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Development of an advanced thermospray interfaced HPLC-AAS system for speciation studies of cadmium species excreted from human body fluids: Interaction between metallothionein cadmium and zinc

Chang, Peter Ping, Ph.D.
The Louisiana State University and Agricultural and Mechanical Col., 1991
DEVELOPMENT OF AN ADVANCED THERMOSPRAy INTERFACED
HPLC-AAS SYSTEM FOR SPECIATION STUDIES OF
CADMIUM SPECIES EXCRETED FROM HUMAN BODY FLUIDS:
INTERACTION BETWEEN METALLOTHIONEIN CADMIUM AND ZINC

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements of the degree of
Doctor of Philosophy

in

The Department of Chemistry

by

Peter Ping Chang

B. S., Shanghai University of Science and Technology, 1982
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DEDICATION

To my mother and my father
for their endless love

and

to my little sister for her sweet smile
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FORWARD

Parts of this dissertation have been presented:


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J. W. Robinson, P. P. Chang, "Interaction between ionic Zn, ionic Cd and metallothionein complexed Zn and Cd".
J. W. Robinson, P. P. Chang, "Detection Sensitivity Enhancement of Flame AAS Using Capillary ID Size Based Thermospray Nebulizer".
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ABSTRACT

A new technique has been developed to reveal different metal species at very low concentrations (lower ppb level) in human body fluids.

Using this technique it was determined that urine contained many different species of cadmium and that perspiration contained many other species of cadmium. This indicated that there were two avenues for the elimination of cadmium from the body. Quantitative data showed that much more cadmium was eliminated through perspiration than urine and that the biological half life of cadmium in the body was less than 1.5 years - not 20-30 years as currently believed.

Further, in vitro experiments showed that zinc ions displaced cadmium from metallothionein complexes at mole ratio as low as 1:100:1,000, with actual concentrations of 0.015:1.0:40 ppm of zinc:cadmium:metallothionein, respectively. These results suggest that zinc ions may displace cadmium from metallothionein in vivo and caution should be exercised in taking zinc tablets.

This new system interfaced high performance liquid chromatography (HPLC) with flame atomic absorption spectroscopy (FAAS) using an advanced thermospray (TSP) nebulizer. The procedure maintained high chromatographic resolution in the system. No sample pretreatment was necessary, eliminating many sources of analytical error.
The TSP nebulizer, an electrothermally heated stainless steel capillary, has the ability to convert HPLC effluents into an aerosol/vapor jet with relatively small and uniform particles of dried solute. The jet can be directly delivered into the flame, therefore, interfacing HPLC with FAAS.

The enhancement of sensitivity and the optimization of the TSP interface led to a study of TSP desolvation mechanism. A new concept of the mechanism was proposed in which a TSP induced solute-enrichment, by way of forming a "solute plug", achieved the desolvation.

Quantitative studies showed that 4.5 ppb of analytical sensitivity and 0.45 ng of absolute sensitivity for cadmium were achieved.

The optimum standard deviation obtained at the 200 ppb level of cadmium was $7.0 \times 10^{-4}$ absorbance. The relative standard deviation at that level was 0.3 %.

The technique should be applicable to the speciation and accurate determination of many metals at low concentration level in body fluids, and other aqueous and non-aqueous solutions.
CHAPTER ONE

GENERAL INTRODUCTION
1. INTRODUCTION

Metals, even in trace quantities, are playing a very important role in environmental and biological systems. Many metals are considered essential to human health, while some heavy metals such as lead, mercury and cadmium are toxic. Intensive research in the areas of toxicology, clinical or environmental chemistry, and related fields have led to the measurement of metal concentrations in these systems. The resulting information has been useful in understanding the actual roles of metals.

It is well documented that the actual toxic impact of metals on the biological and/or environmental systems varies significantly depending upon their chemical and physical forms. Most of the studies, however, were based upon the total concentration of metal either existing in or excreted from those systems. Such data, observed without chemical speciation, can hardly provide adequate information on the actual threat of metal toxicity. Therefore, identification of the chemical forms of the metals is necessary for a better understanding of the metal toxicity in these systems.
2. SIGNIFICANCE OF SPECIATION STUDIES

It is well known that the biological impact of many metals is dependent on their chemical forms. One such element is chromium. Chromium has two important valence forms, i.e. Cr (III) and Cr (VI). The former is a necessary constituent for insulin metabolism and is part of the glucose tolerance factor (1), while the latter is a known carcinogen (2). Another example is silver. Ag (I) is very toxic, while silver in Ag-complex is less toxic.

Another example is mercury. Inorganic mercury compounds such as HgS, are not very toxic. It was used as a drug for eternal life by ancient Chinese pharmacists. But, organic Hg compounds are very toxic. Methyl mercury, for example, because of its stability in vivo, can easily accumulate in the target organs before decomposition and excretion from body and cause toxic effects (3, 4).

The decomposition, transformations and local mobilizations of a metal in the environment are largely altered by different factors. One such factor is pH. Robinson and Deano (5) noted that aluminum had the highest toxicity on fish at pH 4.5, which is the pH of typical acid rain. The predominant form of aluminum at this pH was Al(OH)$_2^+$, which exerted highly toxic effects on aquatic organisms. Robinson and Murungi (6) also observed that aluminum toxicity varied depending on the anion present in
addition to the complexing agents. The anions appeared to affect the polymerization of aluminum, altering the toxicity.

The metal biological activity can also be affected by chemical forms of the metal of interest. The absorption and distribution amongst the body organs, metabolism and excretion of a metal vary widely from compound to compound. For example, the bioavailability of selenium on rats depends on the chemical forms of metal in the diet (7). Studies on the detoxication of cadmium accumulation in kidney reveal the effects of the metal protein complexation (2, 8). Robinson and Choi (9) observed that the calcium compounds excreted from (a) human urine and, (b) human perspiration are quite different.

The objective of metal speciation studies is to identify and measure the individual chemical forms of metal compounds, which actually exist in the biological sample. Whether the chemical forms of the metal are inorganic or organometallic, and the quantitative determination of each form, can hopefully provide us useful information and help reveal the mechanism of toxic effects of the metal.

A. Clinical Significance of Speciation

Previous extensive studies on the total concentrations of metals excreted in human fluids, such as urine and blood, have certain statistically diagnostic significance, but
provide no information about the elimination mechanism of the metal of interest, either by chemical complexation or bio-detoxication (2, 8, 10, 11). Hence, such studies have little or no clinical significance which might lead to improve the treatment of either detoxication or elimination of the metal toxicity. In other words, the total concentration of the toxic metal tells us only the Potential threats but NOT the ACTUAL threats on human health. However, metal speciation studies may be able to provide this information, therefore, it is much more clinically significant.

B. Significance of Metal Inter-Relationship Study

The complexation effects of EDTA on inorganic cadmium compounds have been extensively studied (12). It is also well known that the metal-binding protein, metallothionein, can bind cadmium to form cadmium-thionein complexes (2).

The binding sites of metallothionein are mostly those amino acid terminals, such as cystine and cysteine. The S-S bond in cystine and the S-H bond in cysteine have the capability of combining with metal ions, especially +2 ions, and form metallothionein. Examples include copper, zinc, and mercury (2).

Metals usually show stronger toxic effects in their free ionic forms (13), and lose toxic strengths in complexation. If, for instance, cadmium-metallothionein is
formed in the human body, then the toxicity of cadmium is greatly decreased through complexation and excretion of the metal-binding protein via various routes, such as perspiration and urination. However, other essential metals, zinc and copper, also bond to cystine and cysteine to form Zn- or Cu-metallothionein. Furthermore, it is believed that zinc has a stronger affinity towards the binding sites than cadmium (14). Consequently, therefore, if there are excessive amounts of zinc co-existing in the human body with cadmium, which is usually the case when excessive zinc tablet are taken, zinc may displace cadmium from the binding sites. This leads to the formation of excessive Zn-thionein, and releases ionic cadmium back into the blood stream which is toxic to human health. Figure 1 shows the reaction of zinc replacing cadmium from cysteine, a basic amino acid in metallothionein.

In vivo, it is almost impossible to reveal the mechanism of complexation of these metals in the human body. In vitro, however, the complexation reactions can be monitored, and the formation of different metallothioneins can also be analyzed through the metal speciation. The resulting data could be useful to the better understanding of real benefits of trace metal treatments performed in clinical routines.

The fundamental objective of this study was to develop an analytical method sensitive enough to complete such
FIGURE 1. POSSIBLE REACTION OF ZINC ION REPLACING CADMIUM ION ON THE BINDING SITE, CYSTEINE, OF METALLOTHIONEIN.

