Determination of copper and zinc in blood plasma by ion chromatography using a cobalt internal standard

Edmund Lane,[a] Alexis J. Holden*[a] and Robert A. Coward[b]

[a] *Centre for Toxicology, University of Central Lancashire, Preston, UK PR1 2HE. E-mail: a.j.holden@uclan.ac.uk*
[b] *Dialysis Unit, Royal Preston Hospital, Sharoe Green Lane North, Fulwood, Preston, UK PR2 9HT*

Received 12th November 1998, Accepted 13th January 1999

Ion chromatography was used to detect levels of copper and zinc in blood plasma from renal dialysis patients on continuous ambulatory peritoneal dialysis (CAPD) and haemodialysis (HD). The developed method used cobalt as an internal standard and when combined with the standard additions method improved the overall precision of the results by between −0.3 and 6.0% and −0.8 and 5.7% for copper and zinc, respectively. The method was compared with inductively coupled plasma optical emission spectrometry (ICP-OES) and the results indicated no significant difference between the two methods with or without an internal standard. Without an internal standard, t_{calc} was 0.869 with a t_{crit} of 2.201 ($n = 12$, $P = 0.05$) and with an internal standard, t_{calc} was 0.189 compared to a t_{crit} of 2.201 ($n = 12$, $P = 0.05$). The copper and zinc levels in blood plasma in both dialysis groups were not significantly different to the copper and zinc levels in blood plasma of the control patients. A convenient method of analysis of trace elements in blood such as ion chromatography with UV/VIS detection is useful for determining whether inorganic elements may be disrupted in the body due to changes in the state of health.

Introduction

The trace elements copper and zinc serve chiefly as key components of proteins and of enzyme systems in the human body and are vital for their proper functions.[1] Zinc is involved in the synthesis of nucleic acids and proteins and is necessary for growth.[1] The majority of these enzymes act as antioxidants in biological systems [*e.g.*, Cu, Zn in superoxide dismutase (SOD) and Cu in caeruloplasmin]. Deficiencies or excess of the trace elements may have a detrimental effect on the function of these enzymes.[2,3] Levels of many trace elements are disturbed in various diseases, *e.g.*, abnormal levels of copper and zinc have been found in patients on renal dialysis,[4–8] lowered copper levels are associated with Wilson's disease and hypocupremia is observed with leukaemia and with a number of acute diseases.

The detection of trace elements in blood is an important factor for determining any disruption in trace element status of the body. The method used to determine elemental levels needs to be sensitive enough to detect the amounts present in blood (levels of Cu and Zn ~1 mg dm^{-3}) in an all too often limited sample volume.[3] Analysis of trace elements in biological samples requires: precise and accurate separation of the analyte from the bulk matrix for some measurements, a suitable means of detection which is of the appropriate sensitivity and to be free of contamination. Careful consideration of factors such as diet and disease state are critical in the assessment of the results before any conclusions can be made about cause–effect relationships for the disruption of metal status.[3]

Atomic absorption spectrometry (AAS) is widely used for the detection of many elements. Techniques such as flame atomic absorption spectrometry (FAAS) and electrothermal atomic absorption spectrometry (ETAAS) are suitable for the detection of Zn and Cu in blood serum and plasma. With the development of such routine methods often samples only require dilution.[9] Detection limits for flame AAS have been reported to be as low as 9.4 μg dm^{-3} for zinc[10] and 0.2 μg dm^{-3} for copper when preconcentration techniques have been employed.[11] Detection limits for ETAAS are typically 0.4 μg dm^{-3} and 0.1 μg dm^{-3} for Cu[12] and Zn,[13] respectively. However, for most of the AAS techniques, only one element can be determined at a time in many routine laboratories, although the relatively new Perkin-Elmer SIMAA 6000 method of simultaneous multi-element ETAAS is able to detect up to six elements with the same detection limits as conventional ETAAS analysis due to the very low noise of the solid-state detector.[14] Inductively coupled plasma optical emission spectrometry (ICP-OES) and mass spectrometry (ICP-MS) can be used in the direct analysis of biological fluids. The advantages include simultaneous detection of several elements in the one sample and its ability to overcome matrix interferences.[15] The sample is efficiently desolvated, vaporised, excited and ionised. Many chemical interferences are greatly reduced by the high temperature of 7000–8000 °C. Typical levels of detection range from < 0.5 to 100 mg dm^{-3} for most metals. One of the major advantages of ICP-MS over ICP-OES is a three orders of magnitude higher sensitivity for metal detection although there may be problems due to the matrix.[15] Ion chromatography has been increasingly used for the determination of metals and is becoming an alternative to conventional spectrometric methods.[16,17] The main advantage is that multiple elements can be simultaneously determined in one sample using similar sample pre-treatment methods. Ion chromatography can separate a mixture of ions by their net charge according to the principles of ion exchange. Several detection methods are available including conductance and detection by UV by the use of a post column derivatisation reagent. Ion chromatography is suitable for small sample volumes, with injection volumes of 25 μl per analysis. Detection limits of less than 13 μg dm^{-3} have been reported by a number of workers.[18,19]

