Advances in Instrumental Methods for the Measurement and Speciation of Trace Metals*

JOHN SAVORY, Ph.D.,† and MARY M. HERMAN, M.D.‡

†Departments of Pathology, Biochemistry and Molecular Genetics,
University of Virginia Health Sciences Center,
Charlottesville, VA 22908
‡Clinical Brain Disorders Branch,
Section of Neuropathology
NIMH/NIH,
Bethesda, MD 20892

ABSTRACT

Progress in understanding the role of trace metals in biology has been largely dependent on the development of sensitive, accurate and precise analytical methods. Atomic spectroscopic techniques, particularly atomic absorption, have made the greatest contribution. Key to the success of such analytical techniques has been the simplification of sample processing so that contamination is minimized. Electrothermal atomization has allowed sensitivity limits to be lowered sufficiently so that even ultra-trace metals can be detected. More recently, mass spectrometric detection of metal ions has added to the repertoire of available instrumentation, particularly with the use of inductively coupled plasma to introduce ions into the mass analyzer. These analyzers are suitable for multielement analysis. More conventional mass spectrometric analysis of metal chelates offer an alternate solution but require considerable specimen preparation time. Intracellular localization of trace metals necessitates complex specimen processing prior to analysis on instruments that are highly sophisticated and expensive. Metal speciation is a rapidly growing area of trace metal research, with the major advances coming from coupling of the separation process, such as capillary electrophoresis or high performance liquid chromatography, with the analytical instrument for metal detection. Inductively coupled plasma-mass spectrometry has proved to be an excellent choice for such detection purposes. Refinement of these methods as well as more widely available instruments for microanalysis will add greatly to continued advances in our knowledge of the role of trace metals in biology and medicine.
plethora of problems has been encountered, ranging from the collection of specimens free of contamination to the development of analytical methods with sensitivity limits in the parts per billion and lower range. Measurement of the total concentration of a metal is in itself a challenge; however, the assessment of metal speciation represents an even greater problem. The past 100 years have seen the field progress from inaccurate chemical and physicochemical methods to present-day techniques with exquisite sensitivity capable of highly accurate measurements. Speciation studies have also gained momentum by the development of high-resolution separation methods, which can be coupled on-line with the final analytical measurement. We present here advances in this field which are opening new horizons in the biological role of trace metals in health and disease. This review represents in part an update of a similar survey of advances in trace metal measurements.1

Techniques for the Detection of Metals

Atomic spectroscopic techniques, which include either atomic absorption or emission spectrometry, have for many years been the most commonly used methods for the measurement of trace metals in biological materials. Each has its own merits and deficiencies, depending on the application.

**Atomic Absorption**

Some of the more abundant trace metals present in biological materials, such as zinc and copper, can be detected using flame atomization techniques, which were used in the first atomic absorption spectrometers. Such analyses are relatively straightforward and provide moderately sensitive limits of detection. However, for the more challenging analyses, such as for measurement of nickel in biological fluids, the sensitivity of flame atomic absorption spectrometry (AAS) is only in the ~100 μg/L range, thus eliminating this method for serious investigations. For analyses in the mg/L range, the convenience of flame AAS with a precision in the 1 percent range (relative standard deviation) makes the process attractive.2