(A) CADMIUM IONS BONDED ONTO CYSTEINE TO FORM CADMIUM METALLOTHIONEIN COMPLEX

(B) CADMIUM ION IS REPLACED BY ZINC ION TO FORM ZINC METALLOTHIONEIN COMPLEX, AND RELEASES FREE CADMIUM ION
study, and to use the method to study the concentration at which zinc could replace cadmium in metallothionein.

3. EXPERIMENTAL TECHNIQUES FOR METAL SPECIATION

A. Difficulties of Metal Speciation Studies

Generally, the experimental determination of metal chemical forms in biological sample encounters the following difficulties:

[1] The total analyte concentrations are usually so low that most of the analytical methods are too insensitive.

[2] Samples are often very complex compound mixtures in which the compounds may be closely interrelated.

[3] The compounds in the sample are sensitive to air oxidation or chemical reduction during sample pretreatment, which changes the sample chemical forms.

[4] The compounds in the sample are unstable and will not survive the complicated conventional speciation procedures.

[5] Any analytical methods that may contaminate the sample must be avoided, making the analysis even more difficulty.
In order to eliminate the potential errors caused by sample pretreatment, the analysis has to be performed as quickly and simply as possible, with the least analytical preparation, and without sample pretreatment.

B. Conventional Techniques for Metal Speciation

Many traditional techniques are used in metal speciation studies, but involve complicated sample treatments and tedious multistep analytical processes with numerous interferences. We will discuss a few of them in terms of their metal speciation applications.

[1] Dialysis

Dialysis techniques are widely used for biological sample separation according to the molecular size of metal compounds. A semipermeable membrane with a certain porous size is used through which some molecules, but not others can diffuse. Ions or molecules with size smaller than the pores can diffuse through the membrane, but large molecules such as proteins and metal colloids cannot; therefore they are separated by molecular size.

Slowey and Hood (15) observed that 70% of dissolved Zn in sea water was dialyzable using dialysis membrane along with solvent extraction. Cox et al. (16, 17) suggested that Donnan Dialysis as a rapid metal speciation method. This method, introduced by Blaedel and Haupert (18), using an ion
exchange membrane to transport ions across the membrane under the influence of an ionic strength gradient. If the dialysis is carried out in an acidic receiver the total soluble metal is removed, but into a neutral receiver the free or labile metal forms are dialyzed. In this way, the labile and total metal concentrations can be determined.

Dialysis can be performed without sample preparation. Since it is a membrane transfer process, it may simulate the natural phenomenon.

[2] Ion-Selective Electrode

Ion-selective or p-ion electrodes (ISE) offer a method of directly measuring the ionic form of metal without preseparation of the species from the matrix or chemical constituents. The activity in terms of potential of the ion on each side of the ion-exchange membrane surface is measured. This is related to the concentration of the metal ion by the Nernst equation. The selectivity and the sensitivity of the method depends on the strategies of developing an electrode that is selectively sensitive to one species (19). However, it suffers from insufficient sensitivity and numerous interferences.


Anodic stripping voltametry (ASV), especially differential pulse polarography (DPP), is a highly sensitive
method for metal speciation analysis and can be used to determine the labile (weakly complexed) and bound (strongly complexed) metals. The former are measured at neutral pH and the results represent the free metal ions plus the metal dissociated at the electrode surface from complexes and colloids which are reported biologically available (20). The latter are determined by the difference between the former and the total metal concentration. This is measured later after digestion with acid, or by ultraviolet irradiation.

ASV technique can provide preliminary metal speciation information without extensive sample preparation. However, the method suffers from interferences originating from the sample matrix and electrode contamination.

[4] Chelex 100 Metal Compounds Separation

Chelex 100, a styrene-divinylbezene resin containing imimodiacetic acid groups, has a high affinity towards inorganic metals, especially transition metal ions. Depending upon the stability of the metal compounds, metals weakly complexed or free are bound to the resin, which can later be eluted by nitric acid, while the metals are strongly complexed to organic or inorganic colloids are excluded.

Batley and Florence (21) developed a very complicated metal speciation scheme using Chelex 100 resin, ASV, and UV irradiation to decompose the metal from organic matrix,
which required a tedious multi-step analysis that was not practical.

[5] Thiol Resin for Biological Metal

Thiol resin was reported as a better agent for metal toxicity and bioavailability studies than Chelex 100 resin (22). In vivo, the metal penetration through the biological membrane occurs primarily via metallothionein type proteins to which the metals are complexed with sulfhydryl group in cysteine (23). Therefore, the measurement of the metals bound to thiol resin would mimic the in vivo conditions and suggest a reasonable estimate of the bioavailable metal (24).

[6] Double-Stage Carbon Atomizer AAS

Robinson and Rhodes (25) developed a single graphite rod, double-stage atomizer for gasoline lead speciation which depended on the volatility difference between the forms of organometallic lead, such as tetraethyllead (TEL), trimethyllead (TML), diethyllead (DEL), and tetramethyllead (TML). The atomizer was designed to have two sections in accordance with the two stages. The temperature of the two section were separately controlled. A single sample was introduced into the first, programmed section to vaporize the metal compounds based on their volatilities. Then, the separated metal compounds were pushed by purging gas into
the secondary section at where the metal compounds were atomized. The absorptions of different metal compounds were measured at the end of the secondary section. Later, the data were used to speciate the metal compounds.

The advantage of the method was that it could directly determine the metal in the sample without any pretreatment of the sample. Both liquid and solid samples could be analyzed directly, minimizing the sample loss and contamination from the sample processing.

C. Chromatographic Techniques for Metal Speciation

Among all the separation methods, chromatography is the most powerful one. It provides rapid separation and sensitive detection limits to fit the needs of high resolution metal speciation studies. A few reviews on the subject of chromatographic metal speciation has been published, e.g. by Nickless (26) and Cappon (27).

Chromatography is a method of separating a mixture of compounds into its components by the differences in the interaction of the components with the mobile phase and the stationary phase. The components with different chemical and physical properties interact differently with the mobile phase and stationary phases, leading to separation of the compounds. Such properties include solubility, affinity, ionic strength, charge, and molecular dimensions.
Initially, the effluents from a chromatographic separation were usually identified by other methods. The recent development of coupling chromatography with IR, NMR, and MS has broadened the analytical capability of the method. Its recent coupling with atomic absorption spectroscopy has extended its application to elemental analysis and metal speciations.

In metal speciation studies, the modes of chromatographic separation, the methods of metal compounds detection, and the strategies of chromatography and its detection interfacing are equally important. We will briefly discuss some of each in the following sections.

[1] Gas Chromatography

In gas chromatography (GC) metal speciation, volatile and thermally stable metal components must pass through the GC column. Therefore, metal speciation using GC has been limited to volatile metal compounds such as mercury, tin, lead and arsenic. However, most inorganic metal compounds are not sufficiently volatile under the GC column temperature, and the method is limited in its applications.

[2] Liquid Chromatography

Liquid chromatography (LC) normally operates at ambient temperature and is therefore more suitable for speciation of nonvolatile and volatile metal compounds, large molecular
organometallic compounds, and in particular, thermally unstable biomolecular metal compounds. Liquid chromatography offers a choice of different separation modes and detection compatibility for metal speciation studies.

[3] Separation Modes in LC

The application of LC to metal speciation studies depends on its separation modes and compatibility with detection methods.

(a) Partition Chromatography. Partition chromatography, introduced by Martin and Synge in the early 1940's (28), separates the sample components depending on the partition coefficients of its components between the mobile phase and the stationary phase. The different partitioning results in different migrating rates among the components and thus enable separation.

One special form of partition chromatography is ion pair chromatography, which utilizes a counter ion of opposite charge to the sample that alters the ionic characters of the components. This in turn alters the retention time of the components eluted from the column, and achieves separation. The retention can be further modified by changing the alkyl chain length of the counter ion.

Ion pair chromatography can be very useful in metal speciation because of the ionic nature of metal compounds. The counter ion addition into the biological sample,
however, might change the chemical and physical nature of the sample that could cause unreliable results. Therefore, it was not used in this study.

(b) Ion Exchange Chromatography. Ion exchange chromatography was first named by Small et al. in 1975 (29). The ion-exchange processes are based on exchange equilibria between ions in solution and ions of like sign on the surface of an essentially insoluble, high-molecular-weight solid. Solute ions of the opposite charge are attracted to the stationary phase by electrostatic force.

The first synthetic ion exchange resin was developed in 1935 by B. A. Adams (30). The bonded sulfonic or carboxylic acid active sites were used for cation exchange resins, while amine groups were used in anion exchange resins. Recent developed ion exchange packing materials allowed the method to be applied to a wider sample range of compounds including proteins, amino acids, metals, and organometallic compounds.