The current study involved the development of an ion chromatography (IC) method which would enable the determination of copper and zinc in the same aliquot of a sample. The final method made use of a cobalt internal standard which was used to minimise errors due to sample loading and fluctuations,

which occurred during the running of the column. The peak area results for the copper and zinc peaks were expressed as a ratio of the peak area result from the cobalt peak. Hence, if a varied volume of sample was injected or there was a variation in the flow rate of the mobile phase or the detector did not respond in a uniform manner throughout all the chromatography runs, then the results could still be used. The cobalt peak would be affected in the same manner as the copper and zinc peaks and so the ratio of the peak areas would remain the same although the raw peak area data values would change according to the inconsistencies within the instrument. Hence the use of an internal standard in analyses allows the use of data which may otherwise be deemed worthless. The method of standard additions was also used. To test the developed method a number of blood plasma samples from patients on haemodialysis (HD) and continuous ambulatory peritoneal dialysis (CAPD) as well as control samples were analysed.

Materials and methods

Specimens

Blood samples from eight renal dialysis patients, four on HD and four on CAPD, plus four control samples were obtained from the Royal Preston Hospital. The blood samples were collected from patients on renal dialysis in S-Monovette LH-Metall-Analytik tubes (Sarstedt Ltd., Leicester, UK) which contained lithium–heparin as an anticoagulant. These tubes are recommended by the suppliers for the collection of blood plasma which is to be analysed for analytes such as copper, zinc, lead, manganese, cadmium, iron, aluminium, nickel, selenium, chromium and mercury. The plasma was removed from the blood sample by centrifugation and the samples stored in the refrigerator at 4 °C before use. The time between sample collection and centrifugation was between 1–2 h because of the staggered collection time on the hospital ward. The dialysis subjects ranged in age (35–60 years) and in the number of years that they had received dialysis (2–25 years) (Table 1). The control samples were age matched where possible.

Reagents

All chemicals were of analytical-reagent grade and where possible all analyses were carried out in plasticware to reduce the risk of contamination during storage, transport and analysis. All containers were acid washed by soaking in 10% nitric acid (BDH-Merck, Poole, Dorset, UK) overnight and then in deionised water (Barnstead E-Pure, Fisons, Loughborough, Leicestershire, UK, 17.6 mΩ cm^{-1}) overnight. Stock standard solutions containing 1000 mg dm^{-3} of copper, zinc and cobalt were diluted to 5 mg dm^{-3} working solutions. All these solutions were prepared daily from copper, zinc and cobalt atomic absorption spectrometric standards (Fisher, Loughborough, Leicestershire, UK).

Table 1 Details of the patient sample used in each dialysis and control group

Dialysis type	Male	Years on dialysis	Female	Years on dialysisysis
CAPD	38	1	50	2
	73	10	52	11
HD	54	1	33	3
	70	4	52	1
Control	34	N/A	69	N/A
	50	N/A		
	61	N/A		

Instrumentation

A 1 cm^3 sample of the blood plasma sample was digested with 70% nitric acid in a ratio of 1:3 in pressure sealed Teflon bombs (150 cm^3 CEM, Buckingham, UK). An MDS 8D microwave (CEM) was used at 70% power for a total of 60 min in 3 × 20 min periods allowing the samples to cool for 10 min between each 20 min period. The digested material was diluted to 10 cm^3 with deionised water. For the standard additions method, four 1 cm^3 aliquots of the plasma digest were spiked with increasing amounts of copper (0, 0.2, 0.4, 0.8 cm^3 from the working solution) and zinc (0, 0.2, 0.4, 0.8 cm^3 from the working solution). A 0.6 mg dm^{-3} cobalt internal standard (0.6 cm^3 from the working solution) was also added to each sample. The solutions were made up to 5 cm^3 with deionised water.