The analytical technique that has contributed most to our understanding of trace metal metabolism has been atomic absorption with electrothermal atomization. The concept of using an electric furnace to produce an atomic vapor was proposed in the early days of atomic absorption, but it was many years before truly satisfactory analytical data could be obtained from this approach to trace metal measurements. One of the first reports of the application of electrothermal AAS for the analysis of biological materials was in 1971 from our own laboratory in a study directed towards the measurement of copper in serum3 using a graphite rod atomizer which we constructed. Since that time, electrothermal AAS has been the most effective routine technique for trace metal measurements in a clinical setting. In a review by Slavin,2 electrothermal AAS is stated to be a highly sensitive spectroscopic technique, having detection limits 10 to 100 times lower than flame AAS or inductively coupled plasma (ICP) emission. Analytical sensitivity is often 1,000 times better on an absolute mass basis. Early electrothermal AAS systems were subject to significant interferences when used to analyze biological materials, and reproducibility was not optimal. However, developments in design of the atomization chamber, particularly the stabilized temperature platform, together with background correction, have greatly enhanced performance. Very few biological samples can be analyzed without some form of background correction. The most effective means of performing this is the Zeeman correction, which is significantly more stable than the continuum correction and can adjust for much higher backgrounds. The use of matrix modification has also made a considerable impact on reducing interferences. The chemical reactions involved in the stabilization of specific elements by a matrix modifier are interesting and complex and can be elementspecific. Nickel nitrate has been used extensively as a matrix modifier but cannot be rec-
ommended for clinical laboratories in which nickel measurements are being performed. Experience in our laboratory has led us to use the following matrix modifiers: For aluminum measurements in plasma using the stabilized temperature platform, magnesium nitrate is required, whereas, without the platform, use of nitric acid yields optimal results. For arsenic and selenium, we recommend the use of palladium chloride, and for chromium and nickel, we initially precipitate proteins with nitric acid; the hydrogen ion present in the supernatant has proven to be a suitable matrix modifier. An understanding of the chemical reactions between the analyte, matrix modifier and furnace material has been the subject of many investigations and is important to optimization of the procedure.

Electrothermal AAS is a low throughput technique which takes several minutes for each sample to be analyzed; the improved performance provided by the stabilized temperature platform further slows the rate of analysis. In the authors' laboratory, the most common trace metal assay is the measurement of aluminum in plasma, and one of two methods has been used, depending on clinical requirements.4 For analyses on patients with normal renal function where the plasma aluminum concentrations are generally less than 10 μg/L, the matrix modifier is magnesium nitrate used with the stabilized temperature platform and an oxygen-ash step at 600°C. This method provides a characteristic mass of 16.3 pg/0.0044 A.s, which is the peak area, and a detection limit of 1 μg/L. The characteristic mass is an important measure of the sensitivity of the analysis and is useful for resolving analytical problems. For precise analyses of specimens from hemodialysis patients where aluminum concentrations are considerably higher, we dispense with the time-consuming stabilized temperature platform and use 0.1 mM nitric acid as the matrix modifier. With this method, the characteristic mass is 26.6 pg/0.0044 A.s, but the precision is adequate and the throughput of the analyses is more than twice as fast as the more sensitive method described above. Thus, in each situation, it is important to identify the clinical question that needs to be answered in order to make the most appropriate selection of the analytical technique to be used.

Electrothermal AAS does not readily lend itself to multielement analysis although advances in this field have been made,5-7 and commercially available instruments are now available for this technique. An instrument with these capabilities which appeared on the market recently is the SIMAA 6000 from Perkin-Elmer. This analyzer allows simultaneous determination of up to four elements, which can be increased to six using multielement hollow cathode lamps. An evaluation of this instrument for the simultaneous measurement of cadmium, cobalt, copper and lead has been reported.9 Advances also have been made in sample introduction with flow injection. This technique has been applied to the determination of nickel in biological samples, using an on-line flow injection solvent extraction for electrothermal AAS.9 In this method, nickel pyrrolidine-dithiocarbamate was extracted online into isobutyl methyl ketone, the organic phase separated in the flow injection system and a 50 μL sample injected into the graphite tube of the electrothermal AAS. This system has achieved a detection limit of 4 ng and a precision of 1.5 percent (relative standard deviation). Another example of the application of flow injection analysis is in the determination of mercury in saliva using cold vapor AAS,10 a technique proposed to study mercury release from dental amalgam fillings.

Atomic emission spectrometry: As with atomic absorption spectrometry, atomic emission requires the analyte of interest be converted into an atomic vapor. With a flame or in a graphite furnace, the temperature is approximately 3000°C, and most of the atoms present are in the ground state. Plasma excitation using inductively coupled plasma (ICP) or direct current plasma (DCP) introduces a nebulized sample into the zone of a high temperature inert gas, which can be 6000 to 8000°C. Atomization at this temperature is complete, and matrix effects are minimal. However, the spectra are complex, and a spectrometer with
high resolution is needed to minimize interferences. ICP has a wide concentration range, and detection limits compare favorably with flame AAS. Multielement capabilities are an important feature of this technique, and there are many applications in the literature.11-13