(c) Molecular Exclusion Chromatography. Unlike other forms of chromatography, there is no attractive interaction between the stationary phase and the solute in molecular exclusion chromatography. The separation of the sample components is based on their molecular sizes and shapes. When the components pass through the column, large molecular
species which are unable to penetrate the pores on the surface of column packing materials elute faster than smaller molecules which penetrate the pores are retained longer.

For speciation studies, the separation of metal compounds would be mostly based on the interaction between the compounds and the column stationary phase, not based on the compound size. Therefore, the method was not suitable.

(d) Other chromatographic separation modes are available, but rarely used in metal speciation studies. One such chromatographic separation mode is adsorption chromatography. This method separates components based on the equilibrium between the adsorbed solute and the solution. The separation is achieved by varying degrees of reversible adsorption and desorption of the sample components on the solid stationary phase. In general, nonaqueous solvents are used with silica or alumina as the stationary phase. It is used to separate non-polar substances.

Another technique is affinity chromatography. The separation is achieved by the special affinity of a particular component in the sample, or a few components for the stationary phase. It has been almost exclusively used in biological sample purification, and rarely applied to metal speciation studies.
[4] Reverse-Phase Chromatography

In reverse-phase chromatography, the stationary phase is nonpolar, often a hydrocarbon, and the mobile phase is a relatively polar solvent such as water, methanol, or acetonitrile. The polarity of the mobile phase controls the elution time of components with different polarities. Normally, the mobile phase used in reverse-phase chromatography is a mixture of two different solvents with a certain volume ratio. In some cases, a solvent gradient elution method is applied to improve the separation efficiency.

Reverse-phase chromatography has become the most widely accepted separation method in many different applications. For the separation of metal compounds in biological sample, it is very attractive because of the compatibility of the aqueous solvent with aqueous biological samples. Therefore, it has been used as a major separation method in these metal speciation studies.

[5] Detectors Used for LC

Chromatographic separation requires the detection used be sensitive, reliable and reproducible towards all solutes but highly selective towards one or more classes of solutes. For the studies of metal speciation, however, a metal specific detector is required rather than a general detector.
Among different LC detectors, the use of ultraviolet, infrared, and fluorescence detectors is based on the absorption of UV, IR of the solutes, or on the UV fluorescence of the LC effluents. These detectors are not suitable for metal detection because they do not specifically detect any particular metals unless the metals are complexed. However, they can be used in parallel with metal specific detectors, such as ICP or AAS, to confirm the possible metal compounds in the elution.

Other LC detectors such as refractive index, thermal or electrical conductivity detectors, are not metal specific detectors either because the differences of refractive index or electrical conductance between the metals and mobile phases or between the metal compounds are not significant. They are not suitable as detectors for most metal speciation studies.

Mass Spectrometry (MS) is a most powerful detector for the effluents separated from LC column. It is highly sensitive and provides information related to the molecular structure of the metal compounds once the latter is located. However, it is difficult to locate the metal compounds amongst the multitudes of other compounds in body fluids because MS is not a metal specific detector. Nevertheless, the thermospray interfaced LC-MS system can still provide useful molecular structural information on the possible metal compounds if they are separated with the same LC
system and have been detected with a metal specific detector such as atomic absorption spectroscopy.

[6] LC Detectors Used for Metal Speciation

Atomic Absorption Spectroscopy (AAS or ICP) is the most widely used detection technique for metal determination because of its high sensitivity and selectivity. The combination of liquid chromatography with AAS can be a powerful technique that yields the identity of particular metal compounds separated from the sample. In addition, the specific detection of metal of interest by AAS eliminates much of the complexity due to the presence of other metal organic compounds when conventional, non-selective detectors are used.

Both ICP and AAS have been investigated for detectors used in coupling with chromatography for speciation studies by our group and others (9, 31, 32, 33). An ICP gives relatively poorer sensitivity as a metal detection compared to AAS, but because it has the advantage of multielemental detection ability, it is more versatile than AAS. However, ICP suffers from more problems in interfacing with chromatography because of the plasma instability, inaccuracy caused by interelemental effects, low solvent loading requirement, plus difficulties in handling organic solvents. Overall, AAS offers better compatibility for interfacing with liquid chromatography, especially flame AAS.
D. Introduction to Atomic Absorption Spectroscopy

The fundamental principles of Atomic Absorption Spectroscopy as an analytical technique were based on the concept proposed by Alan Walsh in 1955 (34).

Atomic absorption spectroscopy is the study of the ground state atoms that absorb the resonance radiation and undergo the transition to the excited state. Atoms do not vibrate in the same sense that molecules vibrate and have virtually no rotational energy. Hence, no vibrational or rotational energy are involved in the electronic excitation of atoms. As a result, AA spectra consist of relatively few, very narrow absorption lines that serve as very specific identification criteria of metal detection.

Therefore, atomic absorption occurs when the ground state atoms absorb radiation from the radiation source of an exactly defined wavelength:

\[ A + h\nu \rightarrow A^* \quad \text{(1)} \]

where \( A \) is a ground state atom, \( A^* \) is an excited state atom, \( h\nu \) is the excitation energy.

**Figure 2** shows a block diagram of the key components of an atomic absorption spectrometer. The spectrometer is designed to excite the sample atoms in the atomizer, and then the percentage of the radiation absorbed by the excited atoms is directly related to the metal concentration in the
FIGURE 2. MAJOR KEY COMPONENTS OF A TYPICAL DOUBLE-BEAM ATOMIC ABSORPTION SPECTROPHOTOMETER
sample, which is measured by the optical system.

[1] Radiation Sources in AAS
(a) Commercial Radiation Sources

The radiation source emits radiation with the exactly same wavelength as the wavelength at which the absorption of the ground state atoms of interest taking place. There are two major radiation sources commercially available which are the hollow cathode lamp (HCL) and the electrodeless discharge lamp (EDL).

The hollow cathode lamp consists of a glass cylinder filled with inert gas which is ionized by a high voltage across the anode and cathode. The ionized gas molecules at the anode are attracted and accelerated toward the cathode. There the metal atoms are dislodged from the cathode surface. This process is called sputtering. The sputtered atoms become excited to emit the characteristic spectrum of the metal used to make the cathode. Hollow cathode lamps emit spectra of the metal used to make the cathode with very narrow lines. Therefore, the radiation can be completely absorbed by the atoms, if they are the same element as that in the cathode.

It is difficult to make stable hollow cathodes from volatile elements such as arsenic. An alternative, electrodeless discharge lamp was developed. It consists of an evacuated tube which is filled with argon at atmospheric
pressure, and in which a small amount of the metal of interest in placed. The sealed tube is placed in a radiofrequency cavity in which the argon gas is ionized by the radiofrequency energy and causes excitation of the metal atoms, producing intense emission lines.

Some line broadening occurs in the hollow cathode lamp caused by the Doppler effect and self-absorption. The former causes emission line broadening at high current line emission; while the latter causes decreased line intensity due to the unexcited atoms in the cloud absorbing in the center of the emitted resonance line. The electrodeless discharge lamp offers high line intensity but suffers from short life-time.

(b) Demountable Hollow Cathode Lamp

In order to overcome certain of the problems encountered in HCL and EDL lamps, a demountable hollow cathode lamp was developed and used in Robinson's research group (35).

Figure 3 shows the schematic diagram of a demountable hollow cathode lamp. In the demountable hollow cathode lamp, the excess gaseous metal atoms, which may cause self-absorption, are constantly removed by inert filler gas purging and vacuuming. This allows higher current operation, to yield intense line emission. In addition, the lamp is constantly cooled by cooling-water, to maintain a low
FIGURE 3. A DIAGRAM OF BARNES DEMOUNTABLE HOLLOW CATHODE LAMP
NOTE THAT THE ATOM CLOUDS ARE REMOVED BY FILLER GAS
temperature operation to minimize the Doppler effect.

The lamp is very handy and easy operated. The cathode can be changed within minutes for different metal analyses. It is also inexpensive compared with commercial lamps.

[2] Atomizers

The atomizer in AAS produces atoms in the ground state to absorb the incident radiation from the source. It plays the most important role in the whole AAS system. The efficiency of the atomization is critical because the sensitivity of the determination of the metal is directly proportional to the number of ground state atoms formed in the atomizer.

(a) Flame Atomizer

The flame atomizer has been the most widely used atomization technique since its introduction in 1955 (34). There are two principal types of flame atomizers, a total consumption and a premixed burner. The latter is nowadays exclusively used because of its stability and low noise level.

Figure 4 is a schematic diagram of a typical commercial laminar flow burner. Sample solution is introduced as an aerosol into the flame through a pneumatic nebulizer by fuel/oxidant mixture flow. The thermal energy from combustion is used to desolvate the aerosol, vaporize,
FIGURE 4. A DIAGRAM OF A TYPICAL COMMERCIAL LAMINAR FLOW BURNER (BY PERKIN-ELMER CORPORATION)
decompose the inorganic and organic constituents in the sample, and finally atomize the metal analyte of interest.