Measurements were performed in triplicate on a Dionex DX500 ion chromatography system. A 25 cm HPIC-CS5 cation exchange column (Dionex, Camberley, Surrey, UK) with a 5 cm HPIC-CG5 guard column (Dionex) and a 25 μl sample loop was used. The column packing consisted of a cross-linked styrene and vinylbenzene polymer with sulfonic functional groups. The eluent was 50 mM oxalic acid and 95 mM lithium hydroxide at pH 4.8 (BDH-Merck) at a flow rate of 1 cm^3 min^{-1}. The post-column reagent used was 0.3 mM 4-(2-pyridylazo) resorcinol (PAR) (Dionex) in 1 M acetic acid and 3 M ammonium hydroxide solution at a flow rate of 0.7 cm^3 min^{-1}. The detection was at 520 nm using a Dionex A20 absorbance detector (Dionex).

An inductively coupled plasma optical emission spectrometer (ICP-OES) (Spectro, Halesowen, Worcestershire, UK, Analytical Model P) was used to detect copper and zinc. Copper was detected at 324.8 nm and zinc at 213.9 nm. The sample was introduced into the plasma at a flow rate of 1 cm^3 min^{-1} through silicone tubing *via* a peristaltic pump. Five replicate measurements were made and approximately 0.5 cm^3 of sample was required per sample. The system was flushed with deionised water between individual solutions.

Results and discussion

Copper, zinc and cobalt were easily separated by ion chromatography with elution times of 2.9, 4.8 and 6.6 min, respectively, as shown in Fig 1. With the oxalate eluent used it is reported that only cations lead, copper, zinc, manganese, cobalt, cadmium and nickel are detected. It was hypothesised that either iron(II) or iron(III) may interfere with the analysis of zinc or copper but preliminary experiments suggested that this was not the case. The cobalt internal standard was subjected to the same conditions as the sample, hence any fluctuations in the running conditions would be eliminated by plotting the absorbance ratio between copper and cobalt against the concentration of spiked copper. Similarly for zinc, the absorbance ratio between zinc

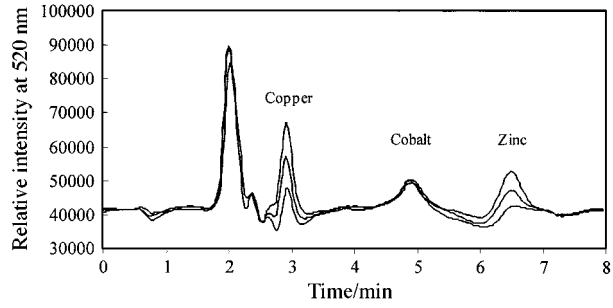

Fig. 1 Chromatograms of spiked blood plasma. Copper, cobalt and zinc eluted at 2.9, 4.8 and 6.6 min, respectively. Each of the three samples was spiked with cobalt (0.6 mg dm^{-3}) and with increasing concentrations of copper and zinc (0, 0.4, 0.8 mg dm^{-3}).

and cobalt against the concentration of spiked zinc was plotted. By using cobalt as an internal standard it is suggested that the ratio of the analyte to the internal standard provides a more accurate determination than the use of the analyte response alone. The precision was further improved by combining the internal standard method with the method of standard additions. This produced an overall improvement in the correlation coefficient of between −0.3 and 6% for copper and between −0.8 and 5.7% for zinc (Fig. 2 and Table 2). For this method to be reliable, the chosen internal standard must be well separated from the components but must appear close to the peaks of interest. Cobalt can be used as an internal standard as it is found in serum at very low levels (< 0.05 μg dm^{-3}),[1] well below the detection limit of IC with VIS detection and ICP-OES. The detection limit was calculated as three times the signal to noise ratio. The signal to noise ratio was calculated by measuring the concentration of the baseline noise in comparison with a peak of a 0.5 mg dm^{-3} sample of the analyte. The detection limits were calculated as 0.09 mg dm^{-3} for Cu and 0.03 mg dm^{-3} for Zn.