Laser fluorescence and laser ionization: The more conventional analytical techniques described above provide the capability of detecting trace metals in the low parts per billion range. Laser fluorescence and laser ionization can stretch detection limits to the parts per quadrillion levels. These methods are not limited by instrumental and system noise and by detection efficiencies much less than unity. For example, the detection efficiency of atomic absorption is much lower than unity since a light beam cannot be focused to a diameter equal to the absorption cross section. Mass spectrometric detection has an overall efficiency no better than 10^{-5} in the atomization-ionization-interfacing. Laser fluorescence and laser ionization techniques approach the ideal. The efficiency of detection must be unity to achieve single atom detection, i.e., every atom produced for those sampled must produce a detectable event. Another important fact is that instrumental and atomizer noises must be less than intrinsic noises. Absolute experimental detection limits for laser excited atomic fluorescence spectrometry (LEAFS) is 0.5–5.0 fg, and for laser enhanced ionization (LEI) is 5–50 fg. These can be compared to electrothermal AAS with a detection limit of 0.2–100 pg, and ICP-MS with a limit of >50 pg. Whether LEAFS or LEI will find clinical applications is questionable, but they have great potential for trace metal research.

Mass spectrometry: Isotope-specific analytical methods or isotope dilution mass spectrometry offer analytical advantages over element-specific methods used in classical analytical chemistry. Because of their high degree of specificity, isotope-specific methods can provide the most accurate measurements of elemental concentrations. Thermal ionization mass spectrometers have been used most frequently and are designed specifically for isotope ratio measurements.14,15 These instruments are not used clinically except as definitive methods, but they have wide applications in the nuclear industry.

Inductively coupled plasma-mass spectrometry (ICP-MS): This technique for the mass spectrometric measurement of elements uses ions extracted from a high temperature plasma. This method is capable of providing both elemental and isotopic measurements with exquisite sensitivity and a wide dynamic range and is capable of multielement analysis. An ICP is interfaced with a mass spectrometer; this initially posed major technical problems since the interface is between a plasma of gas at high temperature and atmospheric pressure and a high vacuum mass spectrometer. These technical problems have been overcome, and ICP-MS is used extensively, particularly in large reference laboratories. This process is suited to the measurement of several elements simultaneously, and throughput is much faster than in electrothermal AAS. Use of nominal mass resolution mass spectrometry has the potential for spectral interferences. This problem is being addressed by the use of high resolution ICP-MS, a technique which is beginning to be applied to the analysis of biological materials.16 In one application, strontium isotope ratio measurements were made in prehistoric bone specimens; excellent precision was obtained for these analyses, being better than 0.03 percent (RSD).17 The application of high resolution ICP-MS to the measurement of aluminum in serum has provided a means for evaluation of the more conventional techniques of electrothermal AAS and low resolution ICP-MS.18 Improved detection limits with high resolution ICP-MS have provided a means of measuring basal levels of aluminum in serum. Another application of high resolution ICP-MS involves the measurement of plasma or serum lead.19 Although the clinical utility of such an assay has yet to be defined, the sensitivity of the technique provides an alternative to the more common whole blood or erythrocyte lead measurement.

Organic mass spectrometry: General-purpose mass spectrometers can also be used for trace metal determinations, although there
have been only a few reports of such applications because of the complicating fact that formation of metal chelates is an important feature of such techniques. In contrast, ICP-MS is a much more direct approach to performing these measurements. Gas chromatography-mass spectrometry (GC-MS) has been successfully used for isotope ratio measurements in biological materials for chromium and selenium. The lack of suitable chelating agents has been a limitation to the wider application of GC-MS. The poor accuracy of the isotope ratio measurements and the exchange of metal in the chelate with metals in the GC-MS system, causing cross-contamination during sequential analyses of samples of widely varying isotopic compositions, have been problems with the use of metal chelates. Some of the studies reported, therefore, have not measured isotope ratios but, rather, have depended on the use of integrated ion currents for quantitation.