The major advantage of the flame atomizer is that, after the sample is burned and evaporated out of the flame, the interferences caused by many organic and inorganic compounds in the sample are eliminated. This is the major concern that has to be taken into account for the metal speciation studies.

The processes of flame atomization were revealed by Robinson (36) in 1961. Table 1 describes the major steps of atomization in flame atomizer. An nitrous oxide/acetylene flame is usually used for most of the metals. In this work, the air/acetylene flame was used. The temperature of an air/acetylene flame is about 2,500°C. A detailed description of the flame AAS processes is provided in Chapter Three.

(b) Graphite Furnace Atomizer

The first graphite furnace atomizer was developed by L'vov in 1961 (37). Robinson, and later Woodriff and Ramelow, described a constant temperature furnace design but it was not developed commercially. Massmann (38) introduced a much simpler furnace in 1968, and that design became the basis of the commercial graphite furnace atomizer. Unfortunately, this design was not nearly as accurate as the designs developed earlier by Woodriff and by Robinson.
<table>
<thead>
<tr>
<th>Physical form of sample in flame</th>
<th>Reaction</th>
<th>Factors controlling reaction</th>
<th>Part of flame</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxide</td>
<td>No reaction or reduction</td>
<td>Stability of metal oxide, flame composition</td>
<td>Outer mantle</td>
</tr>
<tr>
<td>Atoms</td>
<td>Accumulation or oxidation</td>
<td>Flame composition, stability of atoms</td>
<td>Reaction zone</td>
</tr>
<tr>
<td>Solid particles</td>
<td>Disintegration</td>
<td>Stability of compound, anions, flame temperature, ultraviolet light emitted from the flame</td>
<td>Inner cone</td>
</tr>
<tr>
<td>Droplets</td>
<td>Evaporation</td>
<td>Droplet size, solvent, flame temperature, feed rate, combustibility</td>
<td>Base</td>
</tr>
</tbody>
</table>
The graphite furnace atomizer is heated to 3,000°C by electrical current, and a small amount (5 to 50-ul) of sample is atomized to free atoms. The furnace is heated stepwise to evaporate the solvent (dry stage), to decompose organic constituents (char stage), and finally to atomize the metal analyte (atomization stage). An unrivalled high sensitivity, at the picogram level of solute detection, is normally obtained.

The graphite furnace atomizer has the highest detection sensitivity for most of metals amongst all the atomic absorption spectroscopic methods. Normally, its sensitivities for metals are three orders of magnitudes higher than that of the flame atomizer. However, it has higher background molecular absorption noise than the flame atomizer. Many modifications of the furnace designs were proposed, among them, L'vov suggested a "graphite platform" furnace which reduced the background absorption to a significant extent (39). However, the background absorption is still noticeable.

A number of graphite furnaces were designed in Robinson's group for the purpose of metal speciation studies (40, 41). This will be discussed in the following sections.

[3] Interfaces for HPLC with AAS

For the purpose of metal compound speciation studies, aqueous samples normally are involved. Liquid chromatography
is always used, and the high performance liquid chromatography is the method of choice. The choice of metal specific detector was always atomic absorption because of its high sensitivity and reliable detection of the metal of interest. However, the interfacing between the two techniques was not an easy task to accomplish. Some designs for interfacing AAS with chromatography have been investigated. In the past few years, considerable effort has also been made in our group to interface these two techniques for the metal speciation studies. We will discuss them in detail in the following chapters along with the different purposes of the studies.
CHAPTER TWO

DEVELOPMENT OF THERMOSPRAY NEBULIZER INTERFACED
HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-
GRAPHITE FURNACE ATOMIC ABSORPTION SPECTROSCOPY
FOR METAL SPECIATION STUDIES

32
1. INTRODUCTION

The toxic effects of metals depend on the chemical forms of the metal. Such metal compounds are usually at very low total concentrations, often with very limited quantities of biological samples available for analysis. Therefore, metal speciation analysis must be very sensitive.

Speciation studies of a metal involve identifying and measuring the possible chemical forms of that metal which actually exist in the sample. The metal compounds are usually in the presence of many organic, inorganic and organometallic compounds. Consequently, separation of the metal compounds from the other compounds is necessary. Ideally, this is followed by determination of each metal compound with very high sensitivity. Development of such a combination instrument is the top priority of these speciation studies.

Gas and liquid chromatography (GC and LC) are both very powerful chemical separation methods; while atomic absorption spectroscopy (AAS) is one of the most sensitive metal specific detectors. The combination of these two methods is a very attractive analytical tool for trace metal speciation analysis.

Of all the metal specific detection techniques, the graphite furnace atomic absorption spectroscopy (GFAAS) is the most sensitive, having typical absolute sensitivity of
It usually is about 1000 times more sensitive than an AAS system using a flame atomizer (42). It is quite capable of handling very small samples of the order of microliters. Ideally, the sample should not require any pretreatment. This would be a major advantage since many analytical errors are introduced during pretreatment.

Although commercially-made graphite furnace atomizers are designed to handle microliter size liquid samples, they are short-lived because of thermal loss or degradation of carbon. Besides, the discrete sample introduction and separated drying, ashing and atomizing processes are not suitable for the continuous detection of chromatographic effluents. A new furnace design was needed.

A system of interfaced gas chromatography (GC) and GFAAS was developed for the speciation study of human body fluids in our group (41). It was successfully used for determining a few volatile metal compounds such as zinc, mercury and lead. However, the metal contents measured from GC column effluents was always less than the total metal concentration in the sample. This indicated that some nonvolatile compounds were not eluted, and/or some thermal labile compounds were decomposed inside the GC column.

Consequently, an instrument using in-line interfacing of high performance liquid chromatography (HPLC) and GFAAS was proposed. This should eliminate the problems of instability and lack of volatility. The high resolution of
HPLC separation at room temperature and the high sensitivity of GFAAS detection would enable the speciation of either volatile or nonvolatile metal compounds with extremely low metal contents in body fluids. However, this would require the complete redesign of the furnace. Additionally, the interface should be capable of converting the liquid effluents from an HPLC column to vapor before entering the furnace. The research of developing such an analytical system became the main objective of this work.

2. ATOMIZATION MECHANISM IN GRAPHITE FURNACE AAS

The mechanism for free atoms generation in a graphite furnace was first proposed by Campbell and Ottaway in the late 60's based upon the simplified reaction equation (43):

\[
MO (s) + C (s) \rightarrow M (g) + CO (g)
\]

Other researchers explained the entire process of free atom formation in the furnace as first-order process, in which thermal decomposition, reduction and vaporization of the metal oxide or halide might be involved (44-47).

In the commercial graphite furnace, three steps are involved in the generation of free atoms:

(1) **Drying**: the solvent is evaporated from the sample,
(2) **Ashing**: the sample is ashed into residue,

(3) **Atomization**: the residue is atomized to free atoms.

After introducing a sample of microliter size into the furnace, the temperature of the atomizer is raised to a few hundred degrees for a few seconds to evaporate the solvent. The dried sample is then ashed at a higher temperature for another few seconds on the atomizer surface. After ashing, the furnace temperature is rapidly increased to > 2,700°C. Atomization of the residue occurs in a few milliseconds to seconds. The light absorption by the free atoms generated from the solute residue is measured optically (48), and from this measurement and a calibration curve, the concentration is determined.

3. **SENSITIVITY OF GRAPHITE FURNACE AAS**

The higher sensitivity of graphite furnace AAS over that of flame AAS is due to four main reasons:

(1) The dried and ashed sample residue contains mostly the analyte without being diluted by solvent which is always encountered in flame atomizer.

(2) On the graphite surface, the entire dried sample is completely atomized to free atoms that will contribute to absorption. In this way, the absorption peak height or area
is increased in the furnace than in flame, resulting in increased sensitivity.

(3) The entire residue is atomized and because free atoms are confined inside the furnace, analyte loss is minimized.

(4) The average residence time of free atoms within the optical path is increased owing to the atoms are confined inside the furnace through which the optical beam passes. This thereby intensifies the absorption signal.

In contrast, in a flame, there is a sensitivity loss caused by the solvent effect, inefficient atomization, sample loss in transportation, and the brief residence time of atoms in flame. Sensitivities of some typical metal detections from a graphite furnace atomizer and flame atomizer are compared below (49) in Table 2:

| Table-2 Sensitivity for Selected Elements |
|-------------------|---------------------|---------------------|
| Element | Flame AAS (ng/ml) | Graphite AAS (ng/ml) |
| Al    | 30            | 0.005              |
| As    | 100           | 0.02               |
| Cd    | 1             | 0.0001             |
| Cu    | 2             | 0.002              |
| Hg    | 500           | 0.1                |
| Pb    | 10            | 0.002              |
| Zn    | 2             | 0.00005            |
4. ANALYTICAL INTERFERENCES IN GFAAS

However, graphite furnace AAS still has several sources of interference leading to a relative error of 30%.