The results obtained from the ion chromatography analysis were validated by comparing the results with those obtained from ICP-OES. The *t*-test (at 95% confidence limit) revealed no significant differences between the values obtained by the two methods. There was reasonable agreement between both methods, although more samples would indicate if a better correlation was possible. The correlation coefficient for copper was 0.88 with an equation of $y = 1.04x - 0.019$ ($n = 12$) (Fig. 3) and the correlation coefficient for zinc was 0.89 with an equation of $y = 1.08x - 0.022$ ($n = 12$). A Bland–Altman plot is where the difference between the results from the two methods is expressed as a percentage of the mean of the two results for each sample, plotted against the mean concentration of the two measurements. If there is no systematic difference in the results from the two techniques then the data points would oscillate about the zero line on the *y*-axis. The Bland–Altman

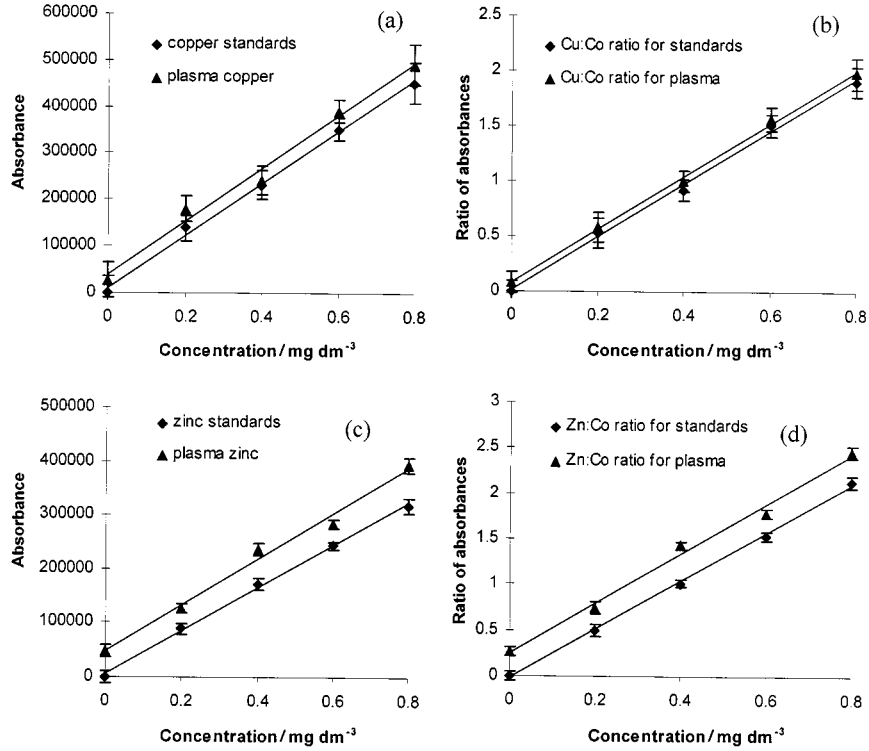

Fig. 2 Comparison of calibration and standard addition graphs of plasma from one haemodialysis patient (* in Table 2) for Cu [(a) and (b)] and Zn [(c) and (d)] with [(b) and (d)] and without [(a) and (c)] the use of an internal standard.

Table 2 Comparison of the correlation coefficient for each patient group when samples were analysed with and without an internal standard (IS). Results marked (*) correspond to data presented in Fig. 2

	Copper			Zinc		
Dialysis type	No IS	With IS	Improvement (%)	No IS	With IS	Improvement (%)
CAPD	0.917	0.976	6.05	0.951	0.943	−0.83
	0.922	0.954	3.35	0.907	0.951	4.64
	0.944	0.991	4.76	0.950	0.977	2.70
	0.954	0.991	3.75	0.921	0.976	5.64
HD	0.923	0.943	2.14	0.945	0.954	0.93
	0.943	0.951	0.82	0.928	0.932	0.43
	0.951	0.977	2.61	0.897	0.942	4.78
*	0.925	0.976	5.23	0.922	0.951	3.05
Control	0.931	0.954	2.41	0.944	0.977	3.33
	0.921	0.932	1.18	0.954	0.976	2.25
	0.945	0.942	−0.33	0.932	0.954	2.31
	0.914	0.951	3.89	0.934	0.991	5.77
Average			2.98 ± 1.89			2.92 ± 2.07

