3.4 Determination of octanol-water partition coefficients (K_{ow}). K_{ow} values were determined by the modified shake flask method. 0.5 g of metallic salt was mixed with 5 ml water and 5 ml 1-octanol in a polypropylene tube for 12 hours at room temperature. The solutions were mixed with magnetic stirring rods for 12 hours and then centrifuged for 10 minutes at 4000 rpm. The metal concentrations in each phase were determined by atomic absorption spectrometry. The experiment was repeated three times for each salt.

27.3.5 Statistics The apparent permeability coefficients of copper and zinc were obtained with sulphate and chloride as counter-ions in the petrolatum and hydrogels, and the metal levels within skin were tested using the non-parametric Kruskal-Wallis test. Kow were compared using the non-parametric Mann-Whitney test. Absorbed amounts (in % of applied dose) were compared using analysis of variance. The comparison of multiple means used Fisher's least significant difference procedure.

28 RESULTS AND DISCUSSION

Describe findings. If appropriate, include table. Sample tables are given below.

28.1 Results Non-entry field.

28.1.1 Octanol/water partition coefficient The Kow values obtained for copper are shown in **Table A6.2(04)-2**.

28.1.2 Skin permeation studies In the fresh receptor fluid, copper concentration as a part of human serum albumin was 605 ± 5 µg/l (5.4 µg per receptor chamber volume). This amount was subtracted from samples to determine the absorbed amounts.

Copper permeation from $CuSO_4$ was hardly affected by the vehicle (formulae A and B, **Figure A6.2(04)-1**). By contrast, permeation from $CuCl_2$ was greater with petrolatum than with hydrogel (formulae C and D). By the 72nd hour, the collected amounts of copper remained below 6% of the applied doses (**Figure A6.2(04)-2**). During the experiment, copper fluxes were very low and variable (**Figure A6.2(04)-3**). They all showed a peak between 1.5 and 6 hours followed by a steady state, with the exception of formula B, where a slow and permanent decrease was observed. The steady-state fluxes were calculated for the following periods: 12-72 hours (formula A); 3-24 hours (formula C); 6-24 hours

(formula D) and tentatively 12-48 hours (formula B).

The steady-state fluxes were used to calculate the apparent permeability coefficients for copper, using the applied concentrations of copper (1.27% in sulphate formula and 1.86% in gel formulae), with 0.82 and 1 for densities of petrolatum and gel, respectively (**Table A6.2(04)-2**).

28.1.3 pH of receptor fluid A slight decrease in pH was observed following gel application (formulae B and D), possibly due to simultaneous percutaneous absorption of the ions, or of the undissociated salts. Absorption from petrolatum formulations (A and C) did not result in any change of receptor fluid pH.

28.1.4 Copper levels in the skin

Dry tissue: The ratio of copper concentrations present in the epidermis & dermis of dry, untreated control skin was 7:1 (**Table A6.2(04)-3**). The copper content of both skin layers rose substantially during the course of the study, the increase was most noticable in the epidermis with formula B and in the dermis with formula D.

Fresh epidermis: The estimated basal concentration of copper within fresh epidermis was twice as great as that within fresh dermis (**Table A6.2(04)-4**). By the end of the study, copper concentrations in the epidermis had increased strongly. Furthermore, the epidermis still contained much more copper than the dermis, with the exception of formula D.

The epidermal load of copper ranged from 2.5 to 6.3 times the cumulative amount found in receptor fluid over the 72 hours of the study. There was no clear-cut relationship with either fluxes or permeability constants.

Fresh dermis: Copper sulphate in hydrogel formula (B) resulted in very low copper storage within the dermis (0.2 µg cm^{-2}). With all three remaining formulae, the amount of copper stored was equal to, lower than, or higher than the amounts collected in receptor fluid over 72 hours (A, C and D, respectively).

28.2 Discussion

Over 72 hours, the cumulative amount of copper recovered in receptor fluid (saline containing 5% serum albumin) was very low (5.5 µg/cm^2 or less), except for copper in petrolatum (22 µg/cm^2). Accordingly, the corresponding K_p values remained in the range of 10^{-6} cm/h. While the amount of copper applied in the experiment was in excess, less than 6% of the total was absorbed.

The time course of transcutaneous copper fluxes always showed a peak between 1.5 and 6 hours, followed by immediate or delayed subsidence. This pattern suggests a decrease in either the partition coefficient or the diffusion coefficient (or in both), in relation to the sorption of the permeant within the stratum corneum.

No relationship was found between the K_p and either the $_{Kow}$ or the type of vehicle.

In the epidermis, copper storage was found to be equal to or greater than the 72-hour absorbed cumulative amount; storage in the dermis was generally lower than the 72-hour absorbed cumulative amount. This finding is in accordance with the well-known high storage capacity of the stratum corneum. Within the epidermis as a whole, storage was generally larger from petrolatum formulations, suggesting the influence of lower partition coefficients. Furthermore, chlorides were stored in generally larger amounts, possibly in relation to their higher K_{ow}.

Within the dermis, chloride was retained in substantial amounts, mostly

when the vehicle was aqueous. This suggested a relation to the higher cumulative quantity found in the receptor fluid following use of an aqueous vehicle, as compared with sulphate formulations. The contrary was observed with sulphates, although they were poorly retained.

As a whole, the added amounts of the permeant found within the dermis and collected from the receptor fluid represented the amount actually absorbed. **Table A6.2(04)-4** shows that some figures increased greatly. For example, copper absorption is multiplied by 1.7 and 7.8 for formulae A and D, respectively.

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1 Materials and methods *Give concise description of method; give test guidelines no. and discuss relevant deviations from test guidelines*

A study was carried out to investigate the simultaneous absorption of copper and zinc sulphates or chlorides through *ex-vivo* sliced human skin taken from three females aged 37 – 45 years old. The study was not designed to follow an internationally accepted guideline, and was not carried out in compliance with GLP.

The following four preparations were tested: (A) 5% copper sulphate and 5% zinc sulphate in white petrolatum; (B) 5% copper sulphate and 5% zinc sulphate in carboxypolymethylene gel; (C) 5% copper chloride and 5% zinc chloride in white petrolatum; (D) 5% copper chloride and 5% (w/w) zinc chloride in hydroxypropylmethylcellulose gel. Formulae A and B contained 1.27% Cu and 1.14% Zn. Formulae C and D contained 1.86% Cu and 2.40% Zn.

Skin samples were sliced with a dermatome to an average thickness of 410 µm and randomised. Samples were then mounted in Franz-type static diffusion cells with a 3.14 cm^2 surface area, and 20 mg/cm^2 of a preparation was applied to the outer skin surface. The receptor fluid, which consisted of isotonic saline containing 5% human albumin, was continuously stirred at 33°C. After 1.5, 3, 6, 12, 24, 48 and 72 hours the receptor fluid was withdrawn and kept for analysis. The receptor chamber was then refilled. Five cells were used for each preparation. After tenfold dilution, the receptor fluids were subjected to flame atomic absorption spectrometry (AAS) for Cu and Zn quantification at 324.7 and 213.9 nm.

After 72 hours, the white petrolatum ointment was washed off the skin surface with toluene. Hydrogels were washed off with distilled water. The whole epidermis was removed from the dermis, dried for 24 hours at 105°C, weighed, and dissolved in nitric acid. The mixtures were then passed through a 0.2 µm nitro-cellulose filter before determination of the copper and zinc. The dry weight of the stratum corneum and viable epidermis and dermis were estimated at 90 and 28% of fresh weight, respectively; the density equal to unity and the volume per cm^2 surface area of fresh epidermis and dermis 5 mm^3 and 36 mm^3, respectively.

The pH of aqueous formulations and receptor fluids was measured. The pH of Formulae B (sulphates) and D (chlorides) were 1.6 ± 0.01 and 4.43 ± 0.01, respectively.

K_{ow} values were determined by the modified shake flask method. 0.5 g of metal salt was mixed with 5 ml water and 5 ml 1-octanol for 12 hours at room temperature. The solutions were then centrifuged for 10 minutes at 4000 rpm. Metal concentrations in each phase were

~~determined by AAS. The experiment was repeated 3 times for each salt.~~

Apparent permeability coefficients of copper and zinc were obtained with sulphate and chloride as counter-ions in the petrolatum and hydrogels, and the metal levels within skin were tested using the non-parametric Kruskal-Wallis test. $_{Kow}$ values were compared using the non-parametric Mann-Whitney test. Absorbed amounts were compared using analysis of variance. The comparison of multiple means used Fisher's least significant difference procedure.

5.2 Results and discussion *Summarize relevant results; discuss dose-response relationship.*

Copper concentration in fresh receptor fluid was 605 ± 5 µg/l. This was subtracted from samples to determine the absorbed amounts.

Copper permeation from $CuSO_4$ was hardly affected by the vehicle, whereas permeation from $CuCl_2$ was greater with petrolatum than with hydrogel. Over 72 hours, the cumulative amount of copper recovered in receptor fluid was very low (4.3, 4.0 and 5.5 µg/cm^2 for preparations A, B and D, respectively), except for copper in petrolatum (preparation C), for which 22 µg/cm^2 was recovered. These amounts correspond to 1.45%, 1.56%, 5.74% and 1.47% of the applied doses for preparations A, B, C and D, respectively. Copper fluxes were very low and variable, all showing a peak between 1.5 and 6 hours followed by a steady state, with the exception of formula B, where a slow and permanent decrease was observed. This pattern suggests a decrease in either the partition coefficient or the diffusion coefficient (or in both), in relation to the sorption of the permeant within the stratum corneum (measured K_{ow} values were very low at $0.85 \times 10^6 \pm 0.07$ for copper as sulphate and $59.7 \times 10^6 \pm 4.77$ for copper as chloride).

A slight decrease in pH was observed following gel application, possibly due to simultaneous percutaneous absorption of the ions, or of the undissociated salts. Absorption from petrolatum formulations did not result in any change of receptor fluid pH.

The ratio of copper concentrations present in the epidermis & dermis of dry, untreated control skin was 7:1. The copper content of both skin layers rose substantially during the course of the study. The increase was most noticable in the epidermis with formula B and in the dermis with formula D. The basal concentration of copper within fresh epidermis was twice as great as that within fresh dermis. By the end of the study, copper concentrations in the epidermis had increased strongly. Furthermore, the epidermis still contained much more copper than the dermis, with the exception of formula D. The epidermal load of copper ranged from 2.5 to 6.3 times the cumulative amount found in receptor fluid over the 72 hours of the study. There was no clear-cut relationship with either fluxes or permeability constants. These findings accord with the high storage capacity of the stratum corneum. Within the epidermis as a whole, storage was generally larger from petrolatum formulations, suggesting the influence of lower partition coefficients. Furthermore, chlorides were stored in generally larger amounts, possibly in relation to their higher K_{ow}.

Within fresh dermis, copper sulphate in hydrogel formula resulted in very low copper storage (0.2 µg cm^{-2}). With all three remaining formulae, the amount of copper stored was equal to, lower than, or higher than the amounts collected in receptor fluid over 72 hours (A, C and D, respectively).

Section A6.2	**Metabolism in mammals**
Annex Point IIA6.2	*Specify section no., heading and species as appropriate*
IUCLID 5.0/04	**A6.2(04), Absorption of copper**

5.3 Conclusion	Measured amounts of copper in receptor fluid were low, accounting for 1.45%, 1.56%, 5.74% and 1.47% of the applied dose for preparations A (petrolatum), B (hydrogel 1), C (petrolatum) and D (hydrogel 2), respectively. Measured $_{Kow}$ values for copper were also very low, at 0.85 x 10^6 ± 0.07 as sulphate and 59.7 x 10^6 ± 4.77 as chloride.
5.3.1 Reliability	*Based on the assessment of materials and methods include appropriate reliability indicator 0, 1, 2, 3, or 4* 2
5.3.2 Deficiencies	Yes. This study was not conducted and/or reported in strict compliance with the principles of GLP. However, this does not compromise the validity of the data generated, or the author's interpretation of that data, given that the study was not carried out for regulatory purposes. No internationally accepted guidelines are available that address the objective of the research presented in this summary. The findings of this study are considered to make a valuable contribution to the 'weight of evidence' approach that has been adopted for the purposes of the current review of copper toxicokinetics. A reliability indicator of 2 has been assigned on this basis. *(If yes, discuss the impact of deficiencies and implications on results. If relevant, justify acceptability of study.)*

Evaluation by Competent Authorities

Use separate "evaluation boxes" to provide transparency as to the comments and views submitted

EVALUATION BY RAPPORTEUR MEMBER STATE

Date	[redacted]
Materials and Methods	[redacted] [redacted]:
Results and discussion	[redacted]
Conclusion	[redacted]
Reliability	[redacted]
Acceptability	[redacted]

Section A6.2	**Metabolism in mammals**
Annex Point IIA6.2	*Specify section no., heading and species as appropriate*
IUCLID 5.0/04	**A6.2(04), Absorption of copper**

Remarks	[redacted]
	COMMENTS FROM ...
Date	*Give date of comments submitted*

Table 1. Deposited amounts of copper and zinc (ìg/cm^2) with each of the 4 formulae

Formula	Counter-ion	Vehicle	Copper	Zinc
A	SO_4	petrolatum	297 ± 39	268 ± 37
B	SO_4	hydrogel1	257 ± 19	230 ± 17
C	Cl_2	petrolatum	385 ± 54	497 ± 70
D	Cl_2	hydrogel 2	373 ± 4	481 ± 5

Table 2. Copper: K o/w, flux at steady state and K_p

Formula	Counter-ion	Vehicle	K o/w x 10^6	Flux µg cm-2 h^{-1} x 10^3	K_p cm h^{-1} x 10^6
A	SO_4	petrolatum	0.85 ± 0.07	56 ± 54 [a]	3.25 ± 3.14 [b]
B	SO_4	hydrogel 1	0.85 ± 0.07	64 ± 80 [a]	4.54 ± 5.67 [b]
C	Cl_2	petrolatum	59.70 ± 4.77	404 ± 310	16.03 ± 12.28
D	Cl_2	hydrogel 2	59.70 ± 4.77	47 ± 20 a	2.26 ± 0.97 [b]

a Difference in flux versus formula C ($p < 0.05$).

b Difference in K_p versus formula C ($p < 0.001$).

Table 3. Copper levels (mg/g dry tissue) in human skin after a 72-hour topical application

Formula	Counter-ion	Vehicle	Epidermis	Dermis
A	SO_4	petrolatum	9.25 ± 2.29 a,b	0.32 ± 0.31 [c]
B	SO_4	hydrogel 1	4.10 ± 1.17 [a]	0.02 ± 0.01 [c]
C	Cl_2	petrolatum	19.09 ± 6.79	0.81 ± 0.70 [c]
D	Cl_2	hydrogel 2	8.20 ± 1.24 a,b	3.73 ± 3.79
Control			0.05 ± 0.04 a	0.007 ± 0.005 [c]

Mean ± standard deviation of 5 determinations.

a Difference with formulation C ($p < 0.0001$).
b Difference with control ($p < 0.0001$).
c Difference with formulation D ($p < 0.01$).

Table 4. Estimated amounts of copper found at 72h within fresh tissue, and of total absorbed amounts (ig/cm^2).

(1) Formula	(2) Counter-ion	(3) Vehicle	(4) Fresh-epidermis	(5) Fresh dermis	(6) Receptor Fluid 72 h	(7) Total absorbed (5+6)
None			0.147	0.071		
A	SO_4	petrolatum	27	3.2	4.3	7.5
B	SO_4	hydrogel 1	12	0.2	4.0	5.1
C	Cl_2	petrolatum	56	8.2	22.1	30.3
D	Cl_2	hydrogel 2	24	37.6	5.5	43.1

Figure A6.2(04)-1 – Figure A6.2(04)-3

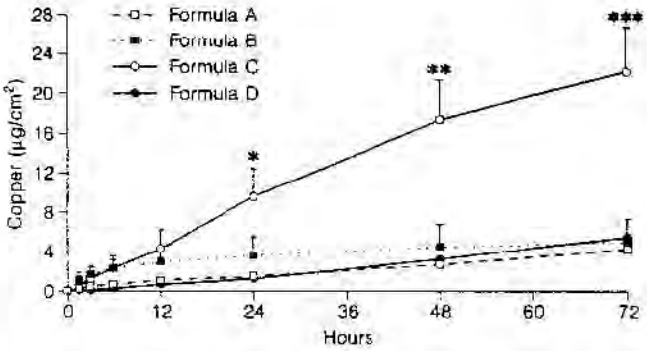


Fig. 1. Cumulative release of copper in receptor fluid versus time. Vertical bars indicate SD. Statistical significance of difference between formula C and the others: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

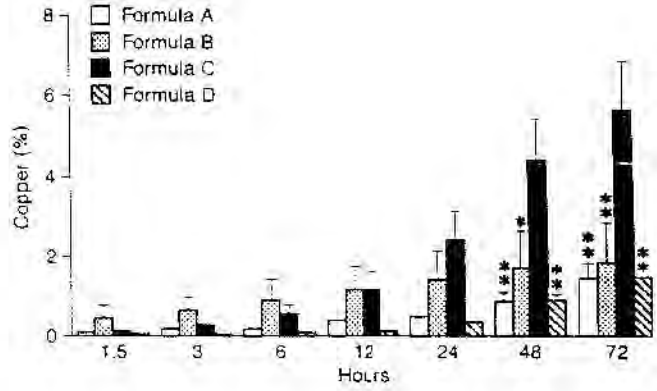


Fig. 2. Cumulated release of copper in receptor fluid versus time, expressed in percent of applied dose. Bars indicate SD. Statistical significance of the difference between formula C and the others: * $p < 0.05$; ** $p < 0.01$.

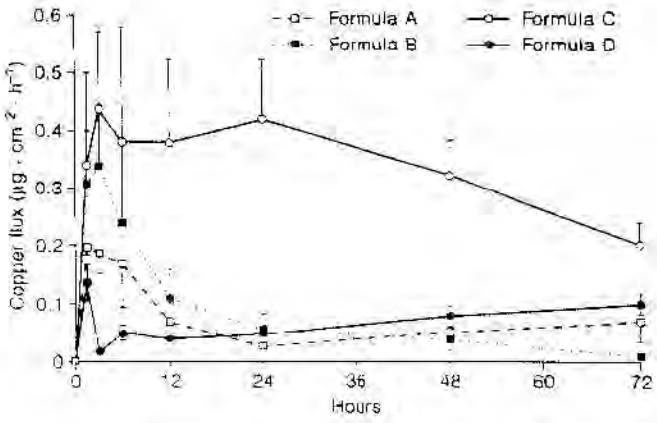


Fig. 3. Time course of copper percutaneous fluxes. Vertical bars indicate SD.

Official use only

29 REFERENCE

1.1 Reference

Author(s), year, title, laboratory name, laboratory report number, report date (if published, list journal name, volume: pages)
If necessary, copy field and enter other reference(s).

Pirot, F., Millet, J., Kalia, Y.N. & Humbert, P. (1996). In vitro Study of Percutaneous Absorption, Cutaneous Bioavailability and Bioequivalence of Zinc and Copper from Five Topical Formulations. Skin Pharmacol. **9:** 259-269 (published).

1.2 Data protection

No
(indicate if data protection is claimed)

1.2.1 Data owner

Give name of company

Public domain

1.2.2 Criteria for data protection

Choose one of the following criteria (see also TNsG on Product Evaluation) and delete the others: No data protection claimed

30 GUIDELINES AND QUALITY ASSURANCE

30.1 Guideline study

No. This was a non-regulatory study carried out to compare in vitro the percutaneous absorption and cutaneous bioavailability of zinc and copper present in 5 topical formulations from differenct salts and vehicles. No guidelines are available to address this objective. This summary addresses only those sections of the report that relate to copper salts.

(If yes, give guidelines; if no, give justification, e.g. "no guidelines available" or "methods used comparable to guidelines xy")

30.2 GLP

No. This was a non-regulatory study.

(If no, give justification, e.g. state that GLP was not compulsory at the time the study was performed)

30.3 Deviations

No. Not applicable to non-guideline studies.

(If yes, describe deviations from test guidelines or refer to respective field numbers where these are described, e.g. "see 3.x.y")

31 MATERIALS AND METHODS

In some fields the values indicated in the EC or OECD test guidelines are given as default values. Adopt, change or delete these default values as appropriate.

31.1 Test material

Zinc 2-pyrrolidone 5-carboxylate (ZnPC); copper 2-pyrrolidone 5-carboxylate (CuPC); zinc oxide (ZnO); zinc sulphate ($ZnSO_4.7H_2O$); copper sulphate ($CuSO_4.5H_2O$).

31.1.1 Lot/Batch number Not available

31.1.2 Specification

Deviating from specification given in section 2 as follows
(describe specification under separate subheadings, such as the following; additional subheadings may be appropriate):

Section A6.2	**Metabolism in mammals**	
Annex Point IIA6.2	*Specify section no., heading and species as appropriate*	
IUCLID 5.0/05	**A6.2(05), Absorption of copper**	
31.1.2.1 Description	*If appropriate, give e.g. colour, physical form (e.g. powder, grain size, particle size/distribution)* Five preparations were tested: Emulsion A (water/oil) was formulated with 0.25% (w/w) ZnPC (Zn^{2+} = 0.05%), 1.5% (w/w) ZnO (Zn^{2+} = 1.21%) and 0.5% (w/w) CuPC (Cu^{2+} = 0.1%). Emulsion B (water/oil) was formulated with 0.25% (w/w) $ZnSO_4$ (Zn^{2+} = 0.06%), 1.5% (w/w) ZnO and 0.5% (w/w) $CuSO_4$ (Cu^{2+} = 0.13%). Emulsion C (water/oil) was formulated with 0.1% (w/w) $ZnSO_4$ (Zn^{2+} = 0.02%), 7% (w/w) ZnO (Zn^{2+} = 5.62%) and 0.2% (w/w) $CuSO_4$ (Cu^{2+} = 0.05%). Ointment D was formulated with 0.1% (w/w) $ZnSO_4$ (Zn^{2+} = 0.02%), 10% (w/w) ZnO (Zn^{2+} = 8.03%) and 0.35% (w/w) $CuSO_4$ (Cu^{2+} = 0.09%). Ointment E was formulated with 10% (w/w) ZnO (Zn^{2+} = 8.03%) and 0.2% (w/w) $CuSO_4$ (Cu^{2+} = 0.05%).	
31.1.2.2 Purity	*Give purity in % of active substance* [redacted]	X
31.1.2.3 Stability	*Describe stability of test material* Not stated	
31.1.2.4 Radiolabelling	*give structural location of radio labelling, give reason if not labelled* The test material was not radiolabelled.	
31.2 Test Animals	*Non-*entry *field*	
31.2.1 Species	Skin samples obtained from humans (see section 3.2.3).	
31.2.2 Strain	Not applicable	
31.2.3 Source	Samples of human abdominal skin were obtained from surgery.	
31.2.4 Sex	Not stated.	
31.2.5 Age	Not stated.	
31.3 Procedures	Non-entry field	
31.3.1 Skin permeation studies	Two series of experiments were done using two different sources of intact human skin. In the first experiment, percutaneous absorption and cutaneous bioavailability of zinc and copper from ZnPC and CuPC were compared with those from $ZnSO_4$ and $CuSO_4$, first using a similar (emulsion B) vehicle, and then in a vehicle of different composition (emulsion C). In the second experiment, the percutaneous absorption and cutaneous bioavailability of zinc and copper from ZnPC and CuPC were compared with those from $ZnSO_4$ (and ZnO) and $CuSO_4$ in ointments D and E. Skin samples were sliced with a dermatome to a thickness of 400 µm as assessed by high frequency (25 MHz) B-scan ultrasound imaging. All dermatomed skin samples were randomised to avoid source effect. Samples were then mounted in Franz-type static diffusion cells with a 3.14 cm^2 surface area. The volume of the receptor compartment was 9 ml, which was filled with 0.9% NaCl solution. The receptor fluid was magnetically stirred throughout the experiment and the temperature maintained at 33°C. 16 mg/cm^2 of each formulation was applied to the	

outer skin surface. The receptor solution was replaced after 2, 4, 12, 24, 48 and 72 hours and kept for analysis. Three cells were used for each formulation. Receptor fluids were subjected to flame atomic absorption spectrometry (AAS) for Cu and Zn quantification at 324.7 and 213.9 nm, respectively, with an acetylene-air flame.

31.3.2 Copper and zinc levels in treated and control skin.

After 72 hours, any remaining formulation on the skin surface was gently removed using cotton swabs. In order to ensure complete removal , the stratum corneum was stripped twice using adhesive tapes. Samples of dermis and whole epidermis (removed from the dermis with forceps), were taken from treated skin using a punch biopsy. A sample of whole skin was also taken for assessment of zinc and copper concentration. All samples were dried for 24 hours at 105°C, weighed, and dissolved in nitric acid (68%) for 96 hours. The mixtures were then passed through a 0.22 µm filter before determination of the copper and zinc. Solutions were diluted 10-fold or 20-fold before analysis by AAS.

31.3.3 Statistics and calculations

Absorbed amounts of zinc and copper through human skin: All distributions were accepted as normal, and the variances between the groups were not found to be significantly different. The significance of the results was evaluated by the analysis of variance (ANOVA). When treatment effects were significant using ANOVA, all possible pairwise comparisons were used with Fisher's protected least significant difference procedure. The chosen level of significance was $p < 0.05$.

Zinc and copper levels in human skin: Mean zinc and copper levels in treated skin were expressed as mg and µg of zinc and copper respectively per gram of dry tissue. Multiple comparisons between metal levels in treated skin to those in control skin were made using the Mann-Whitney test. The relative increase index (RII) was defined as the ratio between zinc or copper concentration in treated skin to that in control skin (%) divided by the applied dose. The RII values were compared using a Mann-Whitney test.

32 RESULTS AND DISCUSSION

Describe findings. If appropriate, include table. Sample tables are given below.

32.1 Results

Non-entry field.

32.1.1 Percutaneous absorption of copper

Percutaneous copper absorption from topical application of five formulations represented less than 6% of the applied dose (range 0.66 – 5.04%), confirming the occurrence of minimal copper diffusion through human skin. The diffusion of copper from all formulations (except emulsion B) appeared to be high for a period of up to 24 hours, as long as copper salts remained solubilised in the vehicle. After 72 hours, the amount of copper absorbed, expressed as % of applied dose) from CuPC in emulsion A was not significantly different from that od $CuSO_4$ in emulsion B. Furthermore, there was no difference between delivery of copper from emulsion A and both ointments D and E. The results also suggested that the delivery of copper was not enhanced by replacing the sulphate counterion with the larger, more hydrophobic PC moiety (**Table A6.2(05)-1**).

32.1.2 Level of copper in skin

All formulations in experiment 1 increased the level of copper on average 4-fold, 2-fold and 5-fold in epidermis, dermis and whole skin respectively, as compared with skin control (**Table A6.2(05)-2**). In experiment 2, a significant increase in the copper level in whole skin

and epidermis was shown by skin treated with emulsion A and ointment D. No corresponding increase in copper level in dermis and in whole skin was shown from the 3 formulations or ointment E, respectively. It was considered that the increase in copper concentration in whole skin may have been due to a build-up of copper in epidermis, in effect forming a copper reservoir, followed by slow diffusion through the dermis.

Multiple relative comparisons between treatments were made using the RII of copper (**Figure A6.2(05)-1**). In experiment 1, the RII of copper in dermis and whole skin, both treated by emulsion C, was significantly higher than those calculated from emulsions A and B. RIIs of copper in epidermis, dermis and whole skin treated by emulsions A and B were not significantly different, so that higher delivery of copper from CuPC as compared to $CuSO_4$ could not be assumed. Furthermore, experiment 2 showed that RIIs of copper in epidermis and dermis treated by the 3 formulations were not significantly different. A significant RII of copper in whole skin treated by the emulsion A and the ointment D was shown as compared to skin treated by ointment E. However, RIIs of copper in whole skin treated by emulsion A and ointment D were not significantly different. As shown in experiment 2, delivery of copper in different layers of skin treated by CuPC in emulsion were not significantly higher than that from $CuSO_4$ in ointment.

32.2 Discussion

Low copper concentrations were measured in the receptor solution, suggesting that percutaneous absorption was minimal across dermatomed skin. Conversely, topical application of formulations produced a significant increase of copper concentrations in epidermis and dermis, as compared to control data.

Bioequivalence of emulsions A and B was shown by means of the similar RII value for copper. However, no bioequivalence of copper from emulsions A and B was shown with ointments D and E. Consequently, a better cutaneous bioavailability of copper from organic salts (CuPC) as compared to mineral salts ($CuSO_4$) was not found by using these emulsions.

Assessments of bioequivalence have been based only on dermatopharmakinetic studies in vitro, but nevertheless confirm minimal percutaneous absorption of copper through normal skin and compare the effects of the nature of the counter-ions and vehicles on cutaneous bioavailability.

33 APPLICANT'S SUMMARY AND CONCLUSION

33.1 Materials and methods

Give concise description of method; give test guidelines no. and discuss relevant deviations from test guidelines

A study was carried out to to compare *in vitro* the percutaneous absorption and cutaneous bioavailability of Zn and Cu present in topical formulations containing different salts and vehicles. The study was not designed to follow an internationally accepted guideline and was not carried out in compliance with GLP.

The following five preparations were tested: Emulsion A (water/oil), formulated (w/w) with 0.25% Zinc 2-pyrrolidone 5-carboxylate (ZnPC), 1.5% zinc oxide (ZnO) and 0.5% copper 2-pyrrolidone 5-carboxylate (CuPC). Emulsion B (water/oil) was formulated with 0.25% zinc sulphate ($ZnSO_4.7H_2O$), 1.5% ZnO and 0.5% copper sulphate ($CuSO_4.5H_2O$). Emulsion C (water/oil) was formulated with 0.1%

$ZnSO_4$, 7% ZnO and 0.2% $CuSO_4$. Ointment D was formulated with 0.1% $ZnSO_4$, 10% ZnO and 0.35% $CuSO_4$. Ointment E was formulated with 10% ZnO and 0.2% $CuSO_4$.

Two series' of experiments were done using 2 different sources of intact human skin. In the first experiment, percutaneous absorption and cutaneous bioavailability of Zn and Cu from ZnPC and CuPC were compared with $ZnSO_4$ and $CuSO_4$, first using a similar (emulsion B) vehicle, and then in a vehicle of different composition (emulsion C). In the second experiment, percutaneous absorption and cutaneous bioavailability of Zn and Cu from ZnPC and CuPC were compared with $ZnSO_4$ (and ZnO) and $CuSO_4$ in ointments D and E.

Skin samples were sliced to a thickness of 400 µm and mounted in Franz-type static diffusion cells, which were then filled with 0.9% NaCl solution. The receptor fluid was magnetically stirred throughout the experiment and the temperature maintained at 33°C. 16 mg/cm^2 of each formulation was applied to the outer skin surface. The receptor solution was replaced after 2, 4, 12, 24, 48 and 72 hours and kept for analysis. Three cells were used for each formulation. Receptor fluids were subjected to flame AAS for Cu and Zn quantification.

After 72 hours, any remaining formulation on the skin surface was removed using cotton swabs. To ensure complete removal , the stratum corneum was stripped twice using adhesive tape. Samples of dermis and whole epidermis, were taken from treated skin by punch biopsy for assessment of Zn and Cu. A sample of whole skin was also taken. All samples were dried at 105°C, weighed, and dissolved in HNO_3. The mixtures were then passed through a 0.22 µm filter and diluted 10- or 20-fold before determination of Cu and Zn using AAS.

Absorbed amounts of zinc and copper through human skin were analysed using ANOVA. When treatment effects were significant, all possible pairwise comparisons were analysed with Fisher's protected least significant difference procedure. The chosen level of significance was $p < 0.05$. Mean Zn and Cu levels in treated skin were expressed as mg and µg of Zn and Cu respectively/gram dry tissue. Multiple comparisons between metal levels in treated skin to those in control skin were made using the Mann-Whitney test. The relative increase index (RII) was defined as the ratio between Zn or Cu concentration in treated skin to that in control skin (%), divided by the applied dose. RII values were compared using a Mann-Whitney test.

33.2 Results and discussion

Summarize relevant results; discuss dose-response relationship.

Percutaneous Cu absorption from topical application of 5 formulations represented less than 6% of the applied dose (range 0.66 – 5.04%), confirming minimal diffusion of Cu through dermatomed human skin. Cu diffusion from all formulations (except emulsion B) appeared to be higher for up to 24 hours, as long as Cu salts remained solubilised in the vehicle. After 72 hours, the % of applied Cu absorbed from CuPC in emulsion A was not significantly different from that of $CuSO_4$ in emulsion B. Furthermore, there was no difference between delivery of Cu from emulsion A and both ointments D and E. The results suggested that the delivery of Cu was not enhanced by replacing the sulphate counter-ion with the larger, more hydrophobic PC moiety.

All formulations in experiment 1 increased the level of Cu, on average 4-, 2- and 5-fold, in epidermis, dermis and whole skin respectively, as compared with control. In experiment 2, a significant increase in the Cu

level in whole skin and epidermis was shown by skin treated with emulsion A and ointment D. No corresponding increase in Cu level in dermis and in whole skin was shown from the 3 formulations or ointment E, respectively. It was considered that the increase in Cu concentration in whole skin may have been due to a build-up of Cu in epidermis, in effect forming a Cu reservoir, followed by slow diffusion through the dermis.

Multiple relative comparisons between treatments were made using the RII of Cu. In experiment 1, the RII of Cu in dermis and whole skin, both treated by emulsion C, was significantly higher than those calculated from emulsions A and B. RIIs of Cu in epidermis, dermis and whole skin treated by emulsions A and B were not significantly different, so that higher delivery of Cu from CuPC as compared to $CuSO_4$ could not be assumed. Furthermore, experiment 2 showed that RIIs of Cu in epidermis and dermis treated by the 3 formulations were not significantly different. A significant RII of Cu in whole skin treated by the emulsion A and the ointment D was shown as compared to skin treated by ointment E. However, RIIs of Cu in whole skin treated by emulsion A and ointment D were not significantly different. As shown in experiment 2, delivery of Cu in different layers of skin treated by CuPC in emulsion were not significantly higher than that from $CuSO_4$ in ointment.

33.3 Conclusion

Copper concentrations measured in receptor solution accounted for 0.66 – 5.04% of the applied dose, suggesting that percutaneous absorption was minimal across dermatomed skin. Conversely, topical application of formulations produced a significant increase of copper concentrations in epidermis and dermis, as compared to control data.

Bioequivalence of emulsions A and B was shown by means of the similar RII value for copper. However, no bioequivalence of copper from emulsions A and B was shown with ointments D and E. Consequently, it was not possible to demonstrate a better cutaneous bioavailability of copper from organic salts (CuPC), compared to mineral salts ($CuSO_4$).

33.3.1 Reliability

Based on the assessment of materials and methods include appropriate reliability indicator 0, 1, 2, 3, or 4

2

33.3.2 Deficiencies

Yes.

This study was not conducted and/or reported in strict compliance with the principles of GLP. However, this does not compromise the validity of the data generated, or the author's interpretation of that data, given that the study was not carried out for regulatory purposes. No internationally accepted guidelines are available that address the objective of the research presented in this summary.

The findings of this study are considered to make a valuable contribution to the 'weight of evidence' approach that has been adopted for the purposes of the current review of copper toxicokinetics. A reliability indicator of 2 has been assigned on this basis.

(If yes, discuss the impact of deficiencies and implications on results. If relevant, justify acceptability of study.)

Evaluation by Competent Authorities

Section A6.2	**Metabolism in mammals**
Annex Point IIA6.2	*Specify section no., heading and species as appropriate*
IUCLID 5.0/05	**A6.2(05), Absorption of copper**

	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	[redacted]
Materials and Methods	[redacted]
Results and discussion	[redacted]
Conclusion	[redacted]
Reliability	[redacted]
Acceptability	[redacted]
Remarks	[redacted]

	COMMENTS FROM ...
Date	*Give date of comments submitted*

Table A6.2(05)-1. Comparisons of percutaneous absorption of Cu2+ in skin treated with 3 different emulsions (experiment 1) and 2 ointments (experiment 2); each value is the mean ± SD of 3 measurements.

Formulations				Cu2+ absorbed (% of applied dose)					
	Salt	**Conc. %**	**Total Cu2+ applied dose µg/cm²**	**2h**	**6h**	**24h**	**48h**	**72h**	**Flux fore2 h ng/cm/h**
Experiment 1									
Em. A	CuPC	0.5	21 ±	0.54 ± 0.23	1.33 ± 0.53	1.67 ± 0.08[1]	1.89± 1.18	2.12± 1.57	6 ±
Em. B	$CuSO_4$	0.5	22 ±	0.52 ± 0.08	0.52 ± 0.08	0.52 ± 0.08	0.66± 1.17	0.66± 1.17	2 ±
Em. C	$CuSO_4$	0.2	12 ±	1.04 ± 0.08[2]	2.28 ± 0.54[3]	2.28 ± 0.54[4]	2.59± 1.01	2.59± 1.01	4 ±
Experiment 2									
Em. A	CuPC	0.5	19 ±	1.26 ± 0.52	1.67 ± 1.15	2.90 ± 1.05	3.60± 1.59	5.04± 2.86	13 ±
Em. D	$CuSO_4$	0.35	15 ±	0.96 ± 0.23	1.58 ± 0.29	2.26 ± 0.46	3.02± 0.76	3.77± 1.00	8 ±
Em. E	$CuSO_4$	0.2	12 ±	0.77 ± 0.16	1.54 ± 0.33	2.41 ± 0.48	3.39± 0.36	3.40± 0.35	6 ±

Fisher's least significance difference test was used in all comparisons.

[1] **$p<0.05$ as compared to B.**

[2] **$p<0.05$ as compared to A and B.**

[3] **$p<0.05$ as compared to A and B.**

[4] **$p<0.05$ as compared to B.**

Table A6.2 (05)-2. Cutaneous bioavailability of copper in treated skin with 3 different emulsions (experiment 1) and 2 ointments (experiment 2); each value is the mean ± SD of 3 measurements, in lg/g.

Formulations	Epidermis	Dermis	Whole skin
Experiment 1			
Em. A	473 ± 253*	4 ± 2 x 10 -1*	10 ± 7*
Em. B	540 ± 334*	6 ±	11 ± 6*
Em. C	600 ± 310*	12 ± 4*	21 ± 15
Control	140 ± 14	$3 \pm 3 \times 10^{-1}$	$3 \pm 3 \times 10^{-1}$
Experiment 2			
Em. A	920 ± 193*	10 ±	33 ± 3*
Oint. D	500 ± 62*	18 ±	25 ± 6*
Oint. E	760 ± 211*	16 ±	11 ± 4
Control	360 ± 60	12 ±	14 ± 1

* $p < 0.05$ as compared to control (Mann-Whitney test).

Figure A6.2(05)-1

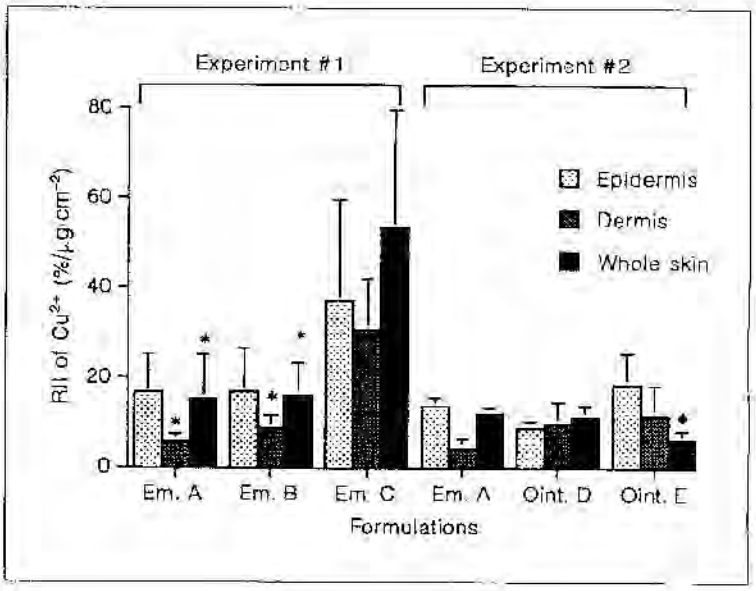


Fig. 3. Comparisons of RII values of copper from skin treated with 3 emulsions (experiment 1) and 2 ointments (experiment 2). Values are normalized by dividing copper level in control skin (percent of zinc increase) and then divided by applied dose of copper (µg·cm^{-2}). Each value is the mean ± SD of 3 determinations. * $p < 0.05$ as compared to emulsion C (Mann-Whitney test). ♦ $p < 0.05$ as compared to emulsion A and ointment D (Mann-Whitney test).

			Official use only
		34 REFERENCE	
1.1	**Reference**	*Author(s), year, title, laboratory name, laboratory report number, report date (if published, list journal name, volume: pages) If necessary, copy field and enter other reference(s).* Turnlund, J.R., Keyes, W.R., Anderson, H.L. and Acord, L.L. (1989). Copper absorption and retention in young men at three levels of dietary copper by use of the stable isotope ^{65}Cu. Am. J. Clin. Nutr. **49**:870-878 (published).	
1.2	**Data protection**	No *(indicate if data protection is claimed)*	
1.2.1	Data owner	*Give name of company* Public domain	
1.2.2			
1.2.3	Criteria for data protection	Choose one of the following criteria (see also TNsG on Product Evaluation) and delete the others: No data protection claimed	
		35 GUIDELINES AND QUALITY ASSURANCE	
35.1	**Guideline study**	No. This was a non-regulatory study carried out in human volunteers. The experimental protocol was reviewed and approved by the Committee for Protection of Human Subjects, University of California, Berkeley, and by the US Department of Agriculture Human Studies Committee. This study was conducted to establish the effect of the level of dietary copper on copper absorption, retention and endogenous faecal losses in young men at three widely different copper levels, with the copper content of the diet as the only variable. *(If yes, give guidelines; if no, give justification, e.g. "no guidelines available" or "methods used comparable to guidelines xy")*	
35.2	**GLP**	No. This was a non-regulatory study carried out in human volunteers. *(If no, give justification, e.g. state that GLP was not compulsory at the time the study was performed)*	
35.3	**Deviations**	No. Not applicable to non-guideline studies. *(If yes, describe deviations from test guidelines or refer to respective field numbers where these are described, e.g. "see 3.x.y")*	
		36 MATERIALS AND METHODS	
		In some fields the values indicated in the EC or OECD test guidelines are given as default values. Adopt, change or delete these default values as appropriate.	
36.1	**Test material**	Cupric oxide	
36.1.1	Lot/Batch number	Not available	
36.1.2	Specification	Deviating from specification given in section 2 as follows *(describe specification under separate subheadings, such as the following; additional subheadings may be appropriate):*	

Section A6.2		**Metabolism in mammals**
Annex Point IIA6.2		*Specify section no., heading and species as appropriate*
IUCLID: 5.0/06		**A6.2(06), Absorption of copper**
36.1.2.1 Description		*If appropriate, give e.g. colour, physical form (e.g. powder, grain size, particle size/distribution)* Acidified aqueous solution
36.1.2.2 Purity		*Give purity in % of active substance* [redacted]
36.1.2.3 Stability		*Describe stability of test material* Not available
36.1.2.4 Radiolabelling		*give structural location of radio labelling, give reason if not labelled* ^{65}Cu
36.2	**Test Animals**	Non-entry field
36.2.1	Species	Human volunteers
36.2.2	Strain	Not applicable
36.2.3	Source	Not applicable
36.2.4	Sex	Male
36.2.5	Age/weight/height at study initiation	*Young adults recommended* Age: 22 to 35 years. Weight: 57 to 93 kg. Height: 165 to 190 cm.
36.2.6	Number of volunteers per group	*Give number* *Specify, if there are differences for example for treatment and recovery groups* 11 (12 originally; one volunteer left the study).
36.2.7	Controls	No
36.3	**Administration/ Exposure**	*(fill in respective route in the following, delete other routes)* Oral administration of radiolabelled cupric oxide in the diet.
36.3.1	Duration of treatment	The total duration of treatment was 90 days. The study was divided into three metabolic periods (MP). Each volunteer received: 1) an adequate-copper diet (1.68 mg/day) for 24 days, followed by 2) a low-copper diet (0.79 mg/day) for 42 days, and then 3) a high-copper diet (7.53 mg/day) for 24 days.

X

36.3.2 Exposure scenario The diet was administered daily, 7 days a week. The diet used throughout the study contained low-copper food items, a liquid formula calorie supplement with added minerals and fiber, and a multivitamin tablet. The food and formula in the diet contained ~0.4 mg Cu before copper was added. A solution containing $CuSO_4$ was added to the liquid formula at each meal to achieve the desired copper content of the total diet.

Cu enriched in the stable isotope ^{65}Cu was fed to subjects for Cu-absorption determinations. The ^{65}Cu was derived from ^{65}CuO that had been dissolved in HCl and diluted with distilled, deionised water. This solution was substituted for the usual Cu solution added to the formula before each meal on days shown in **Table A6.2(06)-1**. ^{65}Cu was added on two consecutive days for the MP2 isotope feedings. This was done to obtain adequate isotopic enrichment of samples used for absorption determinations with minimum increase in the Cu content of the diet after addition of the isotope.

Polyethylene glycol (PEG), a faecal marker not absorbed by the body, was fed at each meal containing the Cu isotope.

36.4 Examinations Non-entry field

36.4.1 Body weight *yes/no (give time periods for determinations).*
Yes. Body weight was monitored over the course of the study.

36.4.2 Faeces collections *yes/no (give time periods for determinations).*
Yes. Complete faeces collections were made throughout the study. Faeces were collected for 3-day periods throughout the study and stored in a freezer. The 3-day collections were later thawed and combined into 6-day pools and homogenised with a blender. Composites were then frozen, lyophilised, crushed to a fine powder and stored in dessicators.

36.4.3 Urine collections *yes/no (give time periods for determinations).*
Yes. Complete urine collections were made throughout the study. The data obtained from these samples are reported in study summary **A6.2(01)**.

36.4.4 Blood collections *yes/no (give time periods for determinations).*
Yes. Blood samples were taken at weekly intervals throughout the study to monitor the health of the subjects and to evaluate Cu status (plasma copper, ceruloplasmin, and erythrocyte superoxide dismutase).

36.4.5 Saliva collections *yes/no (give time periods for determinations).*
Yes. Parotid saliva was collected after the noon meal for determination of copper concentration at the beginning of the study, at the end of each MP, and at the mid-point of MP 2. The data obtained from these samples are reported in study summary **A6.2(01)**.

36.4.6 Sweat collections *yes/no (give time periods for determinations).*
Yes. Sweat was collected for 3 day periods near the end of each MP. The data obtained from these samples are reported in study summary **A6.2(01)**.

36.4.7 Diet collections *yes/no (give time periods for determinations).*
Diets, combined into 1-day composites, were collected at intervals throughout the study and homogenised. Composites were then frozen, lyophilised, crushed to a fine powder and stored in dessicators.

3.5	**Sample processing and analysis**	Non-entry field.
3.5.1	Copper analysis	Cu content of the diet composites and 6-day faecal pools were determined by atomic absorption spectrophotometry. Two replicates of each of each faecal sample and four replicates of each diet composite were analysed. Cu balances were calculated by subtracting faecal Cu from dietary Cu. Following separation and purification of minerals in duplicate faecal samples and feeding solutions using anion-exchange chromatography, ^{65}Cu was determined with a computer-controlled, magnetic sector, thermal ionisation mass spectrometer. The total Cu content of the samples and the ^{65}Cu content were determined by isotope dilution by use of the 65-63 isotopic ratios of each faecal sample, an unenriched faecal sample, and the isotopic diluent; and the weight of the faecal subsample with added isotopic diluent, the weight of the isotopic diluent added, and the total dry weight of the faecal pool. The amount of ^{65}Cu absorbed was calculated by subtracting the amount of ^{65}Cu appearing in the faeces after that an isotope feeding from the amount of ^{65}Cu fed. Endogenous faecal Cu was calculated for the last 18 days of MP1 and MP3 and for the last 36 days of MP2. The first 6 days of the MPs were not included in these calculations because a substantial part of the Cu in the faeces would be from the previous diet. To calculate endogenous Cu, the unabsorbed dietary Cu (total dietary Cu multiplied by the unabsorbed fraction of ^{65}Cu) was subtracted from the total faecal Cu.
3.5.2	PEG analysis	All faecal samples were analysed for PEG by a turbidimetric method. The PEG levels in the samples of most subjects had returned to baseline levels 12 days after the feedings. Therefore samples from the 12th day after each feeding were combined for ^{65}Cu determinations. Intestinal transit was slower for subjects 2 and 3 and additional samples were analysed for them.
3.5.3	Statistical analysis	Statistical analysis was performed with the personal computer version of the Statistical Analysis System (SAS). Analysis of variance (ANOVA) was performed on absorption data comparing the four times absorption was measured and comparing subjects. ANOVA was not applied to combined faecal Cu or balance data because variances were larger for MP3 (high Cu diet) than for the other two MPs. Faecal Cu and Cu balances were plotted with SAS, with error bars representing ± 2 SEM. In addition, data for MP3 were plotted with linear prediction lines and 95% confidence bands because the increase in faecal Cu and decrease in balances were linear.

37 RESULTS AND DISCUSSION

Describe findings. If appropriate, include table. Sample tables are given below.

4.1	**Results**	Cu absorption data are shown in **Table A6.2(06)-2**. Absorption averaged 36.3% in MP1 from the adequate-Cu diet (1.68 mg/d); 56.2% early in MP2 and 55% late in MP2 from the low Cu diet (0.785 mg/d); and 12.4% during MP3 from the high-Cu diet (7.53 mg/d). Absorption differed significantly between MPs ($p<0.0001$) but did not differ significantly among subjects ($p<0.38$) or between the two feedings in MP2. The amount of Cu absorbed averaged 0.61 mg in MP1, 0.44 mg

early in MP2, 0.43 mg late in MP2, and 0.93 mg in MP3.

Average faecal Cu is shown in **Figure A6.2(06)-1**. Faecal Cu during the first 6 days of the study, containing some unabsorbed prestudy dietary Cu, was lower than later in the period. Faecal Cu during the first 6 days of MP2 and MP3 reflected the diet of the previous period, being higher than the rest of the period in MP2 and lower than the rest of the period in MP3. **Figure A6.2(06)-2** is a plot of individual faecal-Cu data in MP3. This indicates that faecal Cu increased linearly with time during MP3, when the diet contained the high level of Cu.

Cu retention for the entire study is depicted in **Figure A6.2(06)-3**. This shows that retention early in each MP reflects previous dietary Cu intake. Average retention during MP1 was most positive during the first 6 day period of the MP and was close to zero after that. Average balance was negative during the first 6 day period of MP2 and the beginning of the low-Cu MP and then it became slightly positive. Average retention was strongly positive during the first 6 day period of MP3. **Figure A6.2(06)-4** shows individual data points during MP3. Cu retention decreased linearly with time during MP3. Cu retention data by MP for individuals are shown in **Table A6.2(06)-3**. Individual retention data for the first 6 days and the following two 18 day periods of MP2 are shown in **Table A6.2(06)-4**. Endogenous faecal losses of Cu averaged 0.61 mg from day 7 to day 24 of the adequate Cu diet (MP1), 0.36 mg from day 7 to day 24 of the low Cu diet (MP2), 0.33 mg from day 25 to day 42 of MP2, and 0.97 mg from day 7 to day 24 of the high-Cu diet (MP3). Endogenous faecal Cu was highest with the high Cu diet, lowest with the low Cu diet, and appears to be related to the amount of Cu absorbed in these periods.

4.2 Discussion

Copper absorption: The Cu absorption data demonstrated that Cu absorption is dependent on dietary Cu intake. The percentage absorbed decreased as dietary Cu increased. The amount absorbed (mg) increased as dietary Cu increased but not in direct proportion to the amount fed. The amount of Cu in the high diet was nearly 10 times the amount in the low Cu diet but the amount absorbed only doubled.

^{65}Cu was fed early and late in the low-Cu period to determine whether absorption changed with time on a low-Cu diet. Absorption was 56.2% early and 55.0% late in MP2 and did not change significantly, suggesting relatively rapid adaptation to the Cu level of the diet. The data from this study suggest that absorption adapts more rapidly to a low Cu intake (protecting against depletion) than it does to a higher Cu intake (which would protect against accumulation of Cu).

Faecal losses: Faecal Cu decreased when dietary Cu decreased and increased when dietary Cu increased. This is due in part to the fact that faeces contain unabsorbed dietary Cu. Faecal Cu during the first 6 days after a dietary Cu change contained a combination of endogenous Cu, unabsorbed dietary Cu from the previous MP , and unabsorbed dietary Cu from the current MP. The proportion from the previous diet depends on the transit time of each individual and is variable. Faecal Cu increased linearly in MP3 throughout the period, suggesting that subjects had not yet adapted to the high dietary Cu.

Cooper retention: Cu balance during the first 6 days of the study was positive, with an average of 0.67 mg/d retained. This suggests that the prestudy diet of the subjects contained less than the 1.68 mg Cu in the diet during MP1. After retaining an average of 0.67 mg/day for the first

6 days, faecal Cu increased and the average for days 13 to 18 was slightly higher than intake. Some of the Cu eliminated during days 13 to 18 would be excess Cu retained earlier in the MP. Average retention for all of MP1 was 0.17 mg/d (**Table A6.2(06)-3**).

All subjects were in negative balance for the first 6 days of the low Cu diet (MP2), reflecting the higher Cu intake of MP1. Average balance became slightly positive for the remaining 36 days of MP2, as shown in **Figure A6.2(06)-3**.

When dietary Cu was increased to 7.5 mg/d in MP3, the men retained an average of 3.9 mg/d during the first 6 day period. Average retention decreased linearly during the remainder of the period, until it was negative during the final 6 days. Because the Cu in the faeces was more than was fed during this time, the negative balance must represent endogenous excretion of excess Cu retained early in the MP. The linear decline in balance would not continue indefinitely, but this 24 day period was not sufficiently long for the men to equilibrate to the high dietary intake. It is likely that negative balance would continue until most of the excess Cu retained previously was eliminated. A somewhat similar trend was observed with the adequate Cu diet, which probably contained more than the prestudy diet of most of the subjects. Balance was strongly positive during the first 6 day period and became slightly negative in at least one of the next three 6 day periods.

Cu retention was calculated using only faecal and dietary data. Urinary Cu and miscellaneous losses were not taken into account. If these were included, balance would be less positive.

Regulation of copper absorption and excretion: Based on the data from this study, especially during MP3, absorption is shown to be the first point of regulation of Cu metabolism and that this is followed by additional regulation through endogenous loss. Endogenous fecal Cu loss appears to play a role in elimination of excess Cu absorbed, suggesting that it is the second point of regulation, compensating for incomplete regulation at the level of absorption. This is demonstrated clearly in both MP3 and, to a lesser extent, in MP1. The data also suggest that adaptation to a lower Cu intake is more rapid than to a higher intake further from the physiological Cu requirement. In this study, Cu retention was more constant after the first 6 days of the low copper diet (MP2) than with the higher Cu diets, particularly the high Cu diet of MP3.

The results of this study indicate that absorption is the most important point of control when the Cu content of the diet is relatively low. The fraction absorbed increases markedly when intake is low and endogenous losses decline. When the diet is high in in Cu, reduced fractional absorption does not entirely prevent absorption of excess Cu and this excess is then eliminated by increased endogenous losses.

4.3 Toxic effects, clinical signs

No effects / describe significant effects referring to data in results table

No effects.

4.5 Recovery of labelled compound

state percentage

Not stated.

5 APPLICANT'S SUMMARY AND CONCLUSION

Section A6.2	**Metabolism in mammals**
Annex Point IIA6.2	*Specify section no., heading and species as appropriate*
IUCLID: 5.0/06	**A6.2(06), Absorption of copper**

5.1 Materials and methods *Give concise description of method; give test guidelines no. and discuss relevant deviations from test guidelines*

Eleven young men were confined to a metabolic research unit for 90 d to determine the effect of the level of dietary copper on absorption and retention. The study was divided into three metabolic periods (MP). Each volunteer received on a daily basis: 1) an adequate-copper diet (1.68 mg/day) for 24 days, followed by 2) a low-copper diet (0.79 mg/day) for 42 days, and then 3) a high-copper diet (7.53 mg/day) for 24 days. A solution containing $CuSO_4$ was added to each meal to achieve the desired copper content of the total diet.

^{65}Cu (as CuO) was fed to subjects in place of the usual $CuSO_4$ solution on one day in MP 1 and MP3 and on two days in MP2. Polyethylene glycol was fed as a faecal marker at each meal containing ^{65}Cu.

Dietary and faecal Cu was determined by atomic absorption spectrometry. ^{65}Cu was determined by mass spectrometry. The amount of ^{65}Cu absorbed was calculated by subtracting the amount of ^{65}Cu appearing in the faeces after an isotope feeding from the amount of ^{65}Cu fed. To calculate endogenous Cu, the unabsorbed dietary Cu (total dietary Cu multiplied by the unabsorbed fraction of ^{65}Cu) was subtracted from the total faecal Cu.

5.2 Results and discussion *Summarize relevant results; discuss dose-response relationship.*

Absorption and retention averaged 36.3 ± 1.3% and 0.17 mg/d, respectively, with an adequate-Cu diet (1.68 mg/d). Absorption averaged 55.6 ± 0.9% and retention averaged -0.316 mg/d for 6 d and 0.093 mg/d for the next 36 d of a low-Cu diet (0.785 mg/d). Absorption averaged 12.4 ± 0.9% with a high-Cu diet (7.53 mg/d) and retention was strongly positive at first, decreasing linearly with time. The study demonstrated that Cu absorption is dependent on dietary Cu level and that Cu balance can be achieved by most young men from a diet of 0.8 mg Cu/d. The apparent regulation of Cu absorption and endogenous losses would tend to protect humans from Cu deficiency and toxicity.

5.3 Conclusion

The amount of copper absorbed inceased as the amount in the diet increased, although absorption was more efficient and a higher proportion was absorbed when intake was low (up to 55.6% of the administered dose). Excretion of endogenous copper is also influenced by dietary intake; when copper intake is low, turnover is slow and little endogenous copper is excreted. As the amount in the diet increases, turnover and endogenous copper excretion increase.

5.3.1 Reliability

Based on the assessment of materials and methods include appropriate reliability indicator 0, 1, 2, 3, or 4

2

5.3.2 Deficiencies

Yes. This study was not conducted and/or reported in strict compliance with the principles of GLP. However, this does not compromise the validity of the data generated, or the author's interpretation of that data, given that the study was not carried out for regulatory purposes. Furthermore, the research was published in a peer-reviewed journal, and has therefore been subject to the prior scrutiny of experts in the field.

No internationally accepted guidelines are available that address the objective of the research presented in this summary.

(If yes, discuss the impact of deficiencies and implications on results. If relevant, justify acceptability of study.)

Evaluation by Competent Authorities

Use separate "evaluation boxes" to provide transparency as to the comments and views submitted

EVALUATION BY RAPPORTEUR MEMBER STATE

Date [redacted]

Materials and Methods [redacted]

[redacted]:

[redacted]

Results and discussion [redacted]

Conclusion [redacted]

Reliability [redacted]

Acceptability [redacted]

Remarks

COMMENTS FROM ...

Date *Give date of comments submitted*

Table A6.2(06)-1

Experimental Design

	Metabolic Period		
	MP1	MP2	MP3
Duration (d)	24	42	24
^{65}Cu fed (day of MP)	13	7 an 8 31 and 32	13
Dietary Cu (mg/d)			
Calculated	2.0	0.8	8.0
Measured	1.68	0.785	7.53
On ^{65}Cu days	1.99	0.900	7.78

Table A6.2(06)-2

^{65}Cu absorption at three levels of dietary Cu*

Subject	MP1	MP2 (early)	MP2 (late)	MP3
		%		
2	42.1	50.6	49.9	12.6
3	34.2	57.5	57.5	10.4
4	40.1	54.3	59.7	7.4
5	29.5	61.0	55.1	14.0
6	34.8	57.7	48.3	11.1
7	34.8	61.3	60.0	14.7
8	38.1	57.0	46.0	16.4
9	33.5	54.4	58.6	7.6
10	40.5	59.3	60.3	12.2
11	30.2	48.9	51.5	14.2
12	41.4	56.6	57.8	16.3
x ± SEM	36.3 ±1.3	56.2 ±1.1	55.0 ±1.5	12.4 ±0.9

* Values for amount absorbed (x ± SEM) were 0.610 ± 0.022, 0.441 ± 0.009, 0.432 ± 0.012, and 0.933 ± 0.068 mg for MP1, MP2 (early), MP2 (late) and MP3, respectively.

Table A6.2(06)-3

Cu retention at three levels of dietary Cu

Subject	MP1	MP2	MP3
		mg/d	
2	0.308	-0.255	1.07
3	0.049	0.106	1.00
4	0.286	-0.028	0.167
5	0.143	0.070	1.97
6	0.039	0.125	1.29
7	0.026	0.044	1.10
8	0.288	0.087	0.988
9	0.249	-0.026	0.762
10	0.322	-0.143	0.547
11	-0.046	0.023	0.128
12	0.169	0.014	1.33
x ± SEM	0.167 ± 0.40	0.002 ± 0.034	0.941 ± 0.160

Table A6.2(06)-4

Cu retention during low-Cu diet (MP2)

	Days of MP2		
Subject	1-6	7-24	25-42
		mg/d	
2	-1.094	-0.116	-0.115
3	-0.118	0.234	0.054
4	-0.673	0.074	0.085
5	-0.815	0.197	0.238
6	-0.047	0.126	0.183
7	-0.292	0.100	0.100
8	0.197	0.151	0.133
9	0.833	0.104	0.112
10	-1.273	-0.029	0.121
11	-0.255	0.022	0.116
12	-0.316	0.013	0.126
x ± SEM	-0.538 ± 0.126	0.080 ± 0.031	0.105 ± 0.026

Table A6.2(06)-5

^{65}Cu absorption at different levels of Cu intake measured in this and earlier studies.

Reference	Number of subjects	Measurements per subject	Cu intake		Absorption
		n	mg/d	%	mg
*	11	2	0.785	55.6	0.44
11	6	3	1.35	41.3	0.56
6	5	1	1.44	41.2	0.59
*	11	1	1.68	36.1	0.61
10	6	4	2.1	34.4	0.72
9	4	3	2.32	33.5	0.78
6	5	1	2.53	33.8	0.86
8	6	2	2.70	27.5	0.74
7	8	1-2	3.26	25.8	0.84
*	11	1	7.53	12.4	0.93

* This study

Figure A6.2(06)-1

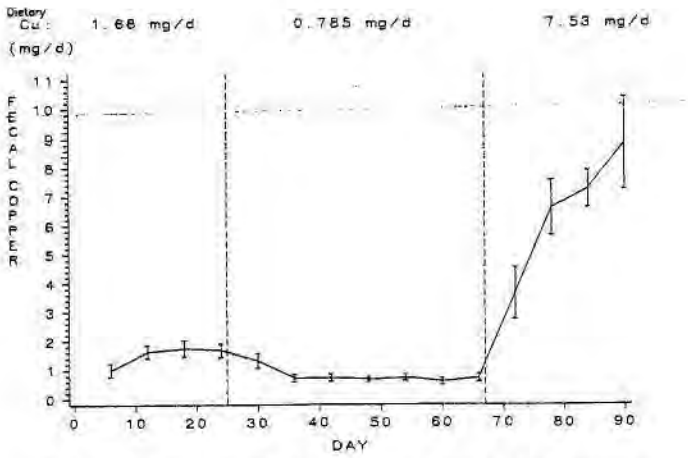


FIG 1. Mean fecal Cu (mg/d) throughout the study. Error bars represent ±2 SEM. Vertical lines show the beginning of each MP.

Figure A6.2(06)-2

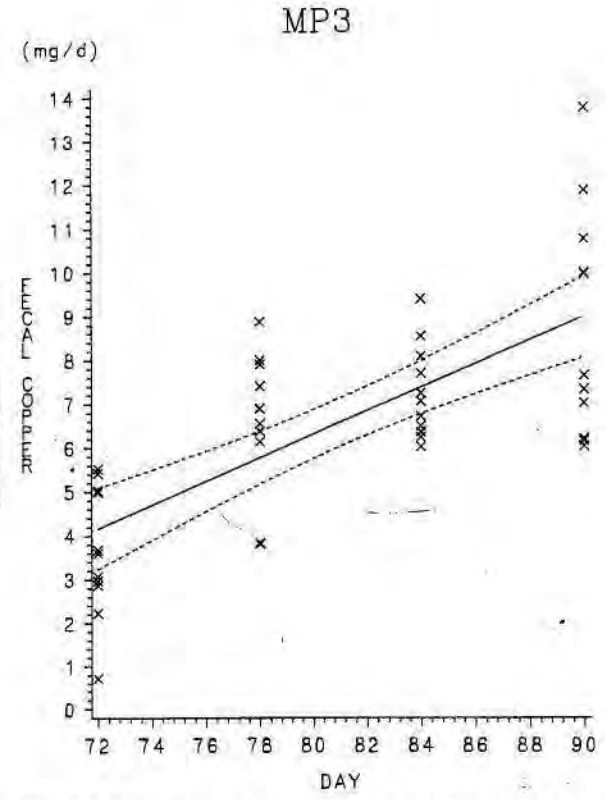


FIG 2. Individual fecal Cu (mg/d) for high-Cu diet (MP3), with linear prediction line and 95% confidence band.

Figure A6.2(06)-3

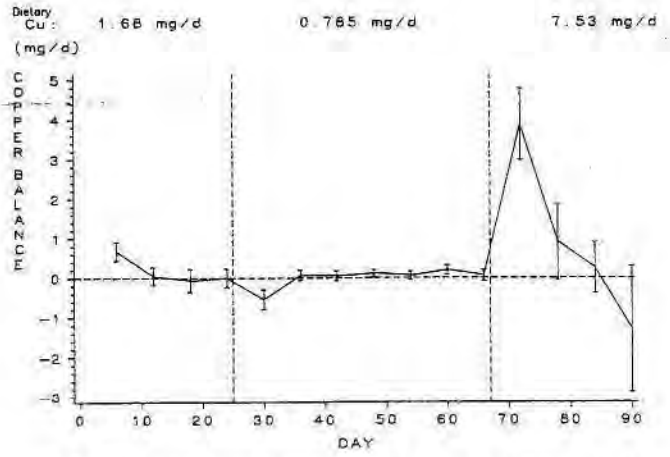


FIG 3. Cu balance (mg/d) throughout the study. Error bars represent ±2 SEM. Vertical lines show the beginning of each MP.

Figure A6.2(06)-4

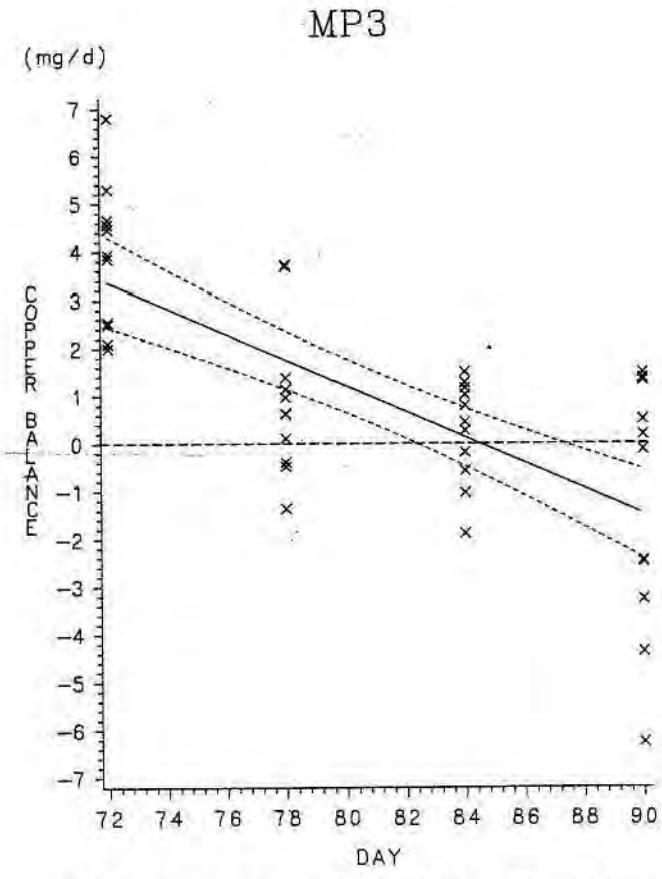


FIG 4. Individual Cu balances for high-Cu diet (MP3), with linear prediction line and 95% confidence band.

			Official use only
		38 REFERENCE	
1.1	**Reference**	*Author(s), year, title, laboratory name, laboratory report number, report date (if published, list journal name, volume: pages)* *If necessary, copy field and enter other reference(s).* Turnlund, J.R., Wada, L., King, J.C., Keyes, W.R. and Lorra, L.A. (1988). Copper Absorption in Young Men Fed Adequate and Low Zinc Diets. Biological and Trace Element Research, **17:** 31 - 41 (published).	X
1.2	**Data protection**	No *(indicate if data protection is claimed)*	
1.2.1	Data owner	*Give name of company* Public domain	
1.2.2			
1.2.3	Criteria for data protection	Choose one of the following criteria (see also TNsG on Product Evaluation) and delete the others: No data protection claimed	
		39 GUIDELINES AND QUALITY ASSURANCE	
39.1	**Guideline study**	No. This was a non-regulatory study carried out in human volunteers to investigate the effect of Zn intake on Cu status. *(If yes, give guidelines; if no, give justification, e.g. "nos guidelines available" or "methods used comparable to guidelines xy")*	
39.2	**GLP**	No. This was a non-regulatory study carried out in human volunteers. *(If no, give justification, e.g. state that GLP was not compulsory at the time the study was performed)*	
39.3	**Deviations**	Yes. Refer to section 5.3.2 for a general discussion of deviations and deficiencies. *(If yes, describe deviations from test guidelines or refer to respective field numbers where these are described, e.g. "see 3.x.y")*	
		40 MATERIALS AND METHODS	
		In some fields the values indicated in the EC or OECD test guidelines are given as default values. Adopt, change or delete these default values as appropriate.	
40.1	**Test material**	Cu^{2+} as copper sulphate. ^{65}Cu.	
40.1.1	Lot/Batch number	Not available	
40.1.2	Specification	Deviating from specification given in section 2 as follows *(describe specification under separate subheadings, such as the following; additional subheadings may be appropriate):*	

Section A6.2	**Metabolism in mammals**
Annex Point IIA6.2	*Specify section no., heading and species as appropriate*
IUCLID: 5.0/07	**A6.2(07), Homeostasis of copper**

40.1.2.1 Description	*If appropriate, give e.g. colour, physical form (e.g. powder, grain size, particle size/distribution)* Aqueous solution	
40.1.2.2 Purity	*Give purity in % of active substance* [redacted]	X
40.1.2.3 Stability	*Describe stability of test material* Not available	
40.1.2.4 Radiolabelling	*give structural location of radio labelling, give reason if not labelled* ^{65}Cu	
40.2 Test Animals	Non-entry field	
40.2.1 Species	Human volunteers	
40.2.2 Strain	Not applicable	
40.2.3 Source	Not applicable	
40.2.4 Sex	Male	
40.2.5 Age/weight/height at study initiation	*Young adults recommended* Age: 21 to 33 years. Weight: average weight 65.5 kg. Height: average height 175 cm.	
40.2.6 Number of volunteers per group	*Give number* *Specify, if there are differences for example for treatment and recovery groups* 6 volunteers were involved in this study.	
40.2.7 Controls	No.	
40.3 Administration/ Exposure	*(fill in respective route in the following, delete other routes)* Oral administration of copper sulphate and ^{65}Cu in the diet.	
40.3.1 Duration of treatment	The total duration of treatment was 75 days.	
40.3.2 Experimental design	The experimental design is summarised in **Table A6.2(07)-1**. The study was divided into three metabolic periods (MP). Each volunteer received: Diet A containing 16.5 mg Zn and 1.36 mg copper for the 12 days of MP1 and the 9 days of MP3; Diet B containing 5.5 mg Zn and 1.34 mg copper for the 54 days of MP2. The Zn:Cu ratios of the adequate and low Zn diets were 12:1 and 4:1, respectively.	
40.3.3 Experimental procedures	The Zn content of the diet was changed by adding 15 g of oysters to diet A. During MP2 (diet B), 0.26 mg of Cu as $CuSO_4$ was added to equal the Cu content of the oysters in diet A. Cu absorption was determined by adding a solution containing 1 mg of ^{65}Cu to the diet on day 10 of MP1 and days 13 and 46 of MP2 (this constituted diet C). The isotope solution was divided into 3 equal portions and added to each meal of the day. Stable isotopes of Zn were also fed on the same days and diet C was modified to contain 2.3 mg of Cu and 15 mg or 5.5 mg of Zn, including the stable isotopes. A solution containing polyethylene glycol (PEG), a faecal marker that is not absorbed, was fed with the isotopes to ensure	

	complete faecal collections and to ensure that isotope measurements included all samples containing unabsorbed isotopes. One gram of PEG was added to each meal on the day of the isotope feeding. Complete faecal collections for 12 to 15 days following each isotope feeding were used for Zn and Cu absorption determinations. Complete faecal collections for the first and last 9 days of MP2 and the 9 days of MP3 were used for Cu balance determinations. Faecal collections from days 10-12 of MP1 and days 10-18 and 19-29 of MP2 were also analysed for additional information on faecal Cu elimination. The Cu content of the diet and faeces were determined by flame atomic absorption spectrophotometry (AAS). Four replicates of 4 composites of each diet were analysed. Faecal samples were analysed in duplicate. To prepare lyophilised samples for Cu analysis, organic material was destroyed by heating samples at 450°C for 24 hours. A few drops of nitric acid were added to the samples, which were dried and returned to the furnace for 5 hours. The minerals remaining in the samples were dissolved in 1.0 N nitric acid and analysed by AAS. A bovine liver reference standard was run each day with the samples. A reference faecal composite was weighed each time samples were weighed and was prepared and analysed with the samples. A Cu standard was added at four levels to a faecal composite and recoveries were calculated. Recovery of standard additions for the faecal composite averaged 101.4% with a CV of 0.63%. Since the balance periods were short, irregular faecal flow could distort the Cu excretion data. Therefore, corrections were made for faecal flow. Average dry weight of all faecal collections listed above was calculated for each subject. The average dry weight for each subject was used to normalise the Cu content of each 3-day collection by multiplying the Cu content by the ratio of the average 3-day dry weight to the dry weight of the three-day sample. PEG was determined in faecal samples using a turbidometric method. Three day faecal pools were combined into larger composites, homogenised and lyophilised. A dilute HCl solution containing ^{65}Cu was weighed accurately into one replicate of each sample. Samples were dried slowly on a hotplate and ashed in a muffle furnace. Cu was separated from the samples and purified by ion exchange chromatography. The ^{65}Cu:^{63}Cu ratios were determined in both replicates by magnetic sector, thermal ionisation mass spectrometry. The total Cu content of the faecal sample and the amount of ^{65}Cu from the isotope feeding which appeared in the faeces were calculated using the ^{65}Cu:^{63}Cu ratios of an unenriched faecal sample, the isotopic diluent (enriched ^{65}Cu), and the two replicates of the faecal sample. The stable isotope was not fed during MP3, but Cu balance data were only available for 3 days of MP1. Therefore, absorption values for MP1 were applied to faecal Cu data from the 9 days of MP3, when dietary Zn was also 16.5 mg/d, to estimate endogenous Cu losses during MP3.
40.3.4 Statistical analysis	Statistical analysis was performed using the Statistical Analytical System (SAS). Cu absorption, faecal Cu and Cu balance were compared between subjects and treatments using analysis of variance (ANOVA). Data were tested for statistical significance at the 5% significance level ($P < 0.05$).

41 RESULTS AND DISCUSSION

Describe findings. If appropriate, include table. Sample tables are given below.

41.1 Results

The results of PEG determinations are shown in **Figure A6.2(07)-1**. This figure demonstrates that transit time was variable in and between subjects.

Faecal Cu content and 9-day Cu balances at the beginning and end of MP2 (days 1-9 and 45-54), and days 1-9 of MP3, are shown in **Table A6.2(07)-2**. In addition to the data shown in **Table A6.2(07)-2**, faecal Cu was determined for the second and third 9-day periods of MP2 and for the last three days of MP1. Averages for the first three 9-day periods of MP2 were 1.10, 1.06, and 1.11 mg/day, and were not statistically different. Faecal Cu for the 3 days of MP1 averaged 1.08 mg/day, which was similar to the other collection periods.

Neither faecal Cu nor Cu balance differed significantly between the 4 balance periods or among subjects. Cu balance was positive for all subjects during all balance periods.

Calculated endogenous Cu losses were similar during the different periods, and averaged 0.25 mg/day early in MP2, 0.24 mg/day late in MP2, and an estimated 0.25 mg/day in MP3.

Cu absorption, shown in **Table A6.2(07)-3**, was significantly higher for MP1, when the diet contained 16.5 mg of Zn, than for early or late in MP2, when the diet contained 5.5 mg of Zn. Cu absorption also differed among subjects. Subject 6 absorbed significantly less Cu than 4 other subjects and subject 2 absorbed significantly more than 2 others. Cu absorption was not correlated with Zn absorption.

41.2 Discussion

The variability of transit time shown in **Figure A6.2(07)-1** demonstrates the need for use of a faecal marker to assure complete collection of unabsorbed isotopes. Failure to make complete collections would result in over-estimation of absorption. Five of the men eliminated all detectable PEG in 6-12 days, and from 3-9 stools. One man excreted PEG for 18 days in up to 15 stools.

Higher Cu excretion was not observed during the first 9-day period following the end of the 16.5 mg Zn diet, nor did faecal Cu decline following the first 9-day period of the low Zn diet. Faecal Cu did not differ significantly between early and late in MP2. Faecal Cu for the final 3 days of MP1 demonstrated that faecal Cu did not appear to be different during MP1 than for later balance periods continued for longer periods of time. Correcting the 3-day faecal Cu values for MP1 for faecal flow showed that a Zn intake of 15 mg/day did not impair Cu retention.

Cu retention was positive in all subjects during all balance periods.

Cu absorption was significantly higher in MP1 when the diet contained 16.5 mg Zn than early or late in MP2 when the diet contained 5.5 mg Zn. However, it was considered unlikely that the Zn intake used in this study was responsible for enhanced Cu absorption. If the men had consumed diets lower in Cu prior to the study, it is possible that the increase in absorption resulted from lower Cu intake prior to participating in the study.

Apparent absorption calculations, based on the difference between dietary and faecal Zn, appear to support enhanced Cu absorption with higher Zn intake. Apparent absorption calculations have traditionally been used to estimate absorption when isotopic labels have not been used. Faecal Cu

during MP3, which was not significantly lower than earlier periods, averaged 0.98 mg, compared to averages of 1.10 – 1.06 mg in early and late in MP2. Apparent absorption calculations include all endogenous faecal Cu excretion with unabsorbed dietary Cu, so apparent absorption is always less than absorption calculated with isotopic labels. Apparent absorption was 27.9% for MP3, compared to 18.6 and 20.8% early and late in MP2.

True Cu absorption would be slightly higher than absorption measured with the stable isotope. The isotopic feeding passes through the stomach and small intestine, where it is absorbed, relatively quickly. All or nearly all of the Cu absorption takes place within hours of feeding. However, The PEG data demonstrate that unabsorbed dietary components remain in the large intesting much longer and are eliminated over a variable number of days. During this period, some of the isotopic Cu that has been absorbed is excreted into the gastrointestinal tract and eliminated with the unabsorbed dietary copper. The extent of this excretion has been estimated from faecal collections following injections of a radioisotope of Cu (Cartwright, G.E. and Wintrobe, M.W., (1964). Am. J. Clin. Nutr. 14, 224). Excretion of the radioisotope, until it was not detectable, averaged 12%. Applying this value to the present study, true absorption, corrected for estimated excretion of 12% of the absorbed isotope into the gastrointestinal tract, would average 54.7% for MP1, 42.3% for MP2 (day 13) and 43.8% for MP2 (day 46).

41.3 Toxic effects, clinical signs

No effects / describe significant effects referring to data in results table No effects.

41.4 Recovery of labelled compound

State percentage

Not stated.

42 APPLICANT'S SUMMARY AND CONCLUSION

42.1 Materials and methods

Give concise description of method; give test guidelines no. and discuss relevant deviations from test guidelines

A study was carried out in humans to investigate the effect of Zn intake on Cu status. Six men were confined to a metabolic unit for 75 days. The study was divided into three metabolic periods (MP). Each volunteer received the following diets: Diet A containing 16.5 mg Zn and 1.36 mg Cu for the 12 days of MP1 and the 9 days of MP3; Diet B containing 5.5 mg Zn and 1.34 mg Cu for the 54 days of MP2. The Zn:Cu ratios of the adequate and low Zn diets were 12:1 and 4:1, respectively.

The Zn content of the diet was changed by adding 15 g of oysters to diet A. During MP2 (diet B), 0.26 mg of Cu as $CuSO_4$ was added to equal the Cu content in diet A. Cu absorption was determined by adding a solution containing 1 mg of ^{65}Cu to the diet on day 10 of MP1 and days 13 and 46 of MP2 (this constituted diet C). Stable Zn isotopes were fed on the same days. Diet C contained 2.3 mg of Cu and 15 mg or 5.5 mg of Zn, including the stable isotopes. A solution containing 1 gram of PEG as a faecal marker was fed with the isotopes to ensure complete faecal collections and that isotope measurements included all samples containing unabsorbed isotopes.

Complete faecal collections for 12 to 15 days following each isotope feeding were used for Zn and Cu absorption determinations. Complete faecal collections for the first and last 9 days of MP2 and the 9 days of

MP3 were used for Cu balance determinations. Faecal collections from days 10-12 of MP1 and days 10-18 and 19-29 of MP2 were analysed for additional information on faecal Cu elimination.

The Cu content of the diet and faeces were acid digested and determined by flame AAS. Four replicates of 4 composites of each diet were analysed. Faecal samples were analysed in duplicate. A bovine liver reference standard was run each day with the samples. A reference faecal composite was weighed each time samples were weighed and was prepared and analysed with the samples. A Cu standard was added at four levels to a faecal composite and recoveries were calculated. (recovery averaged 101.4% with a CV of 0.63%).

Since the balance periods were short, corrections were made for faecal flow. Average dry weight of all faecal collections was calculated for each subject. The average dry weight was used to normalise the Cu content of each 3-day collection by multiplying the Cu content by the ratio of the average 3-day dry weight to the dry weight of the three-day sample.

PEG was determined in faecal samples using a turbidometric method. Three day faecal pools were combined into larger composites. A dilute HCl solution containing ^{65}Cu was weighed into one replicate of each sample. Samples were ashed and Cu was separated and purified by ion exchange chromatography. The ^{65}Cu:^{63}Cu ratios were determined in both replicates by magnetic sector, thermal ionisation mass spectrometry.

The total Cu content of the faecal sample and the amount of ^{65}Cu from the isotope feeding which appeared in the faeces were calculated using the ^{65}Cu:^{63}Cu ratios of an unenriched faecal sample, the isotopic diluent (enriched ^{65}Cu), and the two replicates of the faecal sample.

The stable isotope was not fed during MP3, but Cu balance data were only available for 3 days of MP1. Therefore, absorption values for MP1 were applied to faecal Cu data from the 9 days of MP3, when dietary Zn was also 16.5 mg/d, to estimate endogenous Cu losses during MP3.

Cu absorption, faecal Cu and Cu balance were compared between subjects and treatments using ANOVA. Data were tested for statistical significance at the 5% significance level ($P < 0.05$).

42.2 Results and discussion

Summarize relevant results; discuss dose-response relationship.

PEG determinations showed that transit time was variable in and between subjects. Five of the men eliminated all detectable PEG in 6-12 days, and from 3-9 stools. One man excreted PEG for 18 days in up to 15 stools.

Faecal Cu content at the beginning and end of MP2 (days 1-9 and 45-54), and days 1-9 of MP3 were 1.10, 1.06 and 0.98 mg/day, respectively. Nine-day Cu balances at the beginning and end of MP2 (days 1-9 and 45- 54), and days 1-9 of MP3 were 0.24, 0.28 and 0.38 mg/day, respectively. In addition, faecal Cu was determined for the second and third 9-day periods of MP2 and for the last three days of MP1. Averages for the first three 9-day periods of MP2 were 1.10, 1.06, and 1.11 mg/day, and were not statistically different. Faecal Cu for the 3 days of MP1 averaged 1.08 mg/day, which was similar to the other collection periods. Correcting the 3-day faecal Cu values for MP1 for faecal flow showed that a Zn intake of 15 mg/day did not impair Cu retention. Neither faecal Cu nor Cu balance differed significantly between the 4 balance periods or among subjects. Cu balance was positive for all subjects during all balance periods.

Calculated endogenous Cu losses were similar during the different periods, and averaged 0.25 mg/day early in MP2, 0.24 mg/day late in

	MP2, and an estimated 0.25 mg/day in MP3. Apparent Cu absorption was significantly higher for MP1, when the diet contained 16.5 mg of Zn, than for early or late in MP2, when the diet contained 5.5 mg of Zn (48.1, 37.2 and 38.5%, respectively). True absorption, corrected for estimated excretion of 12% of the absorbed isotope into the gastrointestinal tract, averaged 54.7% for MP1, 42.3% for early MP2 and 43.8% for late MP2. Cu absorption also differed among subjects. Subject 6 absorbed significantly less Cu than 4 other subjects and subject 2 absorbed significantly more than 2 others. Cu absorption was not correlated with Zn absorption.
42.3 Conclusion	Apparent Cu absorption in those fed the diet containing 16.5 mg Zn was higher than in those receiving the diet containing 5.5. mg Zn. Absorption also differed significantly amongst subjects. Faecal Cu did not differ between diets or among subjects. All subjects were in positive Cu balance at both levels of dietary zinc. These results suggested that a dietary Zn intake of 15 mg/day does not increase faecal Cu loss and does not interfere with Cu absorption.
42.3.1 Reliability	*Based on the assessment of materials and methods include appropriate reliability indicator 0, 1, 2, 3, or 4* 2
42.3.2 Deficiencies	Yes This study was not conducted and/or reported in strict compliance with the principles of GLP. However, this does not necessarily compromise the validity of the data generated, or the author's interpretation of that data, given that the study was not carried out for regulatory purposes. Furthermore, the research was published in a peer-reviewed journal, and has therefore been subject to the prior scrutiny of experts in the field. In addition this report has been included in a number of expert reviews of Cu toxicokinetics. No internationally accepted guidelines are available that specifically address the objective of the research presented in this summary. Overall, this is a well-reported study, and its findings are considered to make a valuable contribution to the 'weight of evidence' approach that has been adopted for the purposes of the current review of Cu toxicokinetics. A reliability indicator of 2 has been assigned on this basis. *(If yes, discuss the impact of deficiencies and implications on results. If relevant, justify acceptability of study.)*

Evaluation by Competent Authorities

Section A6.2	**Metabolism in mammals**
Annex Point IIA6.2	*Specify section no., heading and species as appropriate*
IUCLID: 5.0/07	**A6.2(07), Homeostasis of copper**

	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	[redacted]
Reference	[redacted]
Results and discussion	[redacted]
Conclusion	[redacted]
Reliability	[redacted]
Acceptability	[redacted]
Remarks	[redacted]

	COMMENTS FROM ...
Date	*Give date of comments submitted*

Table A6.2(07)-1. Experimental Design

Study period	Duration (days)	Diet	Zinc intake (mg/d)	Copper intake (mg/d)
MP1	12	A	16.5	1.36
MP2	54	B	5.5	1.34
MP3	9	A	16.5	1.36
Isotope feeding days				
MP1 (day 10)		C	15.0	2.31
MP2 (days 13 and 46)		C	5.5	2.31

Table A6.2(07)-2. Faecal Copper and Copper Balance During adequate and Low Zinc diets

	Subjects						
	1	2	3	4	5	6	Mean
Faecal copper[a]	mg/d						
MP2 (day 1 – 9)	1.16	1.31	0.90	0.97	1.09	1.14	1.10
MP2 (day 46 – 54)	1.07	1.08	1.02	1.13	0.96	1.12	1.06
MP3 (day 1 – 9)	[b]	--1.01	1.00	0.95	0.85	1.04	0.98
Copper balance[c]							
MP2 (day 1 – 9)	0.18	0.03	0.44	0.37	0.25	0.20	0.24
MP2 (day 46 – 54)	0.27	0.26	0.32	0.21	0.38	0.22	0.28
MP3	[b]	--0.35	0.36	0.43	0.53	0.32	0.38

[a]adjusted for faecal dry weight.
[b]subject left the study during MP3
[c]$S_{\bar{x}}$ 0.036 for faecal copper and copper balance (MP2), 0.041 (MP3).

Table A6.2(07)-3. Copper absorption (% copper absorbed)

	Subjects						
	1	2	3	4	5	6	Mean
MP1 (day 10)	52.3	57.5	50.6	47.6	41.8	38.7	48.1
MP2 (day 13)	37.1	42.1	41.8	31.2	42.8	28.1	37.2
MP2 (day 46)	40.0	41.4	38.3	37.2	41.3	32.6	38.5
mean	43.1[xy]	47.0[x]	43.6[xy]	38.7[yz]	42.0[xy]	33.1[z]	

$S_{\bar{x}}$ (subject) 2.20

$S_{\bar{x}}$ (MP) 1.56

Means with the same superscript are not significantly different ($p < 0.05$)

Figure A6.2(07)-1

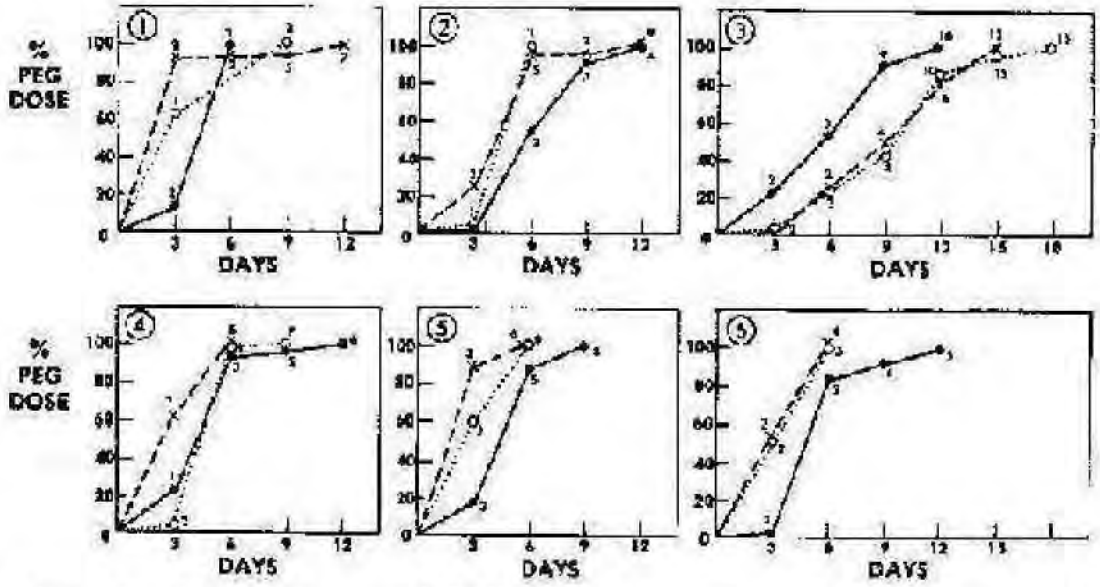


Fig. 1. Cumulative fecal PEG elimination in young men. Numerals on lines = cumulative number of stools: _____ fed MP 1, d 10; . . . . . fed MP 2, d 13; and ------ fed MP 2, d 46.

			Official use only
		43 REFERENCE	
1.1	**Reference**	*Author(s), year, title, laboratory name, laboratory report number, report date (if published, list journal name, volume: pages)* *If necessary, copy field and enter other reference(s).* Turnlund, J.R., Ketes, W.R., Peiffer, G.L. and Scott, K.C. (1998). Copper absorption, excretion and retention by young men consuming low dietary copper determined using the stable isotope ^{65}Cu. Am. J. Clin. Nutr., **67:** 1219 – 1225 (published).	X
1.2	**Data protection**	No *(indicate if data protection is claimed)*	
1.2.1	Data owner	*Give name of company* Public domain	
1.2.3	Criteria for data protection	Choose one of the following criteria (see also TNsG on Product Evaluation) and delete the others: No data protection claimed	
		44 GUIDELINES AND QUALITY ASSURANCE	
44.1	**Guideline study**	No. This was a non-regulatory study carried out in human volunteers to determine the effects of a diet containing inadequate Cu (i.e. an amount at which Cu status could not be maintained) on Cu absorption, excretion and retention. The study protocol was reviewed and approved by the Bionetics Institutional Review Board and by the US Department of Agriculture Human Studies Review Committee. *(If yes, give guidelines; if no, give justification, e.g. "nos guidelines available" or "methods used comparable to guidelines xy")*	
44.2	**GLP**	No. This was a non-regulatory study carried out in human volunteers. *(If no, give justification, e.g. state that GLP was not compulsory at the time the study was performed)*	
44.3	**Deviations**	Yes. Refer to section 5.3.2 for a general discussion of deviations and deficiencies. *(If yes, describe deviations from test guidelines or refer to respective field numbers where these are described, e.g. "see 3.x.y")*	
		45 MATERIALS AND METHODS	
		In some fields the values indicated in the EC or OECD test guidelines are given as default values. Adopt, change or delete these default values as appropriate.	
45.1	**Test material**	Cu^{2+} as copper sulphate. ^{65}Cu as cupric oxide.	
45.1.1	Lot/Batch number	Not available	
45.1.2	Specification	Deviating from specification given in section 2 as follows *(describe specification under separate subheadings, such as the following; additional subheadings may be appropriate):*	

45.1.2.1 Description	*If appropriate, give e.g. colour, physical form (e.g. powder, grain size, particle size/distribution)* Aqueous solution
45.1.2.2 Purity	*Give purity in % of active substance* [redacted] X
45.1.2.3 Stability	*Describe stability of test material* Not available
45.1.2.4 Radiolabelling	*give structural location of radio labelling, give reason if not labelled* ^{65}Cu
45.2 Test Animals	Non-entry field
45.2.1 Species	Human volunteers
45.2.2 Strain	Not applicable
45.2.3 Source	Not applicable
45.2.4 Sex	Male
45.2.5 Age/weight/ height	*Young adults recommended* Mean age (± SD): 26 ± 4 years. Mean weight (± SD): 74.3 ± 8.2 kg at the beginning of the study and 74.1 ± 7.9 kg at the end of the study. Mean height (± SD): 181 ± 6 cm.
45.2.6 Number of volunteers per group	*Give number* *Specify, if there are differences for example for treatment and recovery groups* 11 volunteers were involved in this study.
45.2.7 Controls	No.
45.3 Administration/ Exposure	*(fill in respective route in the following, delete other routes)* Oral administration of copper sulphate and ^{65}Cu in the diet. Intravenous administration of ^{65}Cu.
45.3.1 Duration of treatment	The total duration of treatment was 90 days.
45.3.2 Exposure scenario	The experimental design is summarised in **Table A6.2(08)-1**. The study was divided into 3 metabolic periods (MP). The Cu content of the diet was 0.66 mg/day for 24 days (MP1), followed by 0.38 mg/day for 42 days (MP2) and 2.49 mg/day for 24 days (MP3). The higher Cu contents in diets MP1 and MP3 were achieved by adding a Cu solution to the drink that supplemented each meal. The stable isotope ^{65}Cu was administered orally to 5 of the subjects once during MP1 (days 13 – 14) and MP3 (days 79 – 80), and twice during MP2 (days 31 – 32 and 55 – 56). It was administered intravenously to 6 subjects once during each MP (days 13, 55 and 79 for MP1, MP2 and MP3, respectively).
45.3.3 Isotope preparation and	Cupric oxide containing the stable isotope was refluxed with ultrapure nitric acid and diluted with deionised water to the desired concentrations

administration	for the preparation of standard, oral, intravenous and isotopic diluent solutions. The exact concentrations of these solutions were determined by isotope dilution. The oral solutions contained 0.129 mg ^{65}Cu/g solution in MP1, 0.064 mg/g in MP2 and 0.670 mg/g in MP3. The isotope solutions replaced the natural Cu usually added to the supplement on other days of the study in MP1 and MP3. In MP2, no natural Cu was added to the diet. The ^{65}Cu was fed over 2 days to obtain adequate isotopic enrichment of samples used for absorption determinations with minimum increase in the Cu content of the diet after addition of the isotope in MP2. Polyethylene glycol was fed along with the Cu isotope as a faecal marker. Solutions for i.v. administration were prepared using sterile water for infusion and the pH adjusted to 2.0. Four grams of the solution containing 336 µg ^{65}Cu were infused into arm veins of subjects over 1 minute.
45.4 Examinations	Non-entry field
45.4.1 Body weight	Body weight was monitored throughout the study. The daily energy content of the diet was adjusted as appropriate to ensure that each volunteer would maintain his initial weight.
45.4.2 Faeces collections	Complete faecal collections were made throughout the study. See section 3.5.1 for more information.
45.5 Sample processing and analysis	Non-entry field
45.5.1 Sample collection and processing	Composites of each daily menu were collected 7 times throughout the study for determination of Cu (twice during MP1, three times during MP2 and six times during MP3). Samples of the supplementary drink were saved 6 times during MP1, 9 times during MP2 and 6 times during MP3. Complete faecal collections were made throughout the study and combined into 6-day pools. Diet composites and faecal pools were homogenised, frozen, lyophilised, dried, crushed to a powder and stored.
45.5.2 Preparation of samples for isotope ratio measurements	For each 6-d faecal composite, two 1 g aliquots were weighed into quartz crucibles and a solution containing 10 µg ^{65}Cu was added to one aliquot. After equilibrating overnight, the samples were dried on a hot plate. For every 11 pairs of aliquots weighed in this manner, 2 natural faecal reference composites were weighed. Samples were ashed overnight, treated on a hot plate with a solution of nitric acid, dried, and subjected to a second overnight ashing. Samples were then dissolved in 6 mol ultrapure HCl/litre. Cu was separated and purified by anion-exchange chromatography and samples were concentrated to 0.25 g/l. All diet composites and samples of the supplementary drinks were analyzed in duplicate for Cu. Approximately 2.6 µg ^{65}Cu was added to a 3 g subsample of each composite. Three different amounts of ^{65}Cu were added to the formula composites, depending on the copper content. A rotating menu composite from MP3 was used as a natural diet reference composite. Four subsamples were weighed. After weighing, the samples were ashed, dissolved, purified, and concentrated as the fecal samples.
45.5.3 Isotope ratio determinations	Cu isotope ratios were determined in faecal samples, diet composites, and isotope solutions with a magnetic-sector thermal ionization mass spectrometer. Samples were analysed under computer control, with typical conditions consisting of: evaporation filament current of 1.5 A, filament temperature of 1500 °C. ^{65}Cu ion beam intensity of 300 mV,

analysis time 60 minutes, and single-analysis relative SD of 0.05%.

The ^{65}Cu and total Cu content of the samples were determined by isotope dilution using the 65:63 isotopic ratios of two duplicate aliquots, with and without added ^{65}Cu, unenriched samples, and enriched ^{65}Cu solutions fed, infused, and added as isotopic diluent, along with weights of the sample aliquots and the added isotopic diluent, the concentration of the isotopic diluent, and the total dry weight of the faecal pool or food composite.

45.5.4 Absorption and endogenous excretion calculations

Absorption was calculated as the fraction of fed ^{65}Cu that was not recovered in the stools in the 12 days after the feeding of the oral dose. The recovered ^{65}Cu also included any ^{65}Cu absorbed and then excreted into the gastrointestinal tract during that time. The amount of Cu excreted into the gastrointestinal tract was calculated based on the fraction of the infused dose of ^{65}Cu excreted into the stools and eliminated in the 12 days after the infusion. The average fraction of absorbed Cu that was excreted was then added to the fraction apparently absorbed during each MP, to estimate true absorption during each MP. Slow turnover endogenous losses did not include the absorbed dietary Cu excreted within 12 days. Fast turnover losses, the amount of dietary Cu excreted in 12 days, was based on the 12-day excretion of infused Cu. Total endogenous losses are the total of the two.

45.5.5 Statistical analysis

Statistical analysis was performed with SAS. Descriptive statistics, including means, SDs, and plots, were tabulated and compared. An ANOVA model was used to determine the effects of the 3 dietary Cu intakes on Cu absorption, retention, and excretion. If a significant difference was found, Fisher's least-significant-difference test was used to determine which treatment means differed. The same ANOVA model was used to compare absorption early and late in the copper depletion period. A significance level of 0.05 was used for all statistical tests.

46 RESULTS AND DISCUSSION

Describe findings. If appropriate, include table. Sample tables are given below.

46.1 Results

Non-entry field

46.1.1 Prestudy dietary copper

The average Cu intake of the subjects in the 5 days before the study began ranged from 0.81 to 2.61 mg/day. Overall average Cu intake was 1.68 ± 0.54 mg/day (mean ± SD).

46.1.2 Copper excretion

The excretion pattern of ^{65}Cu after infusions (12 days before the end of each MP) is shown in **Figure A6.2(08)-1**. This shows that the amount of Cu excreted was lowest when dietary Cu was low and increased with higher intakes of dietary Cu.

The fraction of the infused dose excreted into the gastrointestinal tract in the 12 days after the isotope infusion is shown in **Table A6.2(08)-2**. For MP1, 12% of the dose was excreted in the first 6 days after infusion and another 14% was excreted in the next 6 days. In MP2, 6.9% was excreted in the first 6 days and 5.1% in the next 6 days. In MP3, 20% was excreted in the first 6 days and 14% in the next 6 days. Both 6-day and 12-day excretions were significantly lower with the low-copper diet (MP2) than with the higher copper diet of MP3. The 12-day excretion, but not the 6-day, was significantly lower during MP2 than during MP1.

46.1.3 Copper absorption	Cu absorption during each dietary period is shown in **Table A6.2(08)-2**. One of the 5 subjects included in the absorption study had an extremely slow transit time, making it impossible to calculate absorption. His absorption data were not included. The 67% absorbed during the low-Cu diet was significantly higher than the percentage absorbed during MP1 (54%) and MP3 (44%). The amount of Cu absorbed increased with increasing dietary Cu, averaging 0.26 mg/day during MP2, 0.35 mg/day during MP1, and 1.08 mg/day during MP3. Absorption was measured twice during the low-Cu diet, averaging 71% early in this period and 67% later on. This difference was statistically signoficant. True absorption was estimated by adjusting apparent absorption based on faecal monitoring to account for the amount of Cu absorbed and then excreted during the 12-day collection period. The pattern was similar to apparent absorption, but differences were smaller.
46.1.4 Copper retention (balance)	Average Cu retention for each MP is shown in **Table A6.2(08)-2**. Faecal Cu and Cu retention for 6-day periods throughout the study are shown in **Figures A6.2(08)-2** and **A6.2(08)-3**. The influence of previous dietary Cu intake on faecal Cu can be seen early in each MP. After the first 6-day period, faecal Cu more closely reflected dietary intake. Whereas volunteers were in negative balance early in MP1 and MP2, they were no longer in negative balance by the end of the MPs. Balance was highly positive early in MP3, but declined rapidly after the first 6-day period.
46.1.5 Copper turnover	Endogenous Cu losses, calculated for both slow and fast turnover pools in the final 6 days of each MP, and total endogenous losses via the gastrointestinal tract, are shown in **Table A6.2(08)-3**. When dietary Cu was 0.66 mg/day, 0.13 rng/day of the Cu consumed in the previous 12 days was excreted and eliminated in the stools. When intake was 0.38 mg/day, the amount was only 0.036 mg/day, and when intake increased to 2.49 mg/day, it was 0.55 mg/day. Similarly, endogenous losses not attributed to the previous 12-day intake, or slow-turnover Cu, were influenced by Cu intake. When intake was 0.66 mg/day, 0.34 mg/day was excreted into the gastrointestinal tract and eliminated. When intake was 0.38 mg/day, 0.21 mg/day was eliminated and when intake was 2.49 rng/d, the amount eliminated increased to 0.78 mg/day.
46.2 Discussion	The pre-study dietary Cu intake estimate of 1.68 mgCu/day is generally consistent with the Cu excretion data. Faecal Cu and balance data (**Figures A6.2(08)-2** and **A6.2(08)-3**) show the effect of the higher prestudy Cu intake. Subjects were in negative balance during the first part of the study. In part, the fecal losses reflect elimination of previously consumed dietary Cu and higher endogenous losses due to higher prior intake. Two assumptions were made to estimate absorption: excretion of absorbed dietary Cu and endogenous losses. For absorption calculations, it was assumed that the same fraction of both dietary copper and ^{65}Cu administered orally as an extrinsic label was absorbed. Similarly, for excretion of absorbed Cu and endogenous losses, it was assumed that the same fraction of both absorbed dietary Cu and the infused dose was excreted. This latter assumption is much more speculative and subject to limitations. By infusing the isotope, the normal route of entry into the body is bypassed. The Cu isotope was infused over a short period of time and in a different form than that which enters via the gastrointestinal tract. Despite the differences, the approach was considered sufficiently reliable to roughly estimate endogenous losses and to evaluate the effects of different dietary Cu intakes on endogenous losses.

There appeared to be two points of regulation of body Cu stores. Absorption was more efficient with low dietary Cu intake. This lessened the effect of low dietary Cu, but the amount of Cu absorbed with a low-copper diet was still lower than the amount absorbed when dietary Cu was higher. The second point of regulation, excretion of endogenous Cu into the gastrointestinal tract, may be more important in regulating total body Cu. Isotope tracer data showed (**Table A6.2(08)-2**) that when dietary Cu was lowest, 67% of orally administered ^{65}Cu was absorbed, but only 12% of infused ^{65}Cu was excreted into the gastrointestinal tract. When dietary Cu was highest, 44% of orally administered ^{65}Cu was absorbed and 34% of infused ^{65}Cu was excreted. By using the isotopic tracer, it was possible to separate the endogenous Cu into two components. Some of the absorbed dietary Cu was excreted into the gastrointestinal tract relatively rapidly. That fraction and the absolute amount excreted increased as dietary Cu increased. The change in retention of recently absorbed Cu also aided in regulating body stores by retaining more of the absorbed Cu when intake was low and eliminating the excess Cu absorbed when intake was high. In addition, the remainder of Cu in the body appeared to turn over more rapidly as dietary Cu increased. The decreased rate of turnover of the slow turnover pool when intake was low aided in conserving body Cu stores.

Cu absorption, measured twice during MP2, was significantly higher early in depletion (71%) than late in depletion (67%). The difference was so small that it may not be important physiologically, but it could represent adaptation, in which more Cu was absorbed from the depletion diet early, before endogenous losses declined in response to dietary Cu. This would be consistent with the higher fecal losses observed early in depletion than late in depletion.

Both absorption of dietary Cu and conservation of body Cu work toward adapting to a wide range of dietary Cu intakes. However, at the lowest amount of dietary Cu used in this study (0.38 mg/day in MP2), these adaptive mechanisms were not sufficient for the volunteers to maintain Cu status over the course of the MP.

46.3 Toxic effects, clinical signs

No effects / describe significant effects referring to data in results table

No effects reported.

46.4 Recovery of labelled compound

State percentage

Not stated.

47 APPLICANT'S SUMMARY AND CONCLUSION

47.1 Materials and methods

Give concise description of method; give test guidelines no. and discuss relevant deviations from test guidelines

A study was conducted in young men to evaluate the effect of a low-Cu diet on Cu absorption, excretion and retention.

Eleven Young men were confined to a metabolic research unit for 90 days. The study was divided into three separate metabolic periods (MP), with dietary Cu as the only variable. Dietary Cu intake was 0.66 mg/day for 24 days (MP1), 0.38 mg/day for 42 days (MP2) and 2.49 mg/day for 24 days (MP3). Dietary Cu was adjusted by the addition of the appropriate amount of a $CuSO_4$ solution to a supplementary drink. The stable isotope ^{65}Cu was fed to 5 of the subjects once during MP1 (days 13 – 14) and MP3 (days 79-80) and twice, early and late, in MP2 (days 31-32

and 55-56) to determine Cu absorption. ^{65}Cu was infused into a vein of the other 6 subjects once during each MP (days 13, 55 and 79 for MP1, MP2 and MP3, respectively) to estimate excretion of endogenous Cu.

Composites of each daily menu and of the supplementary drink were collected at intervals and frozen. Complete faecal collections were also made throughout the study, combined into 6-day pools and frozen. Diet composites and faecal pools were homogenised, re-frozen, lyophilised, dried, crushed to a powder and stored for subsequent determination of Cu.

Two 1 g aliqots were taken from each faecal composite. A solution containing 10 µg ^{65}Cu was added to one aliquot, allowed to equilibrate overnight and dried on a hotplate. Faecal reference composites were similarly treated. Samples were digested in HNO_3, ashed and dissolved in HCl. Cu was separated and purified by anion-exchange chromatography and samples were concentrated to 0.25 g/l. All diet composites and samples of the supplementary drinks were analyzed in duplicate for Cu. Approx. 2.6 µg ^{65}Cu was added to a 3 g subsample of each composite. Different amounts of ^{65}Cu were added to drink composites, depending on Cu content. Reference composites were similarly treated. Four subsamples were weighed. After weighing, the samples were ashed, dissolved, purified, and concentrated as for the fecal samples. Cu isotope ratios were determined in faecal samples, diet composites, and isotope solutions with a magnetic-sector thermal ionization mass spectrometer.

The ^{65}Cu and total Cu content of the samples were determined by isotope dilution using the 65:63 isotopic ratios of 2 duplicate aliquots, with and without added ^{65}Cu, unenriched samples, and enriched ^{65}Cu solutions fed, infused, and added as isotopic diluent, along with weights of the sample aliquots and the added isotopic diluent, the concentration of the isotopic diluent, and the total dry weight of the faecal pool or food composite.

Absorption was calculated as the fraction of fed ^{65}Cu that was not recovered in the stools in the 12 days after the feeding of the oral dose. The recovered ^{65}Cu also included any ^{65}Cu absorbed and then excreted into the gastrointestinal tract during that time. The amount of Cu excreted was calculated based on the fraction of the infused dose of ^{65}Cu excreted into the stools and eliminated in the 12 days after infusion. The average fraction of absorbed Cu that was excreted was then added to the fraction apparently absorbed during each MP, to estimate true absorption during each MP. Slow turnover endogenous losses did not include the absorbed dietary Cu excreted within 12 days. Fast turnover losses, the amount of dietary Cu excreted in 12 days, was based on the 12-day excretion of infused Cu. Total endogenous losses are the total of the two.

ANOVA was used to determine the effects of the 3 dietary Cu intakes on Cu absorption, retention, and excretion. If a significant difference was found, Fisher's least-significant-difference test was used to determine which treatment means differed. ANOVA was used to compare absorption early and late in the copper depletion period. A significance level of 0.05 was used for all statistical tests.

47.2 Results and discussion

Summarize relevant results; discuss dose-response relationship.

Prestudy dietary Cu: The average Cu intake of the subjects in the 5 days before the study began ranged from 0.81 to 2.61 mg/day. Overall average Cu intake was 1.68 ± 0.54 mg/day (mean ± SD); higher than the intake during MP1. Faecal Cu and balance data show the effect of the higher pre-study Cu intake. Subjects were in negative balance during the first part of the study. In part, the fecal losses reflect elimination of previously

consumed dietary Cu and higher endogenous losses due to higher prior intake.

Cu excretion: The excretion pattern of ^{65}Cu after infusions (12 days before the end of each MP) shows that the amount of Cu excreted was lowest when dietary Cu was low and increased with higher intakes of dietary Cu. For MP1, 12% of the dose was excreted in the first 6 days after infusion and another 14% was excreted in the next 6 days. In MP2, 6.9% was excreted in the first 6 days and 5.1% in the next 6 days. In MP3, 20% was excreted in the first 6 days and 14% in the next 6 days. Both 6-day and 12-day excretions were significantly lower with the lowcopper diet (MP2) than with the higher copper diet of MP3. The 12-day excretion, but not the 6-day, was significantly lower during MP2 than during MPl.

Cu absorption: Cu absorption during each dietary period was assessed. The percentage of Cu absorbed during the low-Cu diet (67%) was significantly higher than during MP1 (54%) or MP3 (44%). The amount of Cu absorbed increased with increasing dietary Cu, averaging 0.26 mg/day during MP2, 0.35 mg/day during MP1, and 1.08 mg/day during MP3. Absorption was measured twice during the low-Cu diet, averaging 71% early in this period and 67% later on. Although statistically significant, this difference was so small that it may not have been important physiologically. It could, however, represent adaptation, in which more Cu was absorbed from the depletion diet early, before endogenous losses declined in response to dietary Cu.

True absorption was estimated by adjusting apparent absorption based on faecal monitoring to account for the amount of Cu absorbed and then excreted during the 12-day collection period. The pattern was similar to apparent absorption, but differences were smaller.

Cu retention (balance): The influence of previous dietary Cu intake on faecal Cu became evident early in each MP. After the first 6-day period, faecal Cu more closely reflected dietary intake. Whereas volunteers were in negative balance early in MP1 and MP2, they were no longer in negative balance by the end of the MPs. Balance was highly positive early in MP3, but declined rapidly after the first 6-day period.

Cu turnover: Endogenous Cu losses, calculated for both slow and fast turnover pools in the final 6 days of each MP, and total endogenous losses via the gastrointestinal tract, were determined. When dietary Cu was 0.66 mg/day, 0.13 rng/day of the Cu consumed in the previous 12 days was excreted and eliminated in the stools. When intake was 0.38 mg/day, the amount was only 0.036 mg/day, and when intake increased to 2.49 mg/day, it was 0.55 mg/day. Similarly, endogenous losses not attributed to the previous 12-day intake, or slow-turnover Cu, were influenced by Cu intake. When intake was 0.66 mg/day, 0.34 mg/day was excreted into the gastrointestinal tract and eliminated. When intake was 0.38 mg/day, 0.21 mg/day was eliminated and when intake was 2.49 rng/d, the amount eliminated increased to 0.78 mg/day.

In conclusion, there were two points of regulation of body Cu stores. Absorption was more efficient with low dietary Cu intake. This lessened the effect of low dietary Cu, but the amount of Cu absorbed with a low-copper diet was still lower than the amount absorbed when dietary Cu was higher. The second point of regulation, excretion of endogenous Cu into the gastrointestinal tract, may have been more important in regulating total body Cu. Isotope tracer data showed that when dietary Cu was lowest, 67% of orally administered ^{65}Cu was absorbed, but only 12% of

infused ^{65}Cu was excreted. When dietary Cu was highest, 44% of orally administered ^{65}Cu was absorbed and 34% of infused ^{65}Cu was excreted. By using the isotopic tracer, it was possible to separate the endogenous Cu into two components. Some of the absorbed dietary Cu was excreted into the gastrointestinal tract relatively rapidly. That fraction and the absolute amount excreted increased as dietary Cu increased. The change in retention of recently absorbed Cu also aided in regulating body stores by retaining more of the absorbed Cu when intake was low and eliminating the excess Cu absorbed when intake was high. In addition, the remainder of Cu in the body appeared to turn over more rapidly as dietary Cu increased. The decreased rate of turnover of the slow turnover pool when intake was low aided in conserving body Cu stores.

47.3 Conclusion Regulation of absorption and of endogenous excretion in response to dietary Cu intake helps to protect against deficiency and toxicity.

47.3.1 Reliability *Based on the assessment of materials and methods include appropriate reliability indicator 0, 1, 2, 3, or 4*

2

47.3.2 Deficiencies Yes

This study was not conducted and/or reported in strict compliance with the principles of GLP. However, this does not necessarily compromise the validity of the data generated, or the author's interpretation of that data, given that the study was not carried out for regulatory purposes. Furthermore, the research was published in a peer-reviewed journal, and has therefore been subject to the prior scrutiny of experts in the field. In addition this report has been included in a number of expert reviews of Cu toxicokinetics.

No internationally accepted guidelines are available that specifically address the objective of the research presented in this summary.

Overall, this is a well-reported study, and its findings are considered to make a valuable contribution to the 'weight of evidence' approach that has been adopted for the purposes of the current review of Cu toxicokinetics. A reliability indicator of 2 has been assigned on this basis.

(If yes, discuss the impact of deficiencies and implications on results. If relevant, justify acceptability of study.)

Evaluation by Competent Authorities

Section A6.2	**Metabolism in mammals**
Annex Point IIA6.2	*Specify section no., heading and species as appropriate*
IUCLID: 5.0/08	**A6.2(08), Homeostasis of copper**

	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	[redacted]
Reference	[redacted]
Materials and Methods	[redacted]
Results and discussion	[redacted]
Conclusion	[redacted]
Reliability	[redacted]
Acceptability	[redacted]
Remarks	[redacted]
[redacted]	

	COMMENTS FROM ...
Date	*Give date of comments submitted*

Table A6.2(08)-1. Experimental Design

	Metabolic period		
	1	2	3
Duration (days)	24	42	24
Dietary copper (mg/day)	0.66	0.38	2.49
Study days in which ^{65}Cu was included in the diet of subjects 1-6	13-14[1]	31-32, 55-56[2]	79-80[1]
Study days in which ^{65}Cu was infused into subjects 7-12	13	55	79

[1]Copper was replaced with ^{65}Cu in the diet.
[2]0.2 mg ^{65}Cu/day was added to the diet.

Table A6.2(08)-2. Copper absorption, excretion and retention

Metabolic period	Dietary copper	Copper absorption (n = 4)		^{65}Cu excretion[2] (n = 6)	True absorption[3] (n = 4)		Copper retention (n = 11)
	mg/day	%	mg/day	%	%	mg/day	mg/day
1	0.66	54	0.35	26[a]	73	0.48	-0.13[a]
2	0.38	67	0.26	12[b]	77	0.29	-0.015[b]
3	2.49	44	1.08	34[a]	66	1.64	511[c]
SE[5]	--	3.2	--	3.8	--	--	39

[1]Means within a column with different superscript letters are significantly different, P > 0.05.

[2]Percentage of dose of ^{65}Cu excreted in 12days after infusion.

[3]Estimated from average absorption of fed ^{65}Cu adjusted for average excretion of infused ^{65}Cu.

[4]Average over entire metabolic period.

[5]Standard error of least-squares mean.

Table A6.2(08)-3. Endogenous gastrointestinal (GI) losses of copper at three intakes of dietary copper

Metabolic period	Dietary copper	Faecal copper[1]	Endogenous GI losses		
			Slow pool[2]	Fast pool[3]	Total[4]
		mg/day			
1	0.66	0.65	0.34	0.13	0.47
2	0.38	0.33	0.21	0.036	0.24
3	2.49	2.17	0.78	0.55	1.33

[1]Last 6 days of each metabolic period (n = 11).

[2]Slow turnover pool (endogenous faecal losses not attributed to excretion of dietary copper in the first 12 days after absorption).

[3]Fast turnover pool (losses of dietary copper consumed and absorbed in the previous 12 days).

[4]Total endogenous losses (faecal copper less unabsorbed dietary copper).

Table A6.2(08)-3. Endogenous gastrointestinal (GI) losses of

Figure A6.2(08)-1

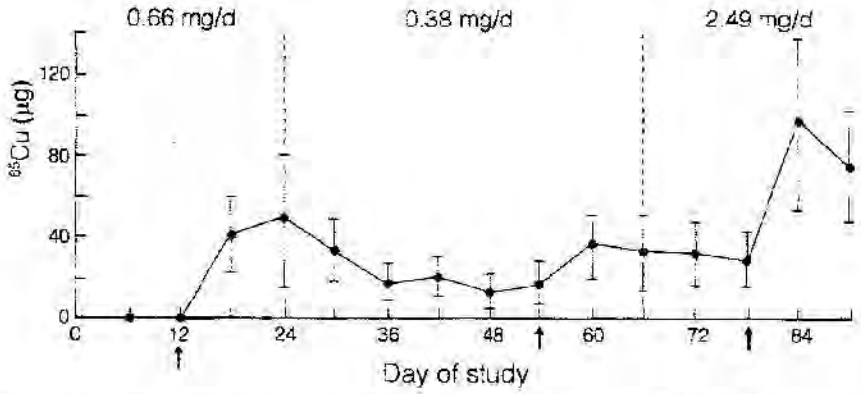


FIGURE 1. Excretion pattern of ^{65}Cu into the gastrointestinal tract after infusion of 0.34 mg of the isotope once in each dietary copper period ($\bar{x} \pm SD$, $n = 6$). Arrows indicate times of isotope administration. Vertical dotted lines represent changes in dietary copper intake, which was 0.66, 0.38, and 2.49 mg/d.

Figure A6.2(08)-2

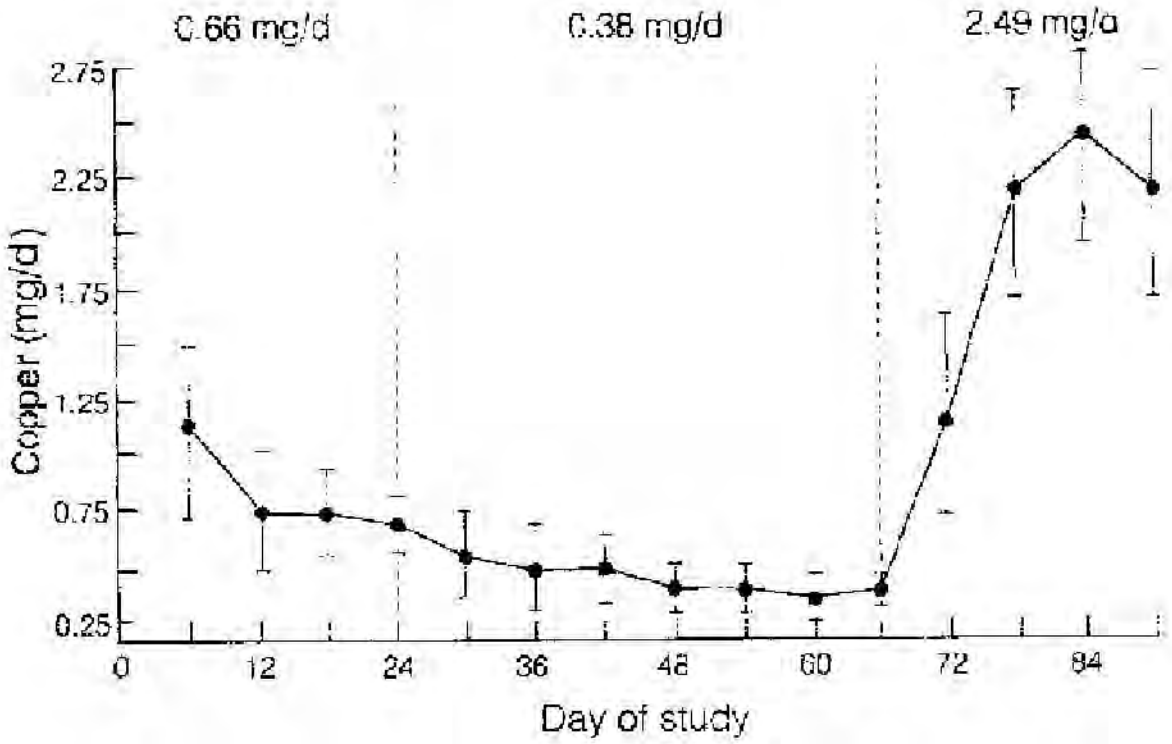


Figure A6.2(08)-3

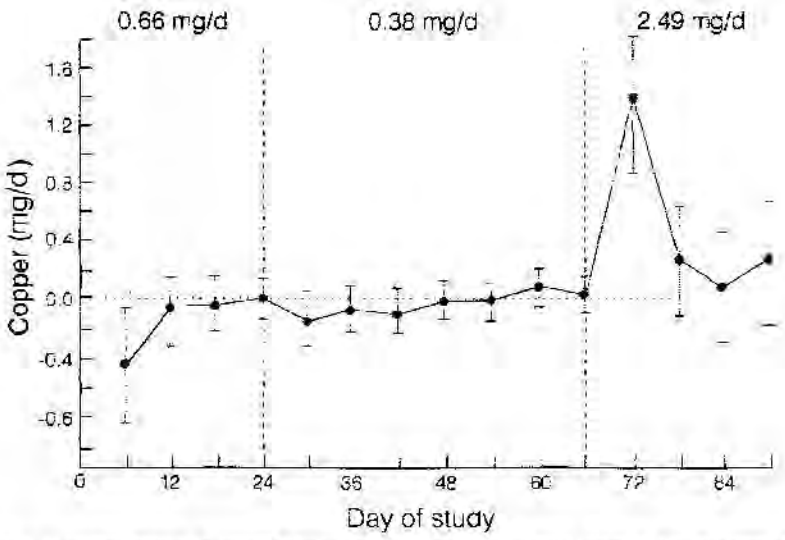


FIGURE 3. Copper balance (mg/d). Pattern of copper balance throughout the study at three levels of dietary copper shown at the top of the graph ($\bar{x} \pm SD$, $n = 11$). Vertical dotted lines represent changes in dietary copper.

			Official use only
		48 REFERENCE	
1.1	**Reference**	*Author(s), year, title, laboratory name, laboratory report number, report date (if published, list journal name, volume: pages)* *If necessary, copy field and enter other reference(s).* Linder M.C., Weiss K.C. and Hai, V.M. (1988). Structure and function of transcuprein in transport of copper by mammalian blood plasma. In: Hurley L.C., Keen C.L., Lonnerdal, B. and Rucker, R.B. (eds). Trace Elements in Man and Animals (TEMA-6). New York: Plenum, 141–144 (published).	
1.2	**Data protection**	No *(indicate if data protection is claimed)*	
1.2.1	Data owner	*Give name of company* Public domain	
1.2.2			
1.2.3	Criteria for data protection	Choose one of the following criteria (see also TNsG on Product Evaluation) and delete the others: No data protection claimed	
		49 GUIDELINES AND QUALITY ASSURANCE	
49.1	**Guideline study**	No. This was a non-regulatory study carried out to investigate the structure and function of the copper transport proteins transcuprein and albumin. *(If yes, give guidelines; if no, give justification, e.g. "no guidelines available" or "methods used comparable to guidelines xy")*	
49.2	**GLP**	No. This was a non-regulatory study. *(If no, give justification, e.g. state that GLP was not compulsory at the time the study was performed)*	
49.3	**Deviations**	No. Not applicable to non-guideline studies. *(If yes, describe deviations from test guidelines or refer to respective field numbers where these are described, e.g. "see 3.x.y")*	
		50 MATERIALS AND METHODS	
		In some fields the values indicated in the EC or OECD test guidelines are given as default values. Adopt, change or delete these default values as appropriate.	
50.1	**Test material**	Cu (II) as either the chloride or as Cu-nitrilotriacetate complex.	
50.1.1	Lot/Batch number	Not available	
50.1.2	Specification	Deviating from specification given in section 2 as follows *(describe specification under separate subheadings, such as the following; additional subheadings may be appropriate):*	

Section A6.2		**Metabolism in mammals**
Annex Point IIA6.2		*Specify section no., heading and species as appropriate*
IUCLID: 5.0/09		**A6.2(09), Distribution of copper**
50.1.2.1 Description		*If appropriate, give e.g. colour, physical form (e.g. powder, grain size, particle size/distribution)* Not available
50.1.2.2 Purity		*Give purity in % of active substance* [redacted]
50.1.2.3 Stability		*Describe stability of test material* Not available
50.1.2.4 Radiolabelling		*give structural location of radio labelling, give reason if not labelled* ^{67}Cu
50.2	**Test Animals**	Non-entry field
50.2.1	Species	Plasma samples used in this study were obtained from rats or humans, as appropriate.
50.2.2	Strain	Not available
50.2.3	Source	Not available
50.2.4	Sex	Not available
50.2.5	Controls	No
50.3	**Investigations**	Non-entry field
50.3.1	Purification and structure of transcuprein.	Partial purification of transcuprein was carried out with a variety of procedures, including ammonium sulphate fractionation, where transcuprein precipitates between 35 – 50% saturation; gel permeation chromatography on Sephadex G150, where it elutes in the void volume and on Ultrogel AcA 34, where it elutes with an apparent molecular weight of 270 kDa; DEAE-cellulose chromatography, in phosphate, pH 7.0, where it elutes at about 0.15M NaCl in a 0 – 0.5M gradient; and pseudoaffinity chromatography (Affigel Blue).
50.3.2	Properties and copper binding.	The specific binding of copper to partially purified transcuprein was measured in a nitrocellulose filter assay, after incubation of the protein with various concentrations of ^{67}Cu, in the presence and absence of a 100-fold molar excess of non-radioactive Cu-NTA at pH 7.0 in 20 mM phosphate-buffered saline. The rate of Cu release from transcuprein was also studied in dialysis and compared with that of albumin. Samples (2.0 ml) of radioactively labelled protein obtained from Sephadex G150 chromatography of ^{67}Cu-treated rat plasma (containing 1.5 – 2 million cpm) were placed in 500 ml buffer and dialysed at 4°C. Aliquots (5.0 ml) were withdrawn periodically and measured for release of radioactive Cu. At the end of dialysis, the ^{67}Cu remaining in the dialysis tubing was also measured.

X

50.3.3 Detection of transcuprein by in-vivo labelling and gel chromatography	The purpose of this study was to determine how to use in-vitro labelling to quantify the amounts of transcuprein present in samples, based on a known albumin content. This was done by: 1. Ascertaining what amounts of Cu (^{67}Cu or ^{64}Cu) could be added to samples in-vitro that would allow detection of transcuprein in the presence of various amounts of albumin. 2. Developing a method of using the proportion of radioactivity (relative to that on albumin) to assess transcuprein content. Cu (II) was added as either the chloride or the NTA complex to 1.0 ml samples of rat or human whole plasma or serum, or to 1.0 ml samples of partially purified rat transcuprein and albumin.
50.3.4 Transcuprein in portal blood.	The purpose of this investigation was to demonstrate that transcuprein is present in portal blood in similar or greater amounts to that in plasma taken from the blood of the vena cava, after passing through the liver. **Figure A6.2(09)-2** shows the radioactive labelling of transcuprein, albumin and low molecular weight components in portal plasma, upon administration of 62 ng Cu (as ^{67}Cu-NTA) to the lumen of a 7 cm, tied-off segment of the upper small intestine in an anaesthetised rat.
	51 RESULTS AND DISCUSSION *Describe findings. If appropriate, include table. Sample tables are given below.*
51.1 Purification and structure of transcuprein.	Tracking transcuprein with ^{67}Cu and by its characteristic elution in Sephadex or Ultrogel, resulted in a preparation containing a major component with Rf 0.41 in disc PAGE (using a 5% separating gel and pH 8.8 Tris-glycine buffer). Three minor protein components were also present, with Rf values of 0.11, 0.24 and 0.60, respectively. The major component had subunits of Mr 80,000 after dissociation and electrophoresis in SDS.
51.2 Properties and copper binding.	Preliminary binding studies indicated that binding of Cu to transcuprein was saturable and half maximal in the range of 10^{-9} M. It was also found that albumin behaved similarly. The results of dialysis indicated that, at pH7-8, Cu was released at a very slow, linear rate. The rate was, however, raised at higher and lower pH values. These studies confirm that the affinity of transcuprein for Cu is similar to that of albumin.
51.3 Detection of transcuprein by in-vivo labelling and gel chromatography	It was found that the amount of Cu added to whole plasma or serum determined the relative proportions that bound to transcuprein and albumin (**Table A6.2(09)-1**). At the lowest concentrations (in the range 1 – 2 ng/ml), almost exactly one third bound to transcuprein and two thirds to albumin. Very little bound to other components that could be separated in gel chromatography. At concentrations almost 1000 times greater than this (2 or more µg/ml), radioactivity on transcuprein was proportionately very low. There was also prominent labelling of low molecular weight components. When samples of partially purified transcuprein and albumin were mixed in various proportions, almost all of the radioactivity became associated with transcuprein at low albumin levels. The reverse was the case in the presence of increasing albumin levels (**Figure A6.2(09)-1**). It was concluded that the amount of radioactivity associated with the transcuprein peak in Sephadex G150 chromatography does reflect the amount of transcuprein present in the sample.

51.4	Transcuprein in portal blood.	More than one third of of the radioactivity in albumin + transcuprein from portal blood was found to be associated with the transcuprein. This is a greater proprtion than is found in samples of vena caval blood labelled in vitro. It was concluded that transcuprein is indeed present in the portal blood, and that it is present in similar or higher amounts than in blood leaving the liver.

52 APPLICANT'S SUMMARY AND CONCLUSION

52.1 Materials and methods

Give concise description of method; give test guidelines no. and discuss relevant deviations from test guidelines

A study was carried out to investigate the structure and function of the copper transport proteins transcuprein and albumin. No guidelines are available to address this objective and the study was not carried out or reported in compliance with GLP.

Partial purification of transcuprein was achieved with a variety of procedures, including ammonium sulphate fractionation; gel permeation chromatography; DEAE-cellulose chromatography; and pseudoaffinity chromatography. The specific binding of copper to partially purified transcuprein was measured in a nitrocellulose filter assay, after incubation of the protein with various concentrations of ^{67}Cu in the presence and absence of a molar excess of non-radioactive Cu.

The rate of Cu release from transcuprein was studied in dialysis and compared with that of albumin. At the end of dialysis, the ^{67}Cu remaining in the dialysis tubing was also measured. The use of in-vitro labelling to quantify the amounts of transcuprein present in samples, based on a known albumin content, was done by 1) ascertaining what amounts of ^{67}Cu or ^{64}Cu could be added to samples in-vitro that would allow detection of transcuprein in the presence of various amounts of albumin, and 2) developing a method of using the proportion of radioactivity (relative to that on albumin) to assess transcuprein content.

The amounts of transcuprein, albumin and low molecular weight components in portal blood were compared with those in plasma taken from the blood of the vena cava, after passing through the liver.

52.2 Results and discussion

Summarize relevant results; discuss dose-response relationship.

Tracking transcuprein with ^{67}Cu and by its characteristic elution in Sephadex or Ultrogel, resulted in a preparation containing a major component with Rf 0.41 in disc PAGE. This component had subunits of Mr 80,000 after dissociation and electrophoresis in SDS.

Preliminary binding studies indicated that binding of Cu to transcuprein was saturable and half maximal in the range of 10^{-9} M. It was found that albumin behaved similarly. The results of dialysis indicated that, at pH7-8, Cu was released at a very slow, linear rate. The rate was raised at higher and lower pH values. These studies confirm that the affinity of transcuprein for Cu is similar to that of albumin.

The amount of Cu added to whole plasma or serum determined the relative proportions that bound to transcuprein and albumin. At concentrations in the range 1 – 2 ng/ml, almost exactly **1/3** bound to transcuprein and **2/3** to albumin. At concentrations ≥2 µg/ml, radioactivity on transcuprein was proportionately very low. When samples of partially purified transcuprein and albumin were mixed in various proportions, almost all of the radioactivity became associated

	with transcuprein at low albumin levels. The reverse was true in the presence of increasing albumin levels. It was concluded that the amount of radioactivity associated with the transcuprein peak in Sephadex G150 chromatography does reflect the amount of transcuprein present in the sample. More than **1/3** of the radioactivity in albumin plus transcuprein from portal blood was found to be associated with transcuprein. This is a greater proprtion than in samples of vena caval blood labelled *in vitro*. It was concluded that transcuprein is present in portal blood at similar or higher amounts than in blood leaving the liver.
52.3 Conclusion	Absorbed copper initially binds reversibly to transcuprein and albumin and is transported via portal blood to the liver. Transcuprein and albumin are also present in vena caval blood.
52.3.1 Reliability	*Based on the assessment of materials and methods include appropriate reliability indicator 0, 1, 2, 3, or 4* 2
52.3.2 Deficiencies	Yes. This study was not conducted and/or reported in compliance with GLP. When compared with generally accepted principles to be applied to toxicokinetics studies, as set out in OECD guideline 417, it is also apparent that methodological details were poorly reported in places, including: • Information on the animals from which samples of plasma were obtained for use in the study (eg. Numbers of animals used and the conditions in which they were housed); • Information on the laboratory techniques applied to isolate and detect transcuprein and albumin and to investigate their various properties. • Information on the specification of the test substance is deficient. These deficiencies do not, however, necessarily compromise the validity of the data generated, or the author's interpretation of that data, given that the study was not carried out for regulatory purposes and that the results obtained are consistent with work published by other researchers. Furthermore, this research (including its methodology) was published in a peer-reviewed publication, and has therefore been subject to the prior scrutiny of experts in the field. No internationally accepted guidelines are available that specifically address the objective of the research presented in this summary. *(If yes, discuss the impact of deficiencies and implications on results. If relevant, justify acceptability of study.)*

Evaluation by Competent Authorities

Section A6.2	**Metabolism in mammals**
Annex Point IIA6.2	*Specify section no., heading and species as appropriate*
IUCLID: 5.0/09	**A6.2(09), Distribution of copper**

	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	[redacted]
Materials and Methods	[redacted]
Results and discussion	[redacted]
Conclusion	[redacted]
Reliability	[redacted]
Acceptability	[redacted]
Remarks	

	COMMENTS FROM ...
Date	*Give date of comments submitted*

Table A6.2(09)-1

Distribution of ^{67} Cu between transcuprein and albumin, upon addition to rat plasma, in vitro.

Copper added (ng) [1]	Percent Radioactivity on	
	Transcuprein	Albumin
1	35	65
20	31	69
200	28	72
2000	16	84
2700	2	98

[1] 0.1 ml 0.9%NaC1 containing various amounts of Cu (as ^{67} Cu-NTA; 1:1 molar ratio) added to 1.0 ml rat plasma 30-90 min before Sephadex G150 chromatography.

Figure A6.2(09)-1

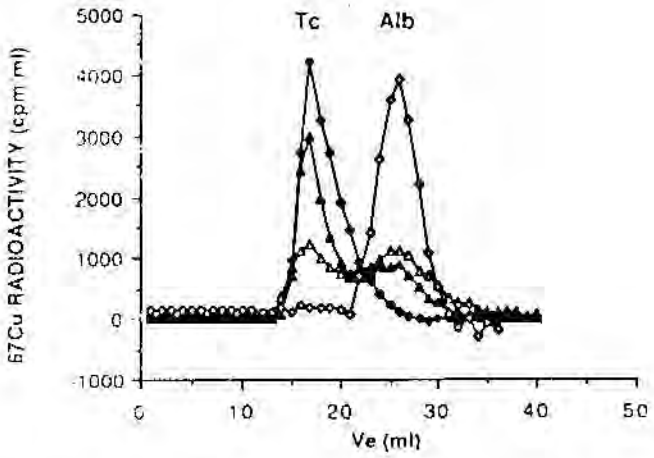


Fig. 1. Distribution of added ^{67}Cu radioactivity after in vitro addition of 2-20 ng Cu to partially purified transcuprein or albumin alone, or to various mixtures of the two. Sample applied to Sephadex G150.

Figure A6.2(09)-2

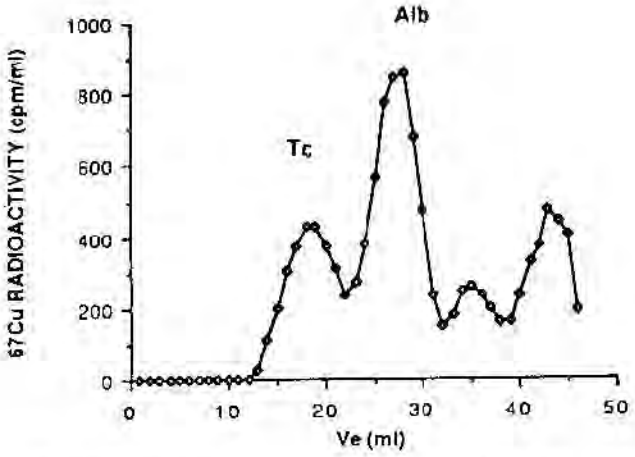


Fig. 2. Elution of copper binding components labeled with ^{67}Cu in portal blood plasma, 30 min after administration of radiocopper to a tied-off intestinal segment of an anesthetized rat. Plasma was applied to Sephadex G150 chromatography. The first peak elutes in the position of transcuprein, the second albumin.

			Official use only
		53 REFERENCE	
1.1	**Reference**	*Author(s), year, title, laboratory name, laboratory report number, report date (if published, list journal name, volume: pages)* *If necessary, copy field and enter other reference(s).* Weiss K.C. & Linder M.C. (1985). Copper transport in rats involving a new plasma protein. Am. J. Physiol. **249**: E77-88 (published).	
1.2	**Data protection**	No *(indicate if data protection is claimed)*	
1.2.1	Data owner	*Give name of company* Public domain	
1.2.2	Criteria for data protection	Choose one of the following criteria (see also TNsG on Product Evaluation) and delete the others: No data protection claimed	
		54 GUIDELINES AND QUALITY ASSURANCE	
54.1	**Guideline study**	No. This was a non-regulatory study carried out to investigate the mechanism of copper transport in blood plasma following absorption from the diet. *(If yes, give guidelines; if no, give justification, e.g. "no guidelines available" or "methods used comparable to guidelines xy")*	
54.2	**GLP**	No. This was a non-regulatory study. *(If no, give justification, e.g. state that GLP was not compulsory at the time the study was performed)*	
54.3	**Deviations**	No. Not applicable to non-guideline studies. *(If yes, describe deviations from test guidelines or refer to respective field numbers where these are described, e.g. "see 3.x.y")*	
		55 MATERIALS AND METHODS *In some fields the values indicated in the EC or OECD test guidelines are given as default values. Adopt, change or delete these default values as appropriate.*	
55.1	**Test material**	$CuCl_2$	
55.1.1	Lot/Batch number	Not available	
55.1.2	Specification	Deviating from specification given in section 2 as follows *(describe specification under separate subheadings, such as the following; additional subheadings may be appropriate):*	

Section A6.2	**Metabolism in mammals**
Annex Point IIA6.2	*Specify section no., heading and species as appropriate*
IUCLID: 5.0/10	**A6.2(10), Distribution of copper**

55.1.2.1 Description	*If appropriate, give e.g. colour, physical form (e.g. powder, grain size, particle size/distribution)* Samples of ^{67}Cu were shipped in 1 – 5 M HCl and diluted in H_2O (for intragastric intubations of rats) or neutralised with NaOH in 0.9% NaCl just before injection.	
55.1.2.2 Purity	*Give purity in % of active substance* [redacted]	
55.1.2.3 Stability	*Describe stability of test material* See section 3.1.2.2	
55.1.2.4 Radiolabelling	*give structural location of radio labelling, give reason if not labelled* ^{67}Cu (see 3.1.2.2)	
55.2 Test Animals	Non-entry field	
55.2.1 Species	Rat	
55.2.2 Strain	Fischer or Sprague-Dawley	X
55.2.3 Source	Simonsosn Labs, Gilroy, CA (Fischer) and Mission Labs, Rosemead, CA (Sprague-Dawley). (Human serum was primarily obtained frozen from Anaheim Memorial Hospital (Anaheim, CA). In some cases, fresh serum was obtained from laboratory volunteers).	
55.2.4 Sex	Female	
55.2.5 Controls	No	
55.3 Investigations and procedures	Non-entry field	
55.3.1 Animals, tissues and treatments	Adult, female Fischer or Sprague-Dawley rats were used in all animal studies. The copper status of the animals was maintained constant by keeping them on the same diet and using rats of similar age, sex, weight and strain. Where checked, tissue and blood analyses of copper gave constant values for different batches of rats. Animals were injected intraperitoneally, or intravenously by tail vein, with 0.1-0.4 ml $^{67}CuCl_2$ (10-300 µCi) in 0.9% NaCl, pH ~4, at various times before death. In some cases 0.2 to 0.3 m1 volumes of $^{67}CuCl_2$ in 0.01 N HCl were given by intragastric intubation. For the time course studies, only high specific activity radioisotope (~1,000 Ci/g) was employed (200-400 µCi/rat; 0.08-0.33 µg). In these studies, groups of six to nine rats were injected at the same and two to three killed at various times thereafter. Plasma was obtained from the vena cava, after anaesthetising with pentobarbital and injecting Na heparin solution. Rats were killed by exsanguination. Fresh plasma was used for most of the experimental	