Certain matrix problems still remain even after sample drying and ashing. Broad-band molecular absorption due to incomplete breakdown of inorganic or organic matrices in the brief atomization time (few seconds) is common, which can be partially corrected by background correction. Furthermore, memory effects are often severe because part of the analyte and matrix apparently diffuse into the porous graphite surface during the atomization. Moreover, chemical interferences remain the same as in the flame AAS. Chemical interferences occur when different chemical forms decompose at different rates. For example, CaSO$_4$ decomposes slower than CaCl$_2$ and gives different absorption signals for the same concentration.

5. GRAPHITE FURNACE DESIGNS FOR DIFFERENT PURPOSES

A. Graphite Furnaces Used in GFAAS

Since the first carbon rod atomizer was built in 1961 by L'vov, the graphite furnace has gone through quite a number of designs before becoming commercialized (50).
The original L'vov carbon rod atomizer was a few orders of magnitude more sensitive than the flame atomizer, but was hard to control and not reproducible. In the meantime, T. S. West, J. W. Robinson, and Massman refined their designs. Increased life-time, improved sensitivity, accuracy and precision, and reduced interferences were achieved with these designs.

Then, in 1969, at the International Conference on Atomic Absorption Spectroscopy, Sheffield, England, two leading papers on the subject of graphite and carbon rod furnaces were presented by T. S. West and J. W. Robinson (50) and riveted researchers' attention. Since then, these furnaces became two major electrothermally heated atomizers used in the field.

B. Graphite Furnaces Used in GC-GFAAS

In order to speciate the metal compounds separated by the GC column, the introduction of the effluents into the furnace must be continuous and direct. New furnaces, and GC-GFAAS interfaces were designed and developed in our group.

As shown in Figure 5, the final version of the furnace had a "T"-shaped configuration. Two hollow graphite tubes were glued together perpendicularly with HCl and furfuryl alcohol mixture. The horizontal tube was aligned with the optical light path where the absorption measurements were made. The base of the vertical tube was connected to the GC
FIGURE 5. A DIAGRAM OF A GC-GFAAS INTERFACE SYSTEM. THE EFFLUENTS FROM THE GC COLUMN ENTER THE VERTICAL ATOMIZER VIA THE PYREX CAPILLARY. THE ABSORPTION OF ATOMS TAKES PLACE IN THE OPTICAL LIGHT PATH IN THE HORIZONTAL PIECE.
column through a Pyrex capillary line. The latter was heated with electric heating tape to 210°C, and its length was kept to a minimum for optimum direct interfacing. The furnace was installed in a water-cooled metal housing, and was heated electrothermally with electrical contact made by two electric leads. One lead was connected to the top of the horizontal tube and the other was connected to the base of the vertical tube.

The furnace was heated to a predetermined high temperature. The effluents from the GC column were directly swept into the furnace through the interface by the carrier gas. The vapor metal compounds were rapidly thermally decomposed upon entering the vertical tube. The latter was maintained at a moderately high temperature <2,000°C (42). The entire products were then swept through a narrow intersection tunnel at the juncture of the two tubes where the temperature was designed to be the highest (>2,100°C). Here the sample metals were atomized. The free atoms were then transported into the horizontal tube. The temperature of this tube was kept at 2,000°C to maintain the sample in the atomized state to absorb the radiation of the resonance wavelength of the metal of interest.

The system functioned well for a few metals such as mercury, and the observed sensitivity showed 2-7 fold enhancement compared to that of the commercial system (41).
A serious limitation was that only volatile metal compounds were detected. The results showed that the metal concentration determined using GC-GFAAS system was always less than the total metal concentration originally known in the sample. This indicated perhaps that certain nonvolatile or thermally unstable metal compounds in the sample were not determined. To overcome the problems, the GC was replaced by HPLC, which was interfaced with GFAAS for the speciation of nonvolatile metal compounds.

C. Graphite Furnace Requirements in HPLC-GFAAS

The prime requirements of a graphite furnace used as a detector for HPLC include: (a) high sensitivity, (b) ability to operate on a continuous basis, and (c) ability to accommodate liquid sample.

For biological sample speciation using reverse-phase HPLC, an aqueous mobile phase is usually used. For optimum resolution, 1.0-ml/min of solvent flow rate is usually used. At this flow rate, 0.056-moles of water will be introduced into the furnace per minute. Under 1-atm and 2500 C, the following reaction,

\[
\text{C (s) + H}_2\text{O (g) }\xrightarrow{\text{CO (g) + H}_2\text{ (g), \quad ----- 3}}
\]

will occur. If the reaction is complete, then 0.67 grams of carbon will be burned per minute. Meanwhile, 0.056-moles of
CO and $\text{H}_2$ gases are produced, generating 420-ml of vapor per second. This huge volume of vapor will cause high pressure in the furnace, and accelerate the sample through the furnace. Consequently, the furnace which weighs less than 1.00 gram of carbon is destroyed by the reaction in less than two minutes.

In such a circumstance, therefore, the furnace has to be made mechanically, thermally, and chemically stronger to survive the reaction, in order to accomplish the continuous detection of HPLC effluents.

6. EXPERIMENTAL

A. Equipment Used


[8] Nebulizer: 0.005" ID, 1/16" OD stainless steel capillary tubing (Alltech) heated by 115-V, 100-W "Firerod" electrical heating cartridge (Waterloo, St. Louis, MO.).

B. Chemicals and Reagents Used
[1] Furnace Material: High density (2.5-g/cm³) graphite of 1" cube by Union Carbide, Co..
[3] Standard Solution: 1,000-ppm CuCl₂ of 1.00-g copper dissolved in HCl acid and diluted to 1.00-liter.

C. Furnace Modification for Even Temperature Distribution

Two important considerations are (a) higher furnace temperature and (b) even temperature distribution. The former provides higher energy to improve atomization efficiency, and the latter enlarges the effective atomization surface area. Further, any local hot spots would cause rapid local burning of the furnace.
The graphite furnace relies on its electrical resistance to be electrothermally heated. Its resistance depends on the cross-sectional area through which the electric current flows. Figure 6-a shows the temperature distribution of the furnace used in GC-GFAAS, which was measured by an optical thermometer:

(1) <600°C, at the large carbon base of the furnace,
(2) <900°C, at the top portion of horizontal piece,
(3) >1,400°C, at the bottom portion of horizontal piece, because the thick carbon/acid glued neck,
(4) 800-1,400°C at the middle portion of vertical piece, and it was not even,
(5) 1,100°C at the narrow neck of two pieces joined section, also was due to thick carbon/acid glued neck.

In practice, it was found that the furnace temperature increased with reduced cross-section area of the graphite tubes, but an uneven temperature distribution still remained as is shown in Figure 6-b.

The attempts made to improve the furnace temperature distribution as follows: Firstly, the single large carbon electric lead was abandoned. It was proposed that this caused a low temperature on top of the horizontal piece. Two carbon extension tubes were placed on both ends of the horizontal piece with the same ID and OD as the latter. The very ends of this extended tubing were tightly fixed into the optical path of the housing, which was also part of the
FIGURE 6. THE TEMPERATURE DISTRIBUTION OF TWO GRAPHITE FURNACES
(A) FURNACE USED IN GC-GFAAS SHOWS LOW TEMPERATURE
(B) NEW FURNACE SHOWS HIGHER TEMPERATURE WITH UNEVEN DISTRIBUTION AT DIFFERENT PORTIONS
top electric lead. These two carbon tubes were built such that resistance was constant throughout, and the electric current flow through the whole tubing with the same electric resistance and heated the whole piece to evenly high temperature.

Secondly, a cup-shaped carbon base was made to be the bottom electric lead which was NOT water-cooled. Only the very end of the vertical tube was inserted into the base. This reduced the contact surface area to a minimum, and ensured high resistance at the entrance of the furnace, thus producing a high temperature.

Thirdly, the narrow outlet neck portion of the vertical tube was modified into a shoulder to maintain a constant cross-section area. The schematic diagram of the whole system is shown in Figure 7.

Figure 8 shows that, after the furnace was heated for 30 seconds, a smooth furnace temperature distribution was obtained. The narrow neck of the furnace reached 3,000°C, which was desirable for the atomization to occur there, and the rest of the furnace was evenly 2,700°C.