Methods involving gas chromatography-mass spectrometry using thermally stable, volatile chelates have been developed in the authors' laboratory. We have investigated the measurement of isotope ratios of chromium, nickel, zinc and copper. The chelating agents acetylacetone, trifluoroacetyl acetone, sodium diethyldithiocarbamate and lithium bis (trifluoroethyl) dithiocarbamate have been used. Experimental conditions for the preparation of chelates and the mass spectrometer operating parameters for precise and accurate measurement of isotope ratios have been optimized with a general-purpose mass spectrometer. Imprecision values of 1 to 4 percent were obtained for measurements of different isotope ratios using chelates containing about 10 ng of metal. The capability of this technique for the accurate determination of natural and altered isotope ratios was also evaluated for these elements using lithium bis (trifluoroethyl) dithiocarbamate as a chelating agent. We have further applied this technique to the measurement of nickel in serum and urine and to the assay of chromium in urine. Both methods again use lithium bis (trifluoroethyl) dithiocarbamate as a chelating agent, with subsequent isotope ratios being monitored following capillary column GC-MS analysis. The memory effect between samples of different isotope ratios has been found to be negligible. The analysis of National Institute of Standards and Technology (NIST) freeze-dried urine reference material SRM-2670, for which acceptable results were obtained, has verified the accuracy of each method.

Robotics: Trace metal analysis is complicated considerably by contamination. Reagent contamination and the need to work in a clean environment are important considerations. In the future, one likely method for the control of contamination during specimen handling is by the use of robotics. Metal contamination from a human operator would be minimized. Robotics offers an excellent means for providing high-quality analyses, increased throughput and reduced costs. Systems would have to be designed with built-in checks for the integrity of analysis in order to ensure that errors will be recognized. At present, few laboratories are using robotics for trace metal measurements. However, as more laboratories become familiar with the potential of robotics, further applications will be developed. The authors have been engaged in robotics development in clinical chemistry for a number of years and foresee wide applications.

Microanalysis: All the analytical techniques heretofore discussed in this review have been for the bulk analysis of biological specimens. In order to obtain distinct localization of elements within tissues, it is necessary to combine microscopy with analytical techniques. Microanalysis will produce correlation between the structure of a microscopic cellular component and its chemical composition. Microanalysis in the transmission electron microscope is most commonly performed employing the energy-dispersive x-ray spectroscopy technique. This method has been used for many years, and several procedures for specimen preparation and analysis have been developed and refined. Transmission electron microscopes with an incorporated electron spectrometer are avail-
able commercially, and permit an avenue of performing electron energy loss spectroscopy (EELS). Electron beam instruments are not the only means of providing elemental microanalysis. The laser microprobe uses irradiation with a laser beam to ionize a specific part of the specimen, followed by analysis of the ions generated by bombardment with a primary ion beam. The proton microprobe uses the x-ray spectrum generated by a beam of high energy protons. As is evident from this brief introduction, all microanalytical techniques are different in principle, instrumentation and performance, and these differences include sensitivity and spatial resolution. Examples of applications of some of these techniques are given for the authors' main area of interest, which is aluminum toxicity.

Energy-dispersive X-ray Analysis (EDS): Energy-dispersive x-ray analysis has been used to localize aluminum deposits within the glomerular basement membrane. Applying this technique, Perl, et al, examined brain tissue from patients with amyotrophic lateral sclerosis and parkinsonism-dementia and detected aluminum in neurofibrillary tangle-bearing neurons. Other workers using this technique have localized aluminum in bone tissues. In experimental animals, the process has been used for the ultrastructural localization of aluminum in the brains of rats given aluminum chloride injections.

Laser microprobe mass analysis (LAMMA): The Laser Microprobe Mass Analyser provides high lateral resolution and extreme sensitivity of detection. The instrument consists of a pulsed Nd-YAG laser (265 nm), an optical microscope, a time-of-flight mass spectrometer with an open secondary electron multiplier, and suitable detection electronics. The principle of LAMMA is based on the excitation of a microvolume of the sample to an ionized state by a focused laser beam. The analytical information is derived from mass spectrometric analysis of these ions. This technique has been applied to localize aluminum in the lysosomes of hepatocytes and Kupffer cells of patients on chronic hemodialysis. We have used LAMMA to study the ultrastructural localization of aluminum in the liver of aluminum maltolate-treated rabbits. LAMMA is a sensitive and powerful technique, but the instrumentation is not widely available, which has limited its application for biomedical research.

