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Trace elements in autopsy tissue

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This article is in essence a compilation of recent trace element studies of human tissue carried out by the Trace Metals Unit at Glasgow Royal Infirmary. The paper deals with the rationale behind the need for tissue analysis, sampling, sample preparation and choice of a frame of reference for reporting trace element concentrations in solid tissues. New multi-element techniques such as ICP:MS (Inductively Coupled Plasma Mass Spectrometry) are becoming available and we present evidence for the validity of results obtained by ICP:MS. The multi-element nature of ICP:MS leads naturally to an explosion of data (multi-variate data) which, although generally advantageous, is open to statistical abuse. This usually takes the form of repeated applications of a univariate test which in turn results in the generation of type 1 errors (i.e. false positives). These errors can be controlled in a variety of ways. In this article we have avoided the false positive by the application of the Bonferroni correction and also by the use of linear discriminant analysis. These statistical treatments both demonstrate that the young and the aged are different in their trace elemental composition. In another study we demonstrate that tissue taken from patients in chronic renal failure and treated by haemodialysis can accumulate higher concentrations of the toxic elements chromium and aluminium. The clinical significance of these accumulations have still to be determined.

WHY ANALYSE TISSUE?

The diagnosis of trace element deficiency or excess is often made by reference to the concentration of the element found in peripheral whole blood or serum, since these are by far the most easily available samples.

Total reliance upon serum concentrations is open to criticism for several reasons:

1. The amount of any trace element in the serum extra-cellular compartment represents only a very small fraction of the total body content. For example, there is about 80 mg of copper in the average 70 kg adult (Cartwright and Wintrobe, 1964) of which around 3 mg or about 4% is in the serum pool. For zinc, Babcock *et al.* (1982) have estimated the whole body content of zinc to be about 1·5 g, but the zinc component of the serum pool is about 2·5 mg. This means that the body compartment sampled to assess the zinc status of a person contains only some 0·2% of the total body zinc.
2. Trace element concentrations in serum can vary independently of the the intracellular concentration depending on quite diverse factors such as fasting, circadian rhythm, acute phase response to infection or injury, medication, disease, and even the season of the year.
3. Trace element concentrations in serum are much lower than those in many soft tissues. This can pose significant problems due to contamination during sample collection and preparation. The concentrations of many biologically important elements present in serum are close to the detection limits of most available techniques, resulting in considerable analytical imprecision.
4. The metabolic actions of both essential and toxic elements take place within cells. To establish the trace element status of an individual person it would be preferable that cellular material should be assayed. It is difficult to obtain samples of soft tissue from living persons, although small amounts can be obtained during surgery in certain circumstances.

Needle biopsy samples can be obtained from liver, bone, muscle and kidney but these yield very small samples which can be unrepresentative of the organ being sampled. The techniques are invasive and not generally available for trace element analysis unless the procedure is required for other diagnostic purposes.

Cells can be obtained from peripheral whole blood and separated into fractions such as erythrocytes, and

various sub-divisions of white cells. Trace element analysis of these cellular fractions is possible but the techniques of cell separation are difficult and subject to error. Specialised laboratories have obtained reproducible results in control populations. However, the number and type of cells in the white cell sub-fractions can be affected by infection and disease independently of nutritional status making the interpretation of trace element concentrations in such cells difficult.

Soft tissue in amounts likely to be representative of the organ being sampled can be obtained at autopsy. (However the provisions of the Human Tissues Act (1961) must be observed.) Even although soft tissue analysis may therefore not be widely used in clinical practice it can be useful in a variety of ways: (1) To relate the actual tissue concentration of an element to the observed serum concentration, and thus aid in the interpretation of serum concentrations in different patient groups. (2) To study marginal nutritional deficiencies or toxic exposure in general populations, people in certain occupations, or patients being treated by a variety of clinical methods. (3) To study patterns of soft tissue concentrations of essential or toxic elements in specific diseases to determine whether the changes are part of the disease process or a secondary consequence thereof.

SAMPLE PREPARATION

Tissue sampling

Autopsy material, 25–50 g wet weight, is removed within 48 hours of death. Samples are taken in the mortuary with stainless steel scalpels, put into clean plastic containers and frozen. Prior to analysis, the samples are partially thawed and the exterior of the sample, and all gross fatty and connective tissue, trimmed off using a titanium knife. At this stage a piece of tissue may be taken 'fresh' for selenium analysis or for assay of any other element that might be considered volatile. The remainder is weighed, refrozen in liquid nitrogen, freeze dried and weighed again. If the sample is sufficiently large it is reduced to a powder in a domestic food blender (plastic and re-equipped with titanium blades). The dried, powdered sample can then be stored until analysis.