This even and high temperature distribution was maintained with a later designed, replaceable, "T"-shaped graphite furnace, which will be discussed in the next section.
FIGURE 7. THE FIRST DESIGN OF THE HPLC-TSP-GFAAS INTERFACE SYSTEM. THE EFFLUENTS FROM HPLC COLUMN ENTER THE ATOMIZER VIA THE TSP.
FIGURE 8. THE HIGH AND EVEN TEMPERATURE DISTRIBUTION ON A NEWLY DESIGNED, REPLACEABLE, "T"-SHAPED GRAPHITE FURNACE.
D. Furnace and HPLC-GFAAS Assembly Modifications

[1] New "T"-shaped Furnace Construction

The construction of the last version of the furnace was again modified for HPLC-GFAAS interfacing. A new, replaceable, two piece "T"-shaped furnace substituted for the glued-up, single piece system, and is shown in Figure 9 with dimensions:

A horizontal piece was made carrying a small perpendicular hollow stem stuck out in the middle like a mushroom. This stem served as the narrow neck of the original "T"-shaped furnace in which atomization took place. This was manually made and was the most difficult part of the construction. Much delicate care was taken whenever the piece was made.

Experimental experience showed that the furnace was always burned by water only at the upper portion of the vertical piece but not the neck. If the vertical piece could be replaced with a new one after being partially burned, the rest of the furnace was reusable. Replacing the vertical piece would be much easier than making a whole new furnace. This led to the idea of making a horizontal piece all-in-one with the atomization section.

The vertical portion of the furnace was machined from 1/4" OD high density graphite rod. A 5/16" ID and 7/8" half-through hole was drilled in a 1" long rod. A 1/8" ID groove ring with 3/32" depth was drilled to accept the stem from
FIGURE 9. THE CONSTRUCTION OF THE NEW, REPLACEABLE, "T"-SHAPED GRAPHITE FURNACE. A HORIZONTAL PIECE WAS MANUALLY MADE AND INSERTED INTO THE NECK OF A VERTICAL PIECE TO FORM A "T"
the horizontal piece. The two pieces were fit tightly into each other to form a replaceable "T"-shaped furnace.

[2] New Furnace Housing Construction

The new, replaceable "T"-shaped furnace was installed into the housing for testing. However, the furnace housing wall, in which a small cavity was made for water cooling, was only 1/4" distance from the 3,000°C furnace and became so hot that thermal vibration of the housing would shake the electric contacts loose. The furnace temperature would then suddenly drop when the electric contacts were lost. The absorption signal then disappeared. A housing with efficient cooling was needed.

Figure 7 shows a new atomizer housing design which had a much larger cooling water capacity and thinner metal body than that used in GC-GFAAS. It allowed a higher water flow rate to effectively carry much more heat from the housing. This design completely eliminated the thermal vibration on the housing, and the problem of electric contact disruption was solved. The furnace then could be kept above 2,700°C for hours without interruption. This was necessary for extended continuous use with HPLC separations.


Figure 7 also shows that the axes of the two carbon electric contact tubes and the horizontal piece were
precisely aligned in the housing. This then perfectly served as an extended optical path. It prolonged the residence time in the optical path of free atoms formed in the furnace, and increased analytical sensitivity.

The housing bottom served as the bottom electric lead. It was electrically isolated from the body with a 1/4" thick boron nitride ring, and was NOT water-cooled for the reason of maintaining a high temperature profile at the furnace entrance. The sample aerosol/vapor mixture leaving the thermospray would encounter a hot zone, and avoid the sample aerosol being recondensed.


In the GC-GFAAS system, a chemically inert atmosphere was achieved by continuously purging nitrogen gas into the housing where the whole furnace was enclosed. The light beam entered the atomizer through quartz windows, which are shown in Figure 5. Before leaving the housing, the gas circulated through the furnace, diluted the free atom concentration, which resulted in decreased sensitivity.

To prevent this dilution, the quartz windows were eliminated leaving the extended horizontal tubes open to the atmosphere. However, the nitrogen which was circulating outside the furnace was adequate to keep it from being oxidized. The vaporization and atomization processes in the furnace were not disturbed by the gas. Absorption by the
free atoms could be measured without them being diluted by nitrogen gas. Figure 7 shows that the new housing design does not have these quartz windows.

E. Thermospray Interface Design for HPLC-GFAAS

In contrast with GC-GFAAS, liquid effluents from a HPLC column are not suitable for direct analysis in the graphite furnace. Therefore, an interface should possess (a) the ability to convert liquid effluents into vapors before entering the furnace, and (b) the ability of interfacing with HPLC system with ease. A thermospray (TSP) nebulizer possesses abilities of being such an interface.

Firstly, the thermospray is obtained by continuously forcing a liquid stream through an electrically heated stainless steel capillary. The thermal energy converts the liquid into vapor, containing a very fine and uniform mist of sample aerosols, spraying out of the capillary tip at very high velocity (>200-m/sec). A cone-shaped sample jet is formed with a narrow diameter of less than 1" at distances less than 4", which can be sent into the furnace for analysis.

Secondly, the spray stem of the TSP nebulizer was made of the HPLC stainless steel capillary connection tubing. It was directly connected to HPLC column giving superior interfacing. It needed no extra connection fittings.
Thirdly, the length of the capillary in TSP nebulizer was very short (less than 3.0\textquotedbl). This enabled minimizing the "dead volume" in the column-detector coupling. This preserved the high resolution of HPLC column.

Finally, biological samples could be separated in the HPLC column at room temperature. The separated compounds are thermally desolvated below 750\textdegree C (TSP operating temperature) passing through the nebulizer in extremely short periods of time, i.e. 6.0 seconds for 0.10-ml sample at 1.00-ml/min flow rate. The chemical forms of these compounds are kept unchanged before the decomposition and atomization in the furnace took place. Based on the retention times, the metal compounds could be identified. The actual molecular identities could be further confirmed using techniques such as HPLC-MS.

[1] Assumption on Thermospray Processes

The mechanism of aerosol generation by TSP nebulizer is not fully understood. Since the first commercial TSP nebulizer was constructed by Vestal et al. in 1983 (51), numerous modifications have been made based on their proposed mechanism. Figure 10 a and b show the major functions of the thermospray:

(a) As the liquid passes along the heated capillary, the temperature of the liquid is increased. (b) There is a
FIGURE 10. THE HYPOTHETICAL PROCESSES OF THE AEROSOL FORMATION INSIDE A TSP NEBULIZER. A RADIAL TEMPERATURE GRADIENT INSIDE THE CAPILLARY WAS BELIEVED TO FORM A SOLUTE CENTRAL CONE.
radial temperature gradient inside the capillary wall, causing a higher liquid temperature near the wall which decreases towards the center. Therefore, solvent in the liquid sample first is vaporized around the wall and the nonvolatile analyte remains in the liquid state. As the temperature continually increases, the layer of vaporized liquid portion gets thicker, and a large volume of vapor is formed. The thermal energy causes further interactions between the vapor particles which increases the vapor pressure inside the capillary drastically. The front of the vapor and the unvaporized liquid solute emerges from the capillary tip. This causes further shattering of the liquid/vapor mixture, and a very fine aerosol jet is formed.

According to this assumption, the solute which remains in the very center of the liquid stream is then concentrated. The aerosol/vapor generated from the capillary tip should form a spray with a solute-concentrated central cone; and the solvent should stay around the edge of the spray which can be eliminated by certain means.

This presumption has been widely accepted, and became the base of our research on the thermospray interfacing HPLC with GFAAS for metal speciation studies.

[2] Construction of Thermospray Nebulizer

The TSP nebulizer home-made in our lab employed an "firerod" electrical heating cartridge of 1.5" in length and
1.0" in outer diameter with a 1/3" hollow stainless steel rod through its axis. It had a maximum 100-W power at 115-V.

A schematic diagram of the TSP nebulizer configuration is shown in Figure 11 with dimensions.

A 1/16" OD, 0.005" ID stainless steel capillary HPLC connection tubing was inserted through the hollow rod of the cartridge. The capillary was silver soldered into the hollow rod, and very precisely centered. The empty space between the rod and the capillary was filled with Boron Nitride/Quartz slurry to maintain high thermal contact. The length of the capillary tip protruding from the rod was very critical. Normally it was necessary to keep it as short as possible to maintain a maximum temperature and achieve the best nebulization efficiency. A 1/8" capillary tip was chosen for the spray position adjustment.

The electrically cut tip of the commercially (Alltech) manufactured capillary showed an even cut surface and a perfectly round orifice, which should be kept untouched. This orifice ensured a smooth outlet for the formation of a perfectly centered, conical shape aerosol spray.