plot is used as Pollock *et al.* [20] suggested it to be more sensitive to potential bias within techniques than the standard regression techniques which can be used. In Fig. 4 if the difference between the results is 10%, this represents that one of the results is 1.1 times the value of the other, if the difference is 66%, then one result is twice the other and if the difference is 100%, one result is three times the other. Using the results from this work the Bland–Altman plot (Fig. 4) demonstrates that there is an overall positive bias for both copper and zinc of 0.006 mg dm^{-3} (0.35%) and 0.128 mg dm^{-3} (7.07%), respectively. This suggests that the results for zinc obtained from the ICP-OES method are higher than those obtained with IC-VIS analysis. This could result from matrix effects in either the IC-VIS or ICP-OES methods. Teixeira *et al.* [21] reported that the use of ICP-OES for the determination of zinc at low concentrations in matrices containing high concentrations of copper was difficult because of the interference by copper in the main emission wavelength of zinc at 213.856 nm.

There were no significant differences in both the copper and zinc levels between CAPD patients and HD patients and there was no significant difference when dialysis groups were compared to the control patients (Tables 3 and 4). It must be stressed that these individual values represent the levels of copper and zinc in different patients and thus the concentrations of copper and zinc may vary from patient to patient due to factors such as sex, age, and time on dialysis. The normal range for copper in serum is 0.8–1.5 mg dm^{-3} and for zinc it is 0.6 –1.3 mg dm^{-3}. Overall, the average control copper levels were lower than the normal levels expected in the blood. The measured copper concentration for this study was 0.501 ± 0.05 mg dm^{-3} for copper and was 1.350 ± 0.05 mg dm^{-3} for zinc in the control samples. This paper is concerned with the development of an alternative analytical method to conventional spectrometric methods for the determination of copper and zinc in biological samples and as such the levels of copper and zinc reported in this study should not be used exclusively as the measured concentration of the zinc in the samples was probably affected by the lapse time between collection and centrifugation as suggested by English and Hambridge.[22] They reported that

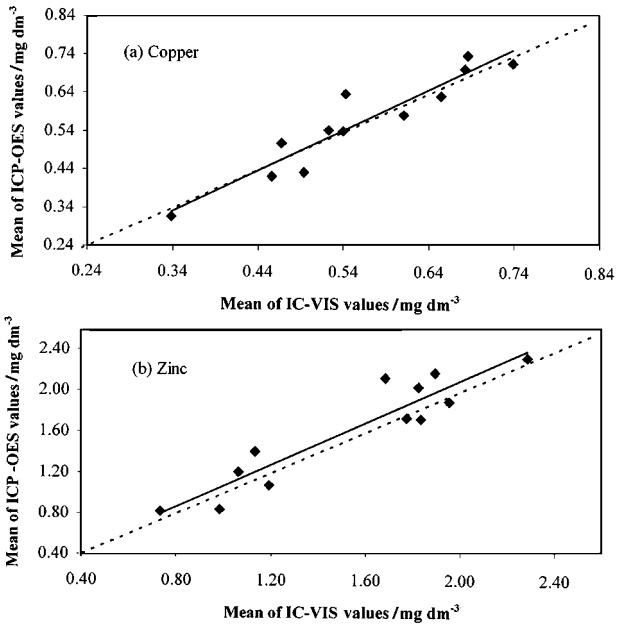

Fig. 3 The correlation between samples run on both ICP-OES and IC-VIS: (a) the correlation coefficient for copper values was $r = 0.88$ and the equation of the line is $y = 1.04\,x - 0.019$ ($n = 12$); (b) the correlation coefficient for zinc was $r = 0.89$ with an equation of the line $y = 1.08\,x - 0.022$ ($n = 12$).

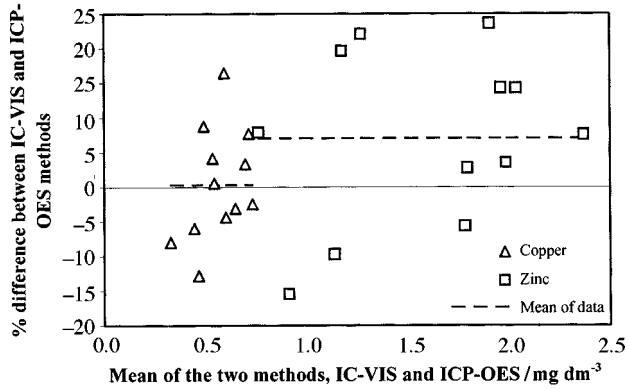

Fig. 4 The Bland–Altman plots for copper and zinc which compares the two methods IC-VIS and ICP-OES. The plots show a small positive bias indicating that the results obtained with ICP-OES are higher than those obtained using IC-VIS.

Table 3 Concentrations of copper and zinc in acid digested blood plasma as detected by ion chromatography with VIS detection using the standard additions method with a cobalt internal standard and with ICP-OES

Method	Dialysis type ($n = 4$)	Copper/mg dm^{-3}		Zinc/mg dm^{-3}	
		Average	Range	Average	Range
IC-VIS	Control	0.501 ± 0.05	0.45–0.67	1.350 ± 0.05	0.73–1.83
	CAPD	0.549 ± 0.05	0.33–0.68	1.576 ± 0.06	0.89–1.95
	HD	0.594 ± 0.05	0.46–0.78	1.348 ± 0.06	0.98–1.79
ICP-OES	Control	0.534 ± 0.03	0.31–0.62	1.417 ± 0.02	0.79–1.95
	CAPD	0.597 ± 0.02	0.51–0.70	1.789 ± 0.03	0.91–2.28
	HD	0.575 ± 0.02	0.43–0.73	1.481 ± 0.02	0.93–2.02

Table 4 One tailed *t*-test for unequal variances was used to compare between the means of the dialysis group with the control groups and between the two methods ($P < 0.05$)

Comparison	n	Copper		Zinc	
		t_{calc}	t_{crit}	t_{calc}	t_{crit}
IC-VIS (with IS) *vs.* ICP-OES	12	0.189	2.201	0.265	2.201
IC-VIS (no IS) *vs.* ICP-OES	12	0.869	2.201	1.170	2.201
CAPD *vs.* control	4	0.448	3.182	0.747	3.182
HD *vs.* control	4	0.938	3.182	0.007	3.182
CAPD *vs.* HD	4	0.427	3.182	0.747	3.182

the level of Zn determined in plasma and serum increased by 6% for the first 2 h after collection which was thought to be attributed to the release of some erythrocyte membrane zinc. A similar effect was not found for copper.

Many factors are involved in determining blood and tissue levels of individual elements in patients with renal failure. The processes involved in renal disease itself can result in either excretion or retention of individual elements whilst dialysis treatment can cause either removal or exposure to these elements. Trace metal disruption causes chronic renal insufficiency in dialysis patients that have been studied but many of these studies are contradictory due to different techniques that have been used. For instance, copper levels have shown conflicting results with high, low and normal levels with some evidence that the levels increase with age and the female sex but not with dialysis duration. Hypozincaemia is also common in patients with end stage renal disease and those on dialysis (haemodialysis and CAPD).

In haemodialysis the blood is pumped at 200–400 $cm^3\ min^{-1}$ into an artificial kidney where it is filtered by a semi-permeable dialysis membrane against the dialysate solution at a flow of 500 $cm^3\ min^{-1}$. Transfer occurs across the membrane of waste products into the dialysate which is drained away. The dialysate solution is made from combining a concentrate with a filtered and reverse osmosis treated tap water.

In CAPD the dialysate (typically 2 dm^3) is introduced into the abdominal cavity *via* a catheter and remains there for 4–6 hours. During this time, excess fluid and waste products are transferred into the dialysate solution from the blood across the peritoneal membrane and subsequently drained out to be replaced by fluid dialysate.

Dialysis fluid (CAPD, haemodialysis concentrate and reverse osmosis treated tap water) has a role in contributing to trace element abnormalities in renal patients. Metal contaminants in dialysis fluid such as copper and zinc should be eliminated by the manufacturing process. Other sources of contamination such as dialysate tubing could be a possible source of metal contamination.[23] Further factors may be important in determining blood and tissue levels, in particular the ongoing ageing process with poor dietary intake and decreased gastrointestinal absorption of zinc, whilst copper absorption in the small intestine is facilitated by zinc deficiency owing to the loss of absorption competition.

Ion chromatography is an attractive alternative to the usually more conventional spectrometric methods for the determination of metals. Ion chromatography offers a more cost effective choice as the running expenses of ion chromatography often are cheaper than those faced by ICP-OES users. The most obvious advantage of this technique is that multiple elements can be determined in one sample of 25 μl volume and complete analysis can be performed when coupled with a suitable detection system or systems. Generally, the sample requires minimal sample pre-treatment.[16] Although it is accepted that microwave digestion seems laborious when compared to the simple dilution methods used with some atomic spectrometric methods, the use of acid digestion to totally breakdown the matrix of a sample in order to ensure all the analyte is recovered offers its own advantages. The selectivity and peak sharpness in IC can be enhanced by the use of complexing agents such as PAR for the detection of cations followed by spectrophotometric detection giving a highly sensitive method. The use of the standard additions method and of an internal standard can improve the precision of the results.

Acknowledgements

Thanks to Dr. Philip H. E. Gardiner and Heather Birtwistle at Sheffield Hallam University for the use of the ICP-OES system. This work was supported by a studentship from the Lancashire Centre for Medical Studies.

References

1 W. Kaim and B. Chwederski, *Bioinorganic Chemistry: Inorganic Elements in the Chemistry of Life, An Introduction and Guide*, Wiley, New York, 1994, pp. 151–161.
2 J. M. Campistol and A. Argiles, *Nephrol. Dial. Transplant*, 1996, **11**, 142.
3 M. Gallieni, D. Brancaccio, M. Cozzolio and E. Sabioni, *Nephrol. Dial. Transplant*, 1996, **11**, 1232.
4 N. J. Emenaker, R. A. Disilvestro and N. S. Nahman, *Am. J. Clin. Nutr.*, 1996, **64**, 757.
5 R. J. Cousins, *Physiol. Rev*, 1985, **65**, 309.
6 P. L. Kimmel, T. M. Philips and S. Q. Lew, *Kidney Int.* 1996, **49**, 1412.
7 L. Yver and D. Blanchier, *Nephrol. Dial. Transplant*, 1987, **2**, 451.
8 C. Canavesse, *Nephron*, 1990, **56**, 455.
9 K. Nomiyama and H. Nomiyama, *Nephrol. Dial. Transplant*, 1989, **4**, (suppl) 114.
10 K. Cundeva and T. Stafilov, *Talanta*, 1997, **44**(3), 451.
11 H. W. Chen, S. K. Xu and Z. L. Fang, *Anal. Chim. Acta*, 1994, **298**(2), 167.
12 P. B. Barrera, R. D. Gonzalez and A. B. Barrera, *Fresenius' J. Anal. Chem.*, 1997, **357**(4), 457.
13 S. D. Huang and K. Y. Shih, *Spectrochim. Acta, Part B*, 1995, **50**(8), 837.
14 M. Hoenig and A. Cilissen, *Spectrochim. Acta, Part B*, 1997, **52**, 1443.
15 J. Schoppenthau and L. Dunemann, *Fresenius' J. Anal. Chem.*, 1994, **349**, 794.
16 C. N. Ong, H. Y. Ong and L. H. Chua, *Anal. Biochem.*, 1988, **173**, 64.
17 M. En-ling and J. Zhu-ming, *Chin. Med. J.*, 1993, **106**(2), 118.
18 H. T. Lu, S. F. Mou, Y. Yan, S. Y. Tong and J. M. Riviello, *J. Chromatogr. A*, 1998, **800**(2), 247.
19 S. Okawa and T. Ishikawa, *Bunseki Kagaku*, 1998, **47**(1), 9.
20 M. A. Pollock, S. G. Jefferson, J. W. Kane, K. Lomax, G. MacKinnon and C. B. Winnard, *Ann. Clin. Biochem.*, 1992, **29**, 556.
21 L. S. G. Teixeira, J. O. N. Reis, A. C. S. Costa, S. L. C. Ferreira, M. D. S. G. Korn and J. B. deAndrade, *Talanta*, 1998, **46**(6), 1279.
22 J. L. English and K. M. Hambridge, *Clin. Chim. Acta*, 1988, **175**, 211.
23 R. Milacic, M. Benedik and S. Knezevic, *Clin. Chim. Acta*, 1997, **265**, 169.

Paper 8/08852G