Digestion procedure

There are as many digestion procedures as there are analytical chemists. Wet digestions involving nitric, sulphuric, hydrofluoric and perchloric acids and hydrogen peroxide, either alone or in some combination are the most commonly used. The digestions can be performed at atmospheric pressure in an open vessel or under pressure in a 'bomb'. Heating can be achieved by electrical resistance or by using a microwave oven. Depending on the analytical technique to be employed the sample may or may not require complete mineralisation. Techniques such as atomic absorption spectrometry (AAS) and inductively coupled plasma mass spectrometry (ICP:MS) require only simple solubilisation of the sample, whereas colorimetric or fluorimetric methods usually require the complete destruction of all organic residues.

Our digestion procedure is as follows: Samples of freeze-dried autopsy material or of appropriate reference materials are weighed (0·500 g) into teflon 'bombs' (60 ml, Savillex Corp., Minnesota, USA) and nitric acid (1 ml, redistilled from a sub-boiling Teflon still) is added together with the indium internal standard (1 ml, 1·25 mg litre^{-1}). The indium standard is added at this point, rather than immediately before mass spectrometry, in order to compensate for any aerosol losses on opening the 'bomb' or losses due to incomplete transfer of the digest. The 'bombs' are placed, six at a time, into a domestic microwave oven and heated for 2 min. at full power (600 W). After cooling, the 'bombs' are opened and the contents washed into an acid-washed plastic vial and made up to volume (25 ml). This digestion procedure does not oxidise all the fat contained in some tissue samples and any undigested globules of fat can be removed with a disposable plastic pipette, or, because fat usually sticks to the wall of the container, the digest can be decanted into a clean container. The incomplete destruction of any fat has little or no effect on the accuracy of the results as judged by the good agreement found with the certified values of the reference materials. Earlier attempts to dissolve the fat by including hydrogen peroxide in the digestion were successful, but were abandoned after the failure of several 'bombs' during the digestion process. We find the present procedure to be rapid, free from reagent contamination and safe when nitric acid alone is used for digestion.

Since volatile forms of selenium present in tissue may be lost during freeze-drying, only fresh frozen tissue is used for this determination. Selenium is determined by molecular fluorescence, which requires complete mineralisation of the sample by an open beaker digestion with nitric and perchloric acids. The method followed was basically that of Ohlsen *et al.* (1975).

REPORTING RESULTS

There are many ways in which trace element concentrations in tissue can be expressed. The usual conventions are μg/g of ash, μg/g wet weight and μg/g dry weight. Early researchers often used the ash weight as a frame of reference. This was convenient as the techniques used in the 1950s were spark source optical emission and mass spectrometry, both of which performed best with dry ashed samples. The choice of frame of reference is often driven by the method of analysis to be

employed. It may be convenient to dry samples to allow long-term storage and use dry weight as a reference. However, possible loss of volatile species such as organo-mercury or selenium compounds must be considered. The wet weight of a sample may be altered as a result of fluid drainage prior to analysis, and for small biopsy samples water evaporation could be significant and render the result reported on a wet weight basis meaningless. The ideal would be to report the amount of the element per unit of *'active cell mass'*. This has led to the use of indices, such as tissue nitrogen, tissue DNA and, for peripheral blood fractions, cell counts. We have tried using the tissue nitrogen content as a measure of active cell mass. In tissue from accident victims we found that the coefficient of variation for element concentrations expressed per gram of nitrogen was slightly larger than when expressed as dry weight or wet weight (Lyon *et al.*, 1989). Presumably the error from Kjeldahl nitrogen analysis added to the total analytical imprecision. The range of concentrations for both iron and zinc in liver tissue of the elderly was found to be much greater than that in young healthy accident victims, see Table 3. Could this result from a greater variability of the active cell mass in the elderly? The zinc and iron concentrations for all accident victims and all the elderly are plotted against the nitrogen content of the tissue in Figs 1a,b. As there is no correlation between zinc or iron concentration and nitrogen content we do not consider tissue nitrogen as a useful frame of reference. We report our results on a μg/g dry weight basis.