The cartridge was electrically heated to its maximum power. This was controlled by adjusting the energy input with a percentage variac. Its power consumption was monitored with amperemeter and voltmeter.
FIGURE 11. THE CONFIGURATION OF THE TSP NEBULIZER. A 0.005" ID STAINLESS STEEL CAPILLARY IS INSERTED IN AND WELDED ONTO THE CENTRAL ROD OF AN ELECTRICAL HEATING CARTRIDGE.
Construction of the Thermospray Interface

Figure 7 actually shows the first interface design. The nebulizer was tightly fit into a cylindrical metal holder with a Teflon ring in between as an insulator. The holder was suspended from a three dimensional adjustable platform on which the furnace housing was set. The distance and the coaxial alignment between the nebulizer tip and the furnace then could be adjusted.

However, on the first experimental trial, the Teflon ring melted at operating temperature (750°C). The nebulizer was out of the proper position, the spray deviated, and the signal disappeared.

Figure 12 shows a redesigned interface. Threads were made inside the holder and outside the cartridge. This enabled the nebulizer to be screwed into the holder, and to coaxially align with the furnace precisely. The distance between the nebulizer and the furnace could also be adjusted. These changes guaranteed that the spray entered the furnace without deviation.

If the thermospray process assumption was correct, then the distance from the nebulizer tip to the furnace would be critical. Suppose the solute/solvent ratio was 9:1 in the center but 1:9 around the edge of the spray cone. At a certain distance, if 90% of the eluate with 90% of the solvent was separated by skimming and withdrawn, 90% solute
FIGURE 12. THE SECOND DESIGN OF THE HPLC-TSP-GFAAS INTERFACE SYSTEM. THE TSP NEBULIZER WAS SCREWED INTO THE HOLDER WHERE THE VAPOR WAS EVENLY WITHDRAWN FROM BOTH SIDES. A LARGE CARBON BASE AND SKIMMER PROTECTED THE FURNACE FROM WATER VAPOR REACTION.
remained in the central cone of the 10% eluate would give a 9 fold increase in detection sensitivity.

The holder also served as a withdrawing chamber for pumping 90% solvent vapor. A skimmer with a small hole (<1.0-mm ID) was installed beneath the furnace entrance, which worked as a jet separator in GC/MS, to eliminate the water vapor. Two outlets on opposite sides of the holder was designed to withdraw evenly the vapor out without disturbing the spray pattern.

Figure 13 shows that an umbrella-shaped, stainless steel disk was welded onto the tip of the capillary to prevent effluent leaking into the heating cartridge and damaging it.

F. Experimental Procedures

A schematic diagram of the complete TSP nebulizer interfaced HPLC-GFAAS system is shown in Figure 14.

First, the AAS system was completely primed to optimum operating conditions. The nebulizer was preheated at 100-W with water pumped through for an hour to ensure its stable status. The furnace entrance was shielded by a metal piece to block the water vapor spray, which was continuously withdrawn by the pump.

Nitrogen was purged continuously into the furnace housing, through the silica and activated charcoal traps, to create an inert atmosphere outside of the furnace.
FIGURE 13. THE CONFIGURATION OF THE SECOND DESIGN OF THE TSP NEBULIZER. A TREADED RING WAS MADE OUTSIDE THE CARTRIDGE. AN UMBRELLA-SHAPED DISK WAS WELDED ONTO THE CAPILLARY TIP TO PROTECT THE CARTRIDGE FROM WATER DAMAGE.
The housing cooling water was turned on before the furnace was heated. The furnace was gradually heated up to 2500°C by increasing the electric current, and kept at that temperature. 0.10-ml of 0.50-ppm CuCl₂ standard was loaded into the sample loop. Copper chloride was used because the solution was stable and copper could be easily detected at low concentration.

Finally, the metal shield was removed. The temperature was quickly raised to above 2,700°C immediately followed by injection of the sample, which was delivered into the nebulizer by the solvent at 1.0-ml/min flow rate. The absorption signals were recorded; and the relationship between concentration and absorbance was determined.

7. RESULTS AND DISCUSSION

A. Graphite Atomizer Tested in HPLC-TN-GFAAS

[1] New Furnace Temperature Distribution

The new furnace could be rapidly heated to 1,000°C within 3 seconds, and reached 2,500°C in less than 15-sec at 12-Volt, 15-A AC current. After one minute, the temperature of the atomizer stabilized with a distribution as shown in Figure 8 of Section 6C of this chapter:

(a) >1,500°C at the bottom of the furnace entrance,
(b) >2,700°C at the vertical and horizontal pieces,
(c) >3,000°C at the juncture of the narrow neck stem. The entire furnace was evenly heated to 2,700°C throughout except 3,000°C (bright white) at the narrow stem. This accomplished the original design objective of a highly effective atomization section. 1,500°C at the furnace entrance was adequate to keep the vapor/aerosol from being recondensed.


With the furnace kept at 2,700°C and the withdrawing pump on, water vapor was allowed to spray into the furnace. A very noisy absorption background was recorded with the pulsing caused by the HPLC pump superimposed. The furnace could be kept at 2,700°C for less than two minutes using a 15-A AC current. Then the current gradually dropped to zero. The temperature of the furnace also gradually decreased, causing the water background signal to drop until it reached zero.

On inspection, the electrical leads to the furnace were found very well connected. The reason for the furnace temperature drop was caused by a gradually burning of the furnace following a reaction between water and graphite. The vertical tube was burned layer by layer by the vapor, and became thinner and thinner until it broke. Soot was formed and pushed by the vapor jet through the narrow neck into the horizontal tube. The soot accumulated there until it clogged.
the atomization section, which resulted in a sudden signal drop.


0.50-ppm standard copper chloride was used to test the system. 0.10-ml of sample was introduced by flow injection. 1.00-ml/min solvent flow rate was used. No column was used. Figure 15 shows a very sharp peak due to copper absorption. It was measured at the 324.8-nm resonance line about 0.5 seconds after injection. The test was repeated at different copper concentrations to prepare a calibration curve. However, it failed because the signals from successive injections gradually decreased with time as the second peak shown in the figure. The examination of the broken furnace revealed the cause: carbon monoxide formation from the carbon-water reaction caused a porous internal furnace surface and the formation of soot. The soot gradually accumulated in the atomization section and reduced the efficiency of free atom formation. Therefore, the absorption signals decreased successively, eventually to zero when the optical pathway was completely blocked up.

Many furnaces with different dimensions were studied, but the results were always the same. Water was the only cause of the failure, but this created a major problem since it was the basic solvent in biological samples and is used
FIGURE 15. THE ABSORPTION SIGNALS OF 0.50-PPM COPPER. THE DECOMPOSITION OF THE FURNACE CAUSED SIGNAL DECREASE OF THE SECOND INJECTION.
SOLVENT: WATER, FLOW RATE: 1.00-ML/MIN,
SAMPLE INJECTED: 100-uL OF 0.5-ppm CuCl₂
in reverse-phase HPLC. Strategies for solving the problem became the main task throughout this work.

B. Attempts to Eliminate Water at the Interface

As has already been discussed in Section 5C of this chapter, the 1.00-ml/min aqueous solvent flow rate used in HPLC would bring 0.056 mole of water vapor into the furnace per minute. By the reaction mentioned in Equation 3, 0.67 gram of graphite will be consumed every minute.

The graphite furnace used in the study weighed only about 0.50 gram. Even if only half of the vapor reacted with the graphite, it would only take 1.50 minutes to burn the whole furnace. Therefore, eliminating water vapor to protect the furnace internal surfaces became a major objective in this work.

[1] Using a Skimmer to Withdraw the Water Vapor

Figure 12 shows that a stainless steel skimmer with a small through-hole was made, and put underneath the entrance of the furnace. The water vapor of effluents sprayed from the TSP nebulizer was separated from the sample components by the skimmer tip. The water vapor was removed using a vacuum pump which sucked it into the water cooled condenser. This was based on the principle of a jet separator used in the GC-MS interfaced system. It is also based on the supposition for thermospray formation that the solute of the
sample is supposed to be concentrated in the center of the spray and could be removed in this skimming-pumping system. This is also shown in Figure 12.

However on testing, even with substantial amounts of water vapor removed, the furnace still did not last more than 5-min. Besides, in contrast to previous results observed with no attempt at water removal, the absorption signals were greatly reduced as soon as the vacuum pump was turned on. It indicated that the pumping actually disturbed the spray pattern and removed not only the water vapor but also the analytes.

After numerous attempts to modify and improve the design, it was decided that we simply did not have the necessary controlled machining to be successful, in spite of the fact that the process has been successfully used in GC-MS and HPLC-MS interfaced with much more sophisticated equipment. The skimmer and vacuum system was therefore abandoned and other techniques to protect the furnace were researched.

[2] Furnace Protected with BN Coating

The examination on the broken furnace showed that only the internal wall of the vertical tube was burned. The furnace external surface lasted extensively in inert atmosphere even at temperature over 3,000°C. Therefore,
protecting the internal surface might extend the lifetime of the furnace.