Electron energy loss spectroscopy (EELS): EELS is a technique complementary to x-ray microanalysis. Information carried by electrons that have originally caused x-ray excitation in the specimen is studied. Electrons that have passed through the specimen are separated according to their kinetic energy by an electron spectrometer in order to form a high resolution energy spectrum. The electron energy loss spectrum, which represents the graphical display of the energy lost as a result of the scatter of electrons by the specimen versus the corresponding electron intensity, can be analyzed directly or indirectly using a signal from a specifically chosen part of the spectrum, which is then passed on to form an image. Even though the original promise of excellent sensitivity has not materialized, the EELS technique provides exceptionally good resolution. We have applied this procedure to the analysis of liver from aluminum maltolate-treated rabbits but have been restricted by sensitivity limitations to the measurement of aluminum in brain tissue.

A major problem with microanalysis of tissue lies in the processing of the specimen. Conventional chemical fixation, as developed for morphological studies, is not ideal for localizing trace elements. Movement of metal ions within the cell is a serious consideration since tissues are left for several days in a fixative. The method of choice in the authors' laboratory has been to use rapid freezing and freeze substitution as a means of tissue processing, but these techniques are difficult and time-consuming.

Although LAMMA and EELS require very expensive sophisticated equipment, both are extremely powerful methods for studying metal localization in tissues. The LAMMA technique permits greater sensitivity than can
be found in EELS; however, the resolution of EELS is superior to that of LAMMA. Our understanding of the toxicity of metals will be enhanced considerably by the application of such localization methods.

**Specimen Handling and Control of Contamination**

It is of prime importance that specimens are collected in a manner which minimizes contamination and that processing and analysis are also carried out in a clean environment. The techniques for providing these clean conditions, which are unique for trace metal measurements, have been reviewed by the authors.

**Advances in the Detection of Chemical Species of Metals in Biological Systems**

A review of the analytical aspects of chemical speciation has been published by Cornelis. The separation and measurement of individual species of trace metals is an important consideration since not all chemical forms of metals possess the same biological activity. Early work from our laboratory in the 1970s, using gel filtration chromatography, provided information on the speciation of calcium in serum. Applying a similar approach, we later identified several species of aluminum in plasma. Physical methods of separation of different species are obviously powerful tools, and new high resolution techniques are replacing the older, more cumbersome methods. In place of gel filtration separation procedures, where individual fractions were collected in a fraction collector and then analyzed by a technique such as electrothermal AAS, the separation system now can be coupled directly to the trace metal analyzer. HPLC coupling to a variety of instruments, including AAS, ICP-MS and ICP-AES, for separation of metallothioneins has been discussed. Much promise is being demonstrated by coupling capillary electrophoresis with ICP-MS for speciation investigations. The high resolution power of capillary electrophoresis has been used for the separation of platinum species with detection limits of 1 µg/L. Two similar reports from the same research group describe the application of capillary electrophoresis coupled with ICP-MS for selenium speciation. Six selenium (Se) species of interest have been separated in a single run and include Se(IV), Se(VI), Se-carrying glutathione, selenomethionine, selenocystine and selenocystamine. Recent literature reports the coupling of fast protein liquid chromatography with ICP-MS. This is an example of advances made in speciation of aluminum over the slow methods which we initially described. Results of this approach again confirm that transferrin is the major aluminum binding ligand in serum.

Not all speciation studies require physical separation of the various species; research in our laboratory using stability constants have demonstrated that transferrin is probably the ultimate carrier of Al in plasma. The results of this alternate approach complement the data on physical separation described above.

**Conclusions**

Many advances have been made in the development of techniques for detection of trace metals in biological materials. Refinement of physical methods have greatly enhanced the sensitivity, precision and accuracy of determinations and have helped to reduce processing steps, thereby diminishing the possibility of contamination. The most reliable techniques are those used in the analysis of biological fluids or bulk tissue. There is still room for improvement for intracellular localization of metals in tissue; the most sensitive methods require extensive tissue preparation and complex and expensive instrumentation. Progress in the speciation of metals in biological materials has been aided by directly coupling the separation process with the metal detection analyzer. Major advances thus have been made in the field of trace metal measurements and speciation, which will contribute greatly to clinical and environment-
tal laboratory services and to research into the exciting field of trace metal metabolism and toxicology.

References