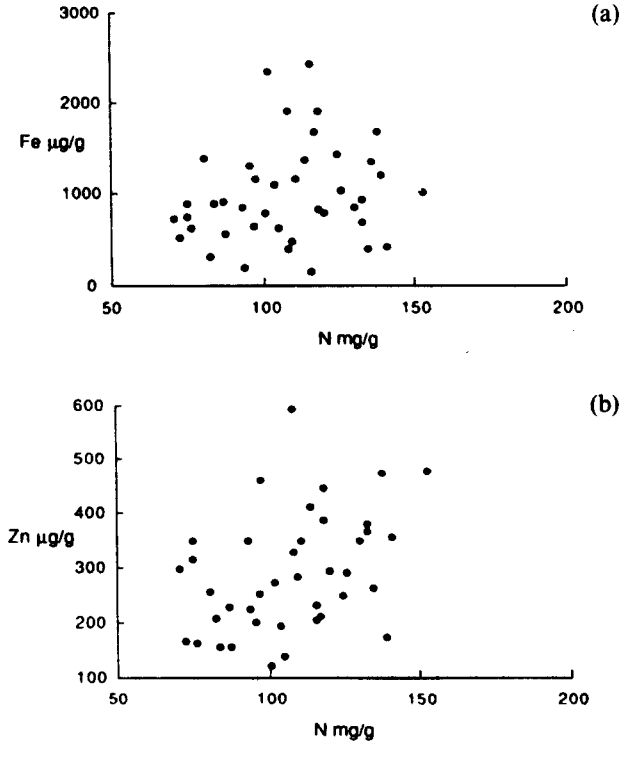

Fig. 1. Plot of (a) iron and (b) zinc concentrations against nitrogen content for young healthy and aged liver samples.

MULTI-ELEMENT ANALYSIS OF TISSUE DIGESTS BY ICP:MS

The inductively coupled argon plasma (ICP) was first used successfully as an ion source for a mass spectrometer by Houk *et al.* (1980) and became commercially available in 1983. This technique is an exciting development in the field of trace element measurement. It combines the sensitivity and multi-element capability of the mass spectrometer with the simple and rapid sample introduction of the ICP torch. It is as simple to obtain a spectrum of much of the periodic table as it is to make a single flame atomic absorption measurement. However, the resulting spectrum does require a degree of interpretation. Because the mass analyser currently used in such systems is a low-resolution quadrupole, isobaric species are not resolved. Polyatomic species (arising from the sample matrix and plasma gas) isobaric with analyte ions can cause seriously erroneous results. The most common examples of such interferences occurring in the biological matrix are shown in Table 1. Some biologically important elements like chromium and selenium cannot be measured directly and may have to wait until another type of plasma such as the helium-ICP is developed. For these reasons we determine chromium by ETA-AAS and selenium by molecular fluorescence.

The validity of any new technique must be established before it can be generally accepted for routine work. This can be done by analysing the appropriate standard reference materials (SRM) and also by comparison with an accepted method employing a different principle.

Analysis of reference materials

The reference materials, NBS:SRM 1577a (bovine liver), IAEA H-8 (kidney) and IAEA H-4 (animal muscle)

Table 1. The main polyatomic ions found in the biological matrix

Mass	Cl^- containing	Other	Isotope
51	$^{35}ClO^+$	$^{34}SOH^+$	^{51}V
52	$^{35}ClOH^+$	ArC^+	^{52}Cr
53	$^{37}ClO^+$		^{53}Cr
54	$^{37}ClOH^+$	ArN^+	^{54}Cr, ^{54}Fe
56		ArO^+	^{56}Fe
63		PO_2^+, $ArNa^+$	^{63}Cu
65		$H^{32}SO_2^+$	^{65}Cu
70	$^{35}Cl_2^+$		^{70}Zn, ^{70}Ge
72	$^{35}Cl^{37}Cl^+$		^{72}Ge
74	$^{37}Cl_2^+$		^{74}Ge, ^{74}Se
75	$Ar^{35}Cl^+$		^{75}As
76		$Ar^{36}Ar^+$	^{76}Se
77	$Ar^{37}Cl^+$		^{77}Se
78		$Ar^{38}Ar^+$	^{78}Se
80		Ar_2^+	^{80}Se
82	$C^{35}Cl_2^+$		^{82}Se

were chosen as being the most suitable for validating the analysis of autopsy tissue by ICP:MS. The agreement with the certified values is good.

Comparison of analytical methods

The traditional way in which analytical chemists compare two methods is to plot the methods against one another for each sample analysed. The association between the two methods is then obtained by performing a simple linear regression on the points. The new method is regarded satisfactory if the product moment correlation r is close to +1, the confidence interval for the slope of the line includes 1 and the confidence interval for the intercept includes 0. Simple regression analysis requires that: (1) there is no random error in the regressor variable (x-axis) (it is usual to plot the established technique on the x-axis) and (2) the data points are evenly distributed along the regression line. The first requirement is never satisfied nor is it always possible to satisfy the second. Bland and Altman (1986) described a statistical test which is specifically designed to assess agreement between two methods. In this test the between-method difference (Δ) for each sample is plotted against its mean value. Ideally, the resulting data points should lie with a normal distribution about the horizontal line through zero difference. The 95% normal range for the differences defines the 'limit of agreement' while the mean difference can be thought of as the 'bias'. This test makes no assumptions nor is it affected by the distribution of the sample concentrations.

Comparison of ICP:MS with AAS

The two statistical tests outlined above are illustrated by applying them to the determination of iron. Tissue samples, liver, kidney, heart and muscle, collected at autopsy from both young healthy accident victims and from elderly subjects ensured that the full range of possible concentrations was covered. The plot for the linear regression is shown in Fig. 2a. The computed regression equation (±2SD) is:

$$Fe_{ICP:MS} = 0{\cdot}965(\pm 0{\cdot}028)\ Fe_{AAS} + 6{\cdot}9(\pm 26)$$

The 95% confidence interval (CI) for the slope is 0·94 to 0·99. As the confidence interval does not include 1 the slope of the regression equation is significantly different from unity ($p < 0{\cdot}05$) and a systematic error in one or other of the methods is indicated. This is not the case. Statistical analysis by the Bland and Altman plot (Fig. 2b) shows that the differences fall into two populations, thus invalidating simple regression analysis. Subsequent plots for the two populations show that up to a mean value of about 400 μg g^{-1} (Fig. 2c) there is very close agreement between the two methods while above 400 the differences suddenly increase (Fig. 2d). We attribute this to the fact that dilutions become

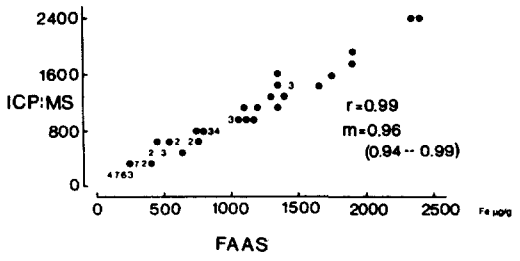

Fig. 2a. Linear regression plot for iron. The numerals refer to the number of overlying points.

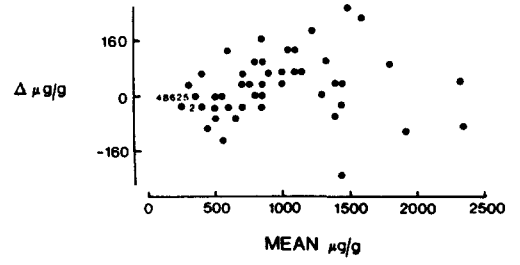

Fig. 2b. Bland and Altman plot for iron. The numerals refer to the number of overlying points.

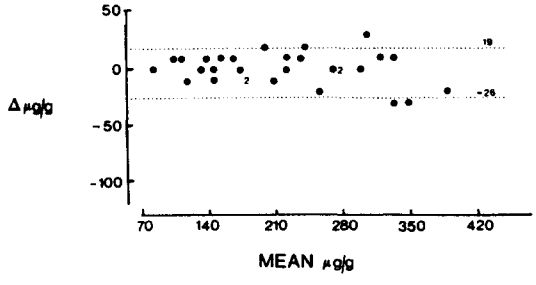

Fig. 2c. Bland and Altman plot for iron <400 μg g^{-1}.

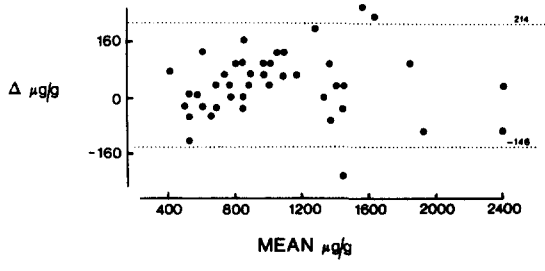

Fig. 2d. Bland and Altman plot for iron (>400 μg g^{-1}) after a ×10 dilution of the digest for FAAS analysis.

necessary for flame AAS at this point. Below 400 μg g^{-1} the mean difference is −3·7 μg g^{-1} (CI, −12 to 4·6) i.e., there is no significant bias, and the limit of agreement is −26 to 19 μg g^{-1}. Above 400 μg g^{-1} there is a significant bias of 34 μg g^{-1} (CI, 6 to 61) and a limit of agreement of −146 to 213 μg g^{-1}. Simple regression failed to reveal this constant bias. A summary of the Bland and Altman plots for Al, Mg, Mn, Cu, Zn, and Cd is shown in Table 2.

As can be seen from the table the level of agreement between the two methods is good. The concentration range that ICP:MS can cope with extends to about six orders of magnitude. This gives an advantage in terms

Table 2. Summary of Bland and Altman plots for Al, Mg, Fe, Cu, Zn and Cd. The bias is AAS–ICP:MS

Element	Limit of agreement ($\mu g\ g^{-1}$ dry wt)	Bias ($\mu g\ g^{-1}$ dry wt)	Comment
Al	−0·36 to +0·39	none	CN+ (mass 27) not a problem
Mg	−114 to +122	none	
Mn	−0·98 to +0·85	none	
Fe < 400	−26 to +19	none	
Fe > 400	−146 to +213	+34	const. bias, dilution error affecting AAS?
Cu	−2·2 to +2·1	none	
Zn	−20 to +10	−8	const. bias, SO_2H^+ affecting ICP:MS?
Cd	−1·7 to +3·9	none	

of ease of use and improved precision at higher concentrations. The positive bias towards AAS for iron only occurs at high concentrations (Fe >400 $\mu g\ g^{-1}$) and can probably be attributed to a constant error involved in the extra step required by flame AAS. The negative bias towards AAS (positive towards ICP:MS) observed for zinc could be the result of an, as yet, unidentified polyatomic species. SO_2H^+ is a possibility, but nebulisation of sulphuric acid solution equimolar with the sulphur concentration of the digests did not produce direct evidence of this species in sufficient quantity to explain this bias.

RESULTS OF PATIENT STUDIES

Relationship between serum and tissue concentrations

Selenium

Serum selenium concentrations have been measured in four groups: elderly outpatients (80 ± 15 yrs), elderly inpatients (75 ± 9 yrs), younger outpatients (51 ± 6 yrs) and in young well controls (<40 yrs). The results are shown in Fig. 3. The mean value of the elderly inpatient group is 0·877 μmol litre^{-1} (CI: 0·81–0·94), the elderly outpatients, 1·16 μmol litre^{-1} (CI: 1·0–1·3), the younger outpatients, 1·29 μmol litre^{-1} (CI: 1·17–1·40) and young healthy controls (<40 yrs) 1·65 μmol litre^{-1} (CI: 1·07–1·29). The tissue selenium results for a different group of elderly patients and younger controls are shown in Table 3. The elderly have lower selenium concentrations in all of the tissues examined, although the differences observed for heart and muscle do not reach statistical significance. For this element the observations in serum are reflected and confirmed by the tissue analyses, suggesting that selenium depletion does occur in the elderly.

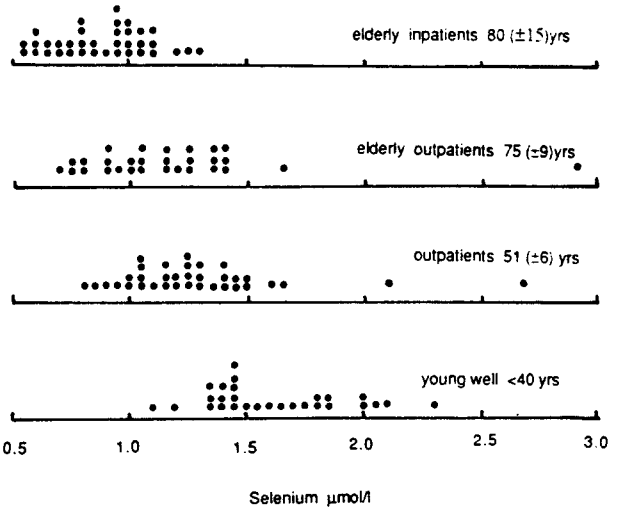

Fig. 3. Serum selenium concentrations in elderly inpatients, elderly outpatients, younger outpatients and the well young.

Table 3. Summary of differences found between the young and the elderly

Element	Units	Organ	Mean (±SD)		p	95% confidence interval
			Young	Elderly		
K	mg/g	Muscle	16·6 (±2·6)	12·1 (±2·6)	$p < 0·001$; t = 5·2	2·8–6·4
K	mg/g	Heart	14·9 (±1·6)	11·5 (±2·1)	$p < 0·001$; t = 5·9	2·2–4·6
Mg	µg/g	Muscle	943 (±143)	724 (±123)	$p < 0·001$; t = 4·7	122–318
Mg	µg/g	Heart	981 (±147)	853 (±128)	$p < 0·001$; t = 4·9	93–228
Mn	µg/g	Kidney	5·9 (±1·6)	2·7 (±1·0)	$p < 0·001$; t = 6·5	2·1–4·2
Zn	µg/g	Liver	201 (±52)	305 (±108)	$p < 0·01$; F^a = 4·2	
Fe	µg/g	Liver	745 (±365)	1060 (±553)	$p < 0·05$; t = 2·2	21–608
Cu	µg/g	Heart	16·2 (±1·5)	13·2 (±2·0)	$p < 0·001$; t = 5·4	1·8–4·2
Al	µg/g	Heart	0·30 (±0·12)	0·65[b]	$p < 0·001$[c]; t = 3·5	
Cr	ng/g	Heart	0·006[b]	0·018[b]		
Se	µg/g	Muscle	0·72 (±0·17)	0·58 (±0·18)	$p < 0·05$; t = 2·5	0·02–0·27
Se	µg/g	Liver	1·8 (±0·28)	1·26 (±0·45)	$p < 0·001$; t = 4·5	0·27–0·72
Se	µg/g	Kidney	5·68 (±1·04)	3·2 (±1·0)	$p < 0·001$; t = 7	1·8–2·3

[a] Fisher's F test for comparing SDs.
[b] Median.
[c] Test performed on log transformed data.

Zinc

Serum zinc concentrations are generally lower in more elderly populations. Stafford *et al.* (1988) found a mean of 10·9 μmol litre^{-1} for a group of 226 geriatric patients and Bunker and Clayton (1989) established a mean of 11·0 μmol litre^{-1} (n = 24) for healthy elderly people and a mean of 11·3 μmol litre^{-1} (n = 20) for the housebound elderly. These levels are lower than the 12–18 μmol litre^{-1} range found in 50 laboratory staff (mean age 36) at the Royal Infirmary. The concentrations of zinc found in the soft tissues of elderly patients were not low relative to our younger control group. Indeed, the results for liver, Table 3, show that many of the elderly have increased zinc concentrations. This suggests that there may be an alteration in the plasma transport and protein binding of zinc in the elderly rather than intracellular depletion.

Iron

Bunker and Clayton (1989) found that many of the housebound elderly have clinical signs of iron deficiency. We do not find tissue iron in the elderly to be low relative to young controls; on the contrary, we find that many elderly patients have increased liver iron concentrations (Table 3). It is possible that the low serum iron concentrations observed in the elderly are also related to changes in the plasma transport and binding of the metal.

Tissue trace metals in renal disease

Patients with end stage renal disease cannot efficiently excrete metals normally lost via urine. Metals may be ingested in the diet or inadvertently given intravenously during dialysis therapy. Changes in blood serum concentrations are only an indirect guide to net tissue accumulation or depletion. Measurements of metals in tissue obtained at autopsy have shown a wide range of abnormalities (Smythe *et al.*, 1982). The changes were considered to be predominantly due to renal failure *per se*, rather than haemodialysis or other therapies. However, our earlier work suggested that contamination of dialysate with metals such as aluminium and chromium results in net transfer of these metals to the ECF of renal patients (Fell, 1986). We now show a progressive increase in soft tissue concentrations of

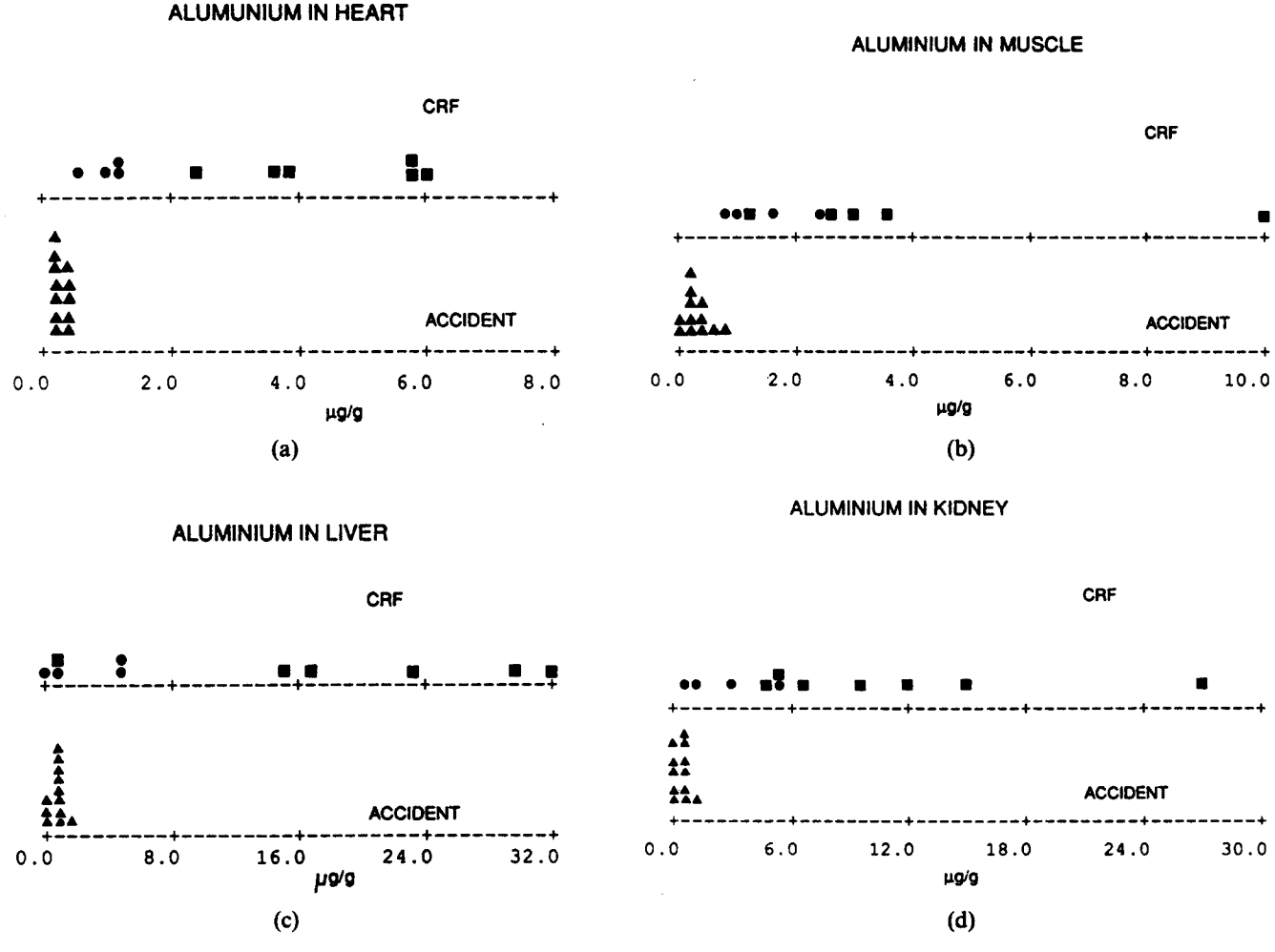

Fig. 4. Comparison of aluminium in tissue from patients with chronic renal failure with young accident victims. (●) undialysed and (■) dialysed: (a) heart, (b) muscle, (c) liver and (d) kidney.

aluminium and chromium when samples obtained from accident victims, chronic renal failure cases (CRF) and patients treated by dialysis are compared, Figs 4 and 5. The clinical signficance of retention of these and other metals is not yet clear.

Tissue trace elements in the elderly

Concentrations of K, Mg, Al, Cr, Mn, Fe, Cu, Zn and Se in autopsy samples of kidney, liver, heart and skeletal muscle from 33 aged subjects (14 male, 19 female; mean age 80 years) have been compared with tissues from 12 young healthy accident victims, mean age 29 years. Comparisons are summarised in Table 3. Potassium and magnesium were found at significantly lower levels in muscle and heart but not in liver and kidney. Selenium was lower in liver and kidney but the differences for muscle and heart did not reach statistical significance. Manganese in kidney was depleted in the elderly. Zinc and iron show no evidence for depletion in any of the tissues but there is evidence for increased deposition in the liver of some individuals.

Copper was lower in heart tissue, some individuals having only about 50–75% of that found in healthy young accident victims. There is a suggestion that copper deficiency may be a risk factor in coronary heart disease (Klevay, 1980).

Both aluminium and chromium have skewed distributions and there is a tendency for increased concentrations in all tissues of most of the elderly subjects, suggesting that these metals are not functioning under homeostatic control as essential elements.

Multiple comparisons

The probability that there is no difference between the two groups, young and old, was determined largely by repeated application of the 't' test. When a univariate test is used repeatedly to make multiple comparisons the chance of throwing up false positive differences has to be taken into account. This can be done by making the Bonferoni correction; this changes the probability level, below which a difference is considered significant, from the usual $p < 0{\cdot}05$ to $0{\cdot}05/(n)$ where n is the number of comparisons. Here, the number of comparisons is 36, so the probability level that has to be reached before we can be confident that there really is a statistically significant difference is $p < 0{\cdot}0014$. Most of the

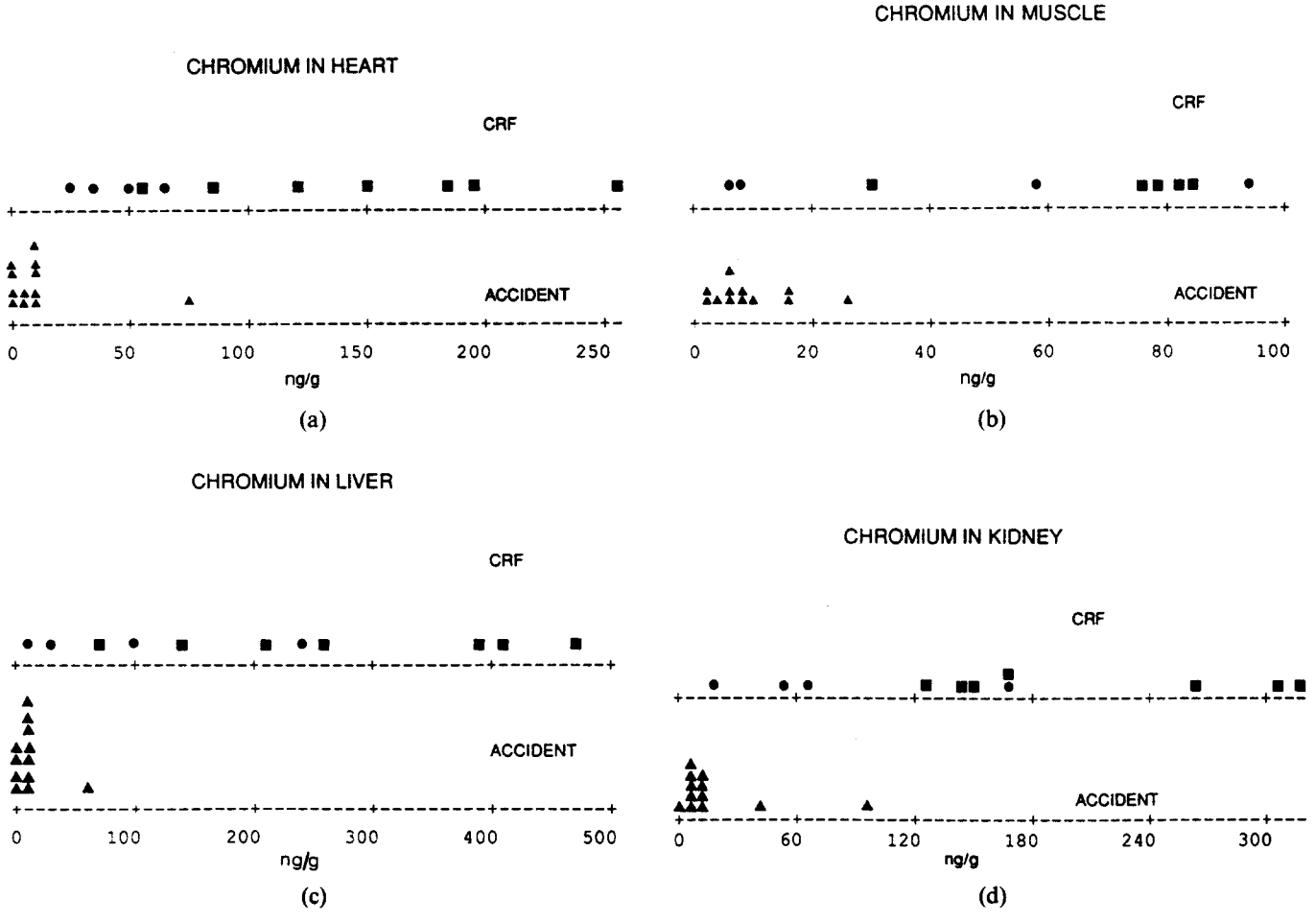

Fig. 5. Comparison of chromium in tissue from patients with chronic renal failure with young accident victims. (●) undialysed and (■) dialysed: (a) heart, (b) muscle, (c) liver and (d) kidney.

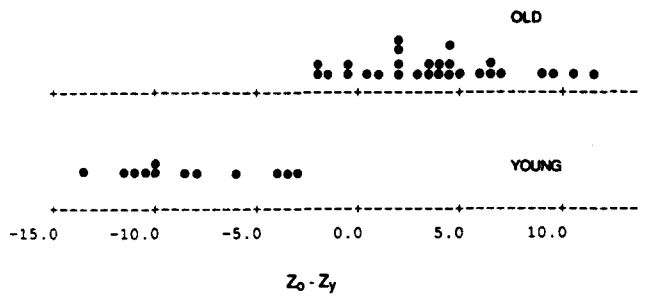

Fig. 6. Plot of the difference in the linear discriminant functions for accident victims and for the aged.

comparisons we find have p values <0·001 and therefore are significant.

An alternative way of treating multivariate data, and so avoiding multiple comparisons, is to use a statistical technique which reduces the number of variables to one. This can be done by linear discriminant analysis. The data from the elderly and young were subjected to the discriminant analysis offered by the MINITAB® statistical package. The function chosen was a linear combination of:

Muscle K + heart K + kidney K + liver K + muscle Se + heart Se + kidney Se + liver Se + heart Cu

In Fig. 6 the difference between the two linear discriminant functions for the two groups is plotted. The difference is obvious and we can be confident that the elderly who have died of disease are different from the young healthy accident victims.

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