Boron nitride, an inorganic refractory material, has many physical and chemical properties similar to graphite (52), such as structure, expansion and contraction coefficients, and thermal conductivity. It is chemically inert to most chemicals even at 2,800°C, and electrically nonconductive. The most impressive feature of it is its inertness in reducing atmospheres even to 3,000°C. This made it a promising candidate for a graphite atomizer protection material.

Figure 16-a shows that a boron nitride coating was applied only to the internal surface of the vertical piece by dipping into a BN slurry. The horizontal piece was uncoated to remain a carbon reducing atmosphere in this atomization zone. The coating was first air-dried for 3 hours, and then oven baked for one hour at 90°C, according to the procedure provided by Standard Oil Engineered Materials (52). A strong and even boron nitride layer was formed inside the vertical tube.

The furnace was put into trial without a skimmer and vapor removal system. The TSP nebulizer was raised towards the entrance of the furnace so that the entire spray of the effluents could be sent into the atomizer.

Figure 17 shows the results of slightly reproducible signals measured with 0.50-ppm copper chloride standards.
FIGURE 16. THE BORON NITRIDE COATING AND TUBINGS PROTECTED THE
GRAPHITE FURNACE FROM WATER SOLVENT VAPOR DAMAGE.
(A) BORON NITRIDE SLURRY COATED FURNACE.
(B) BORON NITRIDE TUBING INSERTED IN THE FURNACE.
(C) TWO MANUALLY MADE BORON NITRIDE TUBING
FIGURE 17. THE ABSORPTION SIGNALS OF 0.50-PPM COPPER CHLORIDE WITH BORON NITRIDE SLURRY COATED GRAPHITE FURNACE. (A) SIGNALS WITHOUT WATER VAPOR PUMPED OFF (B) SIGNALS WITH WATER VAPOR PUMPED OFF. THE REDUCED SIGNAL CAUSED BY SAMPLE LOSS.

SOLVENT: WATER, FLOW RATE: 1.00-ML/MIN, SAMPLES INJECTED: 100-uL OF 0.50-PPM OF CuCl₂ SIGNALS: FROM SUCCESSIVE INDIVIDUAL INJECTION
The furnace lasted a little longer (5-6 minutes), but not long enough for a complete HPLC scan before being burned out.

The broken furnace was examined. The coating material was found to have accumulated inside the narrow neck of the atomizer. This indicated that the coating was gradually driven off by the vapor, and finally blocked the entrance to the atomization zone. Meanwhile, the vapor started to react with the exposed surface, which finally destroyed the furnace and prevented a signal.

[3] Furnace Protected by Larger Graphite Base

It was noticed that the graphite base of the furnace was preferentially burned by reaction with water. This indicated that the base temperature was high enough to initiate the reaction. If the base would decompose with most of the water vapor, then the lifetime of the furnace would be extended. The burned base could be easily replaced with a new one and the furnace could be reused.

Figure 12 in section 6E [3] of this chapter also shows the schematic diagram of a new, larger base with an increased height to elongate the entrance path. This base actually divided the vertical tube into two portions: The base was expected to react with majority of vapor first so the furnace would react with less vapor.
Since the base and the furnace were coaxial, the soot formed from the base was still pushed up to the neck portion and blocked the atomizer. Besides, the larger base reduced the entrance temperature, and decreased the effective atomization surface, causing the signal to drop. Further, the vapor actually reacted with both portions identically, and the much thinner furnace was always consumed faster than was expected. The result showed little improvement on furnace lifetime.

[4] Furnace Protected by Boron Nitride Tubes

The boron nitride coating did protect the furnace to a certain extent. Solid boron nitride has all the features of the slurry plus high mechanical strength, and lower porosity, machinability, and water insolubility which are desirable for the carbon furnace protection.

As shown in Figure 16-b, two solid boron nitride tubes were manually made as protection shields for the neck of the atomization section and the vertical piece of the furnace. Both tubes were of 1/32" thin walls with exact OD's to be tightly fit into the furnace. This ensured the best thermal contact, which was mandatory for effective atomization.

These tubes were inserted into the furnace vertical and neck portions. The exposed graphite horizontal piece provided a reducing atmosphere for atomization, while the BN tubes protected portions which were to provide efficient
vaporization, decomposition, and partial atomization. The furnace and the BN tubes fit into each other so well that there was no mechanical or thermal cracking after repeatedly heating for one hour at 3,000°C.

The preliminary tests of the furnace showed an almost indefinite furnace lifetime at over 3,000°C with water vapor being sprayed into it through the skimmer. No evidence of burned horizontal tube and soot formation were found. Then, the skimmer was removed, and the system was tested with copper chloride standard. But, unfortunately, after the furnace was heated for a few minutes above 2,700°C with the entire effluents directly sprayed into it from the TSP nebulizer, the very juncture of the narrow neck on the horizontal piece was burned through. The electric current and the signal dropped suddenly to zero.

Figure 18 shows the picture of such a burned furnace. The furnaces were mostly burned at their upper portion of the vertical piece, even with the BN tubing protection.

Figure 19 shows the final external appearance of the whole HPLC-TSP-GFAAS system.

8. CONCLUSIONS

The failure to protect the graphite furnace from being burned by reaction with water marked the end of this two and half years research.
The TSP nebulizer interface to a conventional reverse-phase HPLC with graphite furnace AAS has an unconquered problem of water reacting with graphite to burn the furnace. Unless other separation methods were adopted to reduce the quantity of water vapor introduced into the furnace, such as microbore HPLC with less than 50-ul/min flow rate and a larger graphite furnace operating at a lower heating temperature (53, 54), this problem will remain. Such equipment was not available to us, and the study was abandoned.

9. SUMMARY

At this point, our studies of metal speciation of human body fluids turned to the use of TSP nebulizer interfaced HPLC with flame AAS detection. The latter has a large solvent uptake capacity and is not destroyed in the process. However, the sensitivity of flame AAS is much less than graphite furnace AAS. Therefore, increasing the sensitivity of flame AAS detection by any means became the most important task to be accomplished before the metal speciation in biological sample could be performed.
CHAPTER THREE

DEVELOPMENT OF THERMOSPRA Y NEBULIZER INTERFACED HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-FLAME ATOMIC ABSORPTION SPECTROMETRY SYSTEM FOR SPECIATION STUDIES OF CADMIUM COMPOUNDS IN HUMAN BODY FLUIDS
1. INTRODUCTION

Flame atomic absorption spectroscopy (FAAS) is one of the most common metal detection techniques and yields accurate, sensitive and selective data. Its typical sensitivity levels are from a few ppm to a few hundred ppm, depending upon different element, and absolute sensitivity levels are $10^{-8}$-g. The sensitivity is defined on 1% absorption (55, 56). It is capable of handling liquid sample, and thus can be used as a liquid chromatographic detector. The high detection limits of commercial FAAS depend on a high sample introduction rate on a continuous base with a sample introduction rate at 5.5-6.5-ml/min.

When it is interfaced to HPLC, however, the technique suffers from reduced sensitivity because the rate of sample introduction is controlled by the low solvent flow rate. In addition, the sample may be separated into numerous individual components with correspondingly reduced metal concentration. Each component is further diluted by the mobile phase. Therefore, it can only be suitable as an HPLC detector for the metal speciation studies if the sensitivity of FAAS is improved. Examples of the sensitivities for seven different metals analyzed using FAAS are listed in Table-2 in Chapter Two.

In the commercial FAAS system, the sample introduction process to the flame atomizer is a most inefficient process.
This directly governs the sensitivity. During nebulization, almost 95% of the sample is drained away, and only about 5% of the sample is delivered into the flame. The nebulization, desolvation and transportation are the processes which critically control sample introduction in FAAS. Improvement of these processes is necessary to enhance the sensitivity of FAAS.

The TSP nebulizer converts the liquid sample into a jet spray of vapor/aerosol mixture with very fine droplets, which can provide better nebulization than the pneumatic nebulizer used in commercial FAAS. It is compatible with HPLC as a sample introduction device to FAAS when it is used as an interface. Some increases in sensitivity have already observed for some metal analyses (57, 58) by using the interface, but further improvement was necessary for cadmium speciation studies in human body fluids because the total cadmium concentrations ($10^{-8}$-g/ml) are extremely low. This task involved the redesign of the TSP nebulizer interface which was the main objective throughout this study.

2. ATOMIZATION PROCESSES IN FLAME AAS

In flame atomization, a solution of the sample is sprayed into the flame by means of a nebulizer, which converts the liquid sample into fine droplets of aerosol.
The aerosol is swept by fuel and oxidant mixture into the burner. Upon entering the flame, it undergoes the following processes:

(a) Sample desolvation to desolvate the analyte,
(b) Volatilization of the solutes,
(c) Fusion of the vaporized analyte,
(d) Decomposition of the analyte salt,
(e) Finally, atