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VOLUME EXCLUSION ERRORS AND THE DETERMINATION OF SERUM ZINC

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Summary

Volume exclusion gives rise to positive errors in the results of plasma or serum zinc determinations by methods in which protein is precipitated and the zinc extracted into solution. The error is amplified if two extraction steps are employed, and may be +20% or more. The same effects must be expected in any analysis involving comparable extraction procedures, whatever the substance being determined.

Introduction

Greaves and Boyde compared plasma zinc levels in a control group with those in patients suffering from various skin ailments [1]. The mean level in the control group was unusually high (18.1 μmol/l or 1180 μg/l). This cannot be attributed to contamination in view of the exceptional care taken to identify and correct for traces of zinc in apparatus and materials. Nor does this circumstance, per se, affect the validity of the inter-group comparisons, since the assays were conducted simultaneously by the same method and by a single analyst, who remained in ignorance of the source of individual samples until after the series was complete. Nevertheless, it seemed likely that there was a systematic error in the method affecting all samples, and the nature of this error has now been identified.

Materials and methods

Reagents and apparatus

Analytical reagent grade zinc sulphate, hydrochloric acid, zinc granules and

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trichloroacetic acid (TCA) were obtained from Merck. Glass distilled deionised water (passed through a column of mixed cation and anion exchange resin prior to glass-distillation) was used throughout the investigation. The glass (pyrex) centrifuge tubes, polythene volumetric flasks, storage bottles and all other glassware and plastic-ware used were washed overnight with nitric acid (approx. 1.6 mol/l), and then rinsed copiously with glass-distilled water.

Preparation of standards (flame atomisation)

The stock standard (stored in polythene bottles) was prepared by dissolving 1 g zinc granules in 40 ml hydrochloric acid, approx. 6 mol/l, and diluting to 1 litre. For use as working standards, solutions containing 200—1600 μg/l were freshly prepared by dilution on the day of use.

Blood samples

Whole blood was collected through the courtesy of the Hong Kong Red Cross Blood Transfusion Service. After sufficient had been obtained from each normal donor for transfusion purposes (250—400 ml), the plastic delivery tube was cut and blood allowed to flow direct into a centrifuge tube. This was kept at 5°C for 24 h before centrifugation and removal of serum, which was stored at −20°C if not assayed immediately. Dry, acid-washed, zinc-free glassware was used throughout. Fresh and frozen samples gave identical results.

Protein precipitation and extraction of zinc for analysis

(a) 2 ml of serum was mixed with 1 ml 0.6 mol/l TCA ('Whirlimixer', Fison's Scientific) then heated at 90°C in a waterbath for 10 min and centrifuged for 20 min at 3000 rev./min (M.S.E. 'Minor' centrifuge). The clear supernatant was decanted into a pre-weighed 5-ml glass beaker and the volume calculated from measurements of weight and density. To the precipitate remaining in the centrifuge tube was added 1 ml 0.6 mol/l TCA, the mixture again thoroughly mixed, heated and centrifuged as before, and the supernatant collected and weighed as before. Two further extractions were carried out in the same way, but using 1 ml water instead of the TCA and omitting the heating step. Finally, the centrifuge tube containing residual (wet) precipitate was weighed, dried to constant weight in an evacuated desiccator over phosphorus pentoxide (about 7 days), and the weight loss determined.

(b) 2 ml of serum was mixed with 2 ml of 0.6 mol/l TCA, heated to 90°C for 15 min and centrifuged as under (a). The supernatant was then transferred to an acid-washed container for analysis.

(c) Flame atomisation. Each of the supernatants obtained by procedures (a) and (b) above was analysed for zinc with the Varian Techtron 1200 atomic absorption spectrophotometer in flame atomisation mode, using the above standards and the following instrument settings.

Wavelength	213.8 nm
Spectral band width	0.5 nm
Zinc lamp current	5.0 mA
Air line pressure	28.0 pounds per square inch (p.s.i.)
Flow rate of air	6.0 units (arbitrary units on the instrument)
Acetylene line pressure	10.0 p.s.i.
Flow rate of acetylene	1.75 units (arbitrary units on the instrument)

(d) Non-flame atomisation. The determinations were made using the same spectrophotometer with its burner head replaced by a carbon rod atomiser workhead (CRA Model 63, Varian Techtron). The following voltage and time settings were established for the human serum samples.

Dry stage:
Voltage setting: 1.75 to 2.0 units
Time settings: 35 sec (serum samples); 30 sec (aqueous standards)
Ash stage:
Voltage setting: 5.0 units
Time setting: 20 sec
Step atomise stage:
Voltage setting: 6.0 units
Time setting: 5 sec
Inert gas used: Nitrogen; line pressure 20 p.s.i.; flow rate 6.5 arbitrary units on CR gas control unit
Fuel used: Hydrogen; line pressure 10 p.s.i.; flow rate 1 arbitrary unit on CR gas control unit
Cooling source: Running tap water at a flow rate of 2 l/min

The graphite furnace used in the present study was of the cup design. Serum samples were diluted 10-fold with deionised, glass distilled water, and 2-μl aliquots were introduced into the carbon cup by means of a 2-μl Eppendorf micropipette. The disposable plastic tips were decontaminated beforehand in 3 mol/l HNO_3 followed by thorough rinsing with glass-distilled deionised water. Standards containing 40—200 μg/l Zn were made by dilution on the day of use from a zinc sulphate stock solution of 1 g/l Zn.

Determination of density

Density was measured where required by weighing the solution delivered by a calibrated 0.5-ml pipette.

Computation of results

1. Even after 4 extractions, it is clear that some zinc remains in the water in the precipitate. This amount was estimated by assuming that the concentration of zinc in this residual water was the same as in the 4th extract and that the amount of this residual water could be calculated from the weight loss on drying. The amount involved is small, and any error introduced through attempting this correction is probably even smaller. The serum zinc concentration by "exhaustive extraction" was taken as the sum of the amount of zinc in extracts 1—4 (calculated from volume and analyzed concentration of each) plus the correction just mentioned, divided by the volume of the original sample (2 ml = 0.002 l).

2. Procedures b and c above yield an analysed zinc concentration in the TCA extract. This result is multiplied by 2 to give the serum zinc concentration by "single extraction".

3. Procedure d above yields an analysed concentration of zinc in a diluted serum sample. This result is multiplied by 10 to give the serum zinc concentration by "carbon cup".

4. When working with non-lyophilised plasma samples, Prasad et al. [2] carried out two extractions as for the first two extractions in Procedure (a), above (the only exception being that in this work we prolonged the heating stage to

10 min, instead of 5 min). They then combined these two extracts, determined the zinc concentration of this mixed solution by flame atomic absorption spectroscopy and multiplied the result by 2 to obtain the plasma zinc concentration. This last, computational step has been confirmed by Dr. Oberleas (personal communication). We wished to study the progress of extraction of zinc from the precipitate, and therefore proceeded differently. If the volumes of these two extracts are designated D and F and the concentrations of zinc in each as $[Zn]_D$ and $[Zn]_F$, the amounts extracted are $D[Zn]_D$ and $F[Zn]_F$ respectively. We may thus calculate the analysed zinc concentration which would have been obtained by following the procedure of Prasad et al., from the expression $(D[Zn]_D + F[Zn]_F)/(D + F)$.

To calculate the zinc concentration by "double extraction" this result is simply multiplied by 2.

Recovery experiments (double extraction)

(i) To 2 ml serum in a test tube was added 10 μl zinc acetate solution containing 0.9 μg Zn. (ii) As for i, but using zinc chloride (1.0 μg) in 0.6 mol/l TCA. (iii) The first extraction was carried out with 1.0 ml 0.6 mol/l TCA containing 1 μg Zn.

Zinc space

Two ml of serum was mixed with 0.1 ml $ZnCl_2$ solution (1000 μg/l) containing approximately 40 000 counts/min $^{65}Zn^{2+}$ (Radiochemical Centre, Amersham). To this was then added 2 ml 0.6 mol/l TCA, the whole mixed carefully with a glass rod and centrifuged as under procedure (a). 1.0 ml of the supernatant was taken for counting in comparison with samples in which water was substituted for serum in the above procedure.

Results

Preliminary experiments showed that the presence of HCl, sulphate or TCA in zinc solutions made very little difference to flame absorbance. Solutions containing HCl were avoided in the case of carbon-cup atomisation, because of the risk of volatilisation. We could detect no significant contamination from any of the selected reagents or the (specially-washed) apparatus. Zinc standards made up from Zn granules or $ZnSO_4$ behaved identically.

TABLE I

COMPARISON OF ASSAY RESULTS ON 43 SERUM SAMPLES

	Zn concentration	
	Mean ± S.D. (μg/l)	% above (1)
(1) Exhaustive extraction	844 ± 125	0.00
(2) Carbon cup	863 ± 112	2.2
(3) Single extraction	888 ± 107	5.2
(4) Double extraction	1025 ± 157	21.3

TABLE II

PROGRESS OF EXTRACTION

Extract No.	% of total zinc extracted		Mean volume (ml)
	Mean in extract	Cumulative mean	
1	59.5	59.5	1.593
2	21.9	81.4	1.094
3	10.7	92.1	0.926
4	4.6	96.7	0.923
Residual water in washed precipitate	3.3	100	0.657

Comparison of methods

Table I presents a comparison of assay results by the four different methods, on the same set of 43 serum samples. Exhaustive extraction and carbon cup atomisation give the best agreement. Taking the former as the standard for comparison, single extraction gives results 5% higher and double extraction 21% higher.

Progress of extraction

For each individual sample, the progress of extraction of zinc fitted well to the assumption that, after the first incubation with TCA, all the extractable zinc was uniformly distributed over all the water present. This is not conclusive, however, since small deviations would be difficult to detect by this rather insensitive means.

Table II shows the mean percentage yield of zinc at each stage of exhaustive extraction and also the mean volume of each extract (there was considerable variation between samples). There was an overall deficit in recovery of water which was traced in part to evaporative losses, which should have minimal effect on the calculated results for initial zinc concentration, and in part to incomplete drying of the final washed precipitate (as checked in later experiments by use of the "drying pistol"), so that the mean figure for water retained in the precipitate is believed to be 0.1—0.2 ml too low. The effect of this is that the results by exhaustive extraction are believed to be about 0.5% low, but no correction has been applied for this as the basis is not sufficiently certain.

Zinc space

The ratio of (counts in plasma extract/counts in simple dilution) averaged 1.055 (8 pairs) and it made no difference whether the plasma was incubated with active Zn^{2+} overnight, or not. This suggests that in the plasma extract the active Zn^{2+} (and hence also extracted Zn^{2+}) was distributed in a volume of $4.1/1.055 = 3.886$ ml and therefore the volume not accessible to Zn^{2+} in aqueous solution was $4.1 - 3.886 = 0.214$ ml.

Recovery

Recoveries by the flameless method were virtually quantitative (98—100%). Recoveries by double extraction were more variable than would be pre-

dicted from the ordinary analytical precision. Procedure (iii) gave 121.8 ± 21.54% (22 experiments, mean ± S.D.). If the three worst results are excluded the conclusion is 121.9 ± 13.1%. Procedures (i) and (ii) gave results somewhat higher and somewhat more variable still.

Our experience suggests that recovery experiments in which zinc is added to serum should be interpreted with great caution. The variability of results might be accounted for by variable adsorption of zinc to the glass centrifuge tubes and/or by uneven distribution of added zinc. Alkaline zinc salts are, of course, effective protein precipitants: it seems possible that precipitation might occur in the vicinity of an added droplet which contained zinc at a relatively high concentration, and circumstances could be envisaged in which this might lead either to high or to low recovery figures. Some such phenomena may have led to Prasad et al. [2] finding recoveries close to 100% for double extraction. Procedure (iii) was used in an attempt to overcome these difficulties, but is itself not entirely free from objections. It will be noted that the zinc space experiments were with a relatively large volume of added zinc solution at low concentration. This would not have been entirely appropriate for work on analytical recovery.

Discussion

A possible error in the estimation of water-soluble species, arising from volume exclusion at a deproteinisation step, has been known for many years [3], but is usually ignored. It may be explained as follows, using a simple derivation which ignores volume changes and minor non-aqueous constituents. Suppose that a sample of serum (or other fluid) contains A ml protein plus B ml water. The concentration of a substance of interest, X, may then be expressed $x/(A + B)$, where x is the quantity of substance in the sample. To this sample we add C ml of protein precipitant, making a total volume of $A + B + C$. The actual overall concentration is $x/(A + B + C)$ and the dilution factor is $(A + B + C)/(A + B)$. We now separate the precipitated protein by filtration or centrifugation and estimate the concentration of X in the clear supernatant or filtrate: if our analysis was well done the result should be $x/(B + C)$. It is universal practice to calculate the result to be reported by multiplying this concentration by the above dilution factor,

$$[\mathrm{X}]_{\text{reported}} = \frac{x}{B + C} \times \frac{A + B + C}{A + B}$$

The result is inevitably too high, the difference being

$$\frac{x}{A + B} \cdot \frac{A + B + C}{B + C} - \frac{x}{A + B} = \frac{x}{A + B}\left(\frac{A + B + C}{B + C} - 1\right)$$

Expressed as a proportion of the original serum concentration, this becomes

$$\frac{A + B + C}{B + C} - 1 = \frac{A}{B + C}$$

The size of the error obviously depends on the actual values of A, B and C. Two examples will serve. (a) 1 ml of blood is deproteinised by the Folin acid-

tungstate method. A = (say) 0.2 ml, B = 0.8 ml, C = 9 ml. The error = 0.2/9.8 = 2% (negligible for routine purposes). (b) 2 ml of serum is deproteinized by Method (b) above. A = 0.21 ml, B = 1.79 ml, C = 2 ml. The error = 0.21/3.79 = 5.5%. The error is now big enough to be serious.

The situation is no different if separation of the protein-free filtrate from the precipitate is incomplete. We assay a sample of the total volume $B + C$, and assume that any fluid which remains in contact with the precipitate is of the same composition as that which we have managed to separate off.

Suppose, however, that we suspect X remains in part bound to the precipitate and therefore treat the precipitate with a second aliquot of the precipitant, intending to elute the residual X. Provided that we succeed in removing all the fluid from the precipitate on each occasion, no error is introduced, but if part of the fluid remains entrained within the precipitate the result is quite different as will now be shown. Our model is as before, except that after separation of the protein-free fluid a volume Q of the fluid remains entrained within the precipitate. Then the volume separated off is $B + C - Q$, the quantity of X in the separated fluid is $x(B + C - Q)/(B + C)$ and the quantity in the residual fluid is $xQ/(B + C)$. We now add a volume E of precipitant, and the X redistributes over the total volume of fluid available for it, namely $E + Q$, giving a concentration of $xQ/(B + C)(E + Q)$. We again separate off protein-free fluid. If on this occasion a volume R remains behind, entrained in the precipitate, the volume separated is $Q + E - R$, and the quantity separated is $xQ(Q + E - R)/(B + C)(E + Q)$. The total quantity of X separated off is now $x(B + C - Q)/(B + C) + xQ(Q + E - R)/(B + C)(Q + E)$, and the total volume is $(B + C - Q) + (Q + E - R)$ giving a concentration of $(x/(B + C))((B + C - Q) + Q(Q + E - R)/(Q + E))/((B + C - Q) + (Q + E - R))$. The size of the consequent error now depends on the dilution factor used. We have no "universal practice" to guide us, indeed the case may be unique. Prasad et al. [2] assumed in effect that the dilution factor is $(A + B + C + E)/(A + B)$. If we apply this, the error is

$$\frac{x}{B + C}\left(\frac{(B + C - Q) + Q(Q + E - R)/(Q + E)}{(B + C - Q) + (Q + E - R)}\right)\frac{A + B + C + E}{A + B} - \frac{x}{A + B}$$

and the proportionate error is

$$\frac{(A + B + C + E)((B + C - Q)(Q + E) + Q(Q + E - R))}{(B + C)(Q + E)(B + C + E - R)} - 1$$

If in this equation we substitute reasonable round figures (say, A = 0.2, B = 1.8, $C = E = Q = R$ = 1.0), the predicted proportionate error is $1.17 - 1$, or +17%. The potential error arising from volume exclusion has been greatly amplified!

Some readers may find it simpler to consider instead the residual fluid left in contact with the precipitate after the second extraction. This contains a concentration of X which is below the ideal value of $x/(B + C + E)$ (that was the objective of a second extraction). Therefore the combined extracts must have a concentration above the ideal value, i.e. this is not a representative sample.

If allowance is made for loss by evaporation at each of the two heating steps, the volumes lost being q and r respectively, the expression for proportionate

error becomes

$$\frac{(A+B+C+E)((B+C-Q-q)(E+Q-r)+Q(E+Q-R-r))}{(B+C-q)(E+Q-r)(B+C-q+E-R-r)}-1$$

Substitution in this expression of our actual results for extract volumes during "exhaustive extraction" together with best estimates for evaporative loss, volume of residual water in the washed precipitate and space non-available to zinc, gives, by chance, the same result as before (+17.0%), and indeed it will be found that the predicted error is markedly insensitive to changes in the values of parameters substituted. Even taking $B = 0$, equivalent to the assay procedure of Prasad et al. using lyophilised plasma samples, gives roughly the same predicted error, about +20%.

The above derivation has assumed that in and after the first extraction step, substance X becomes uniformly distributed over all the water present, and nowhere else, but it would be easy to modify the theory to take account of non-uniformity of distribution, adsorption to precipitated protein, etc. In the case of Zn^{2+} it will probably be thought more likely that some would remain bound to protein. Our results, both the agreement of "exhaustive extraction" with "carbon cup" results, and the pattern of Zn yield in successive extracts, suggest that such binding is not extensive. It cannot be denied, however, that a small and perhaps metabolically or diagnostically significant fraction might remain bound.

We recommend that the results of Greaves and Boyde [1] and any others by the same method or calibrated against it should be corrected by employing the factor 100/120, which is an approximate mean based on the three estimates of positive error (comparison of assay results, recoveries and theoretical). This gives a mean normal value of 15.08 μmol/l or 986 μg/l, much closer to the usually accepted results. If the correction is applied to the results of Prasad et al. [2], who introduced this particular procedure, we arrive at 775 μg/l as the mean normal level (for plasma, not serum).

Application of this correction, of course, has no effect on the validity or otherwise of the inter-group comparison of Greaves and Boyde, since all results are to be corrected by the same factor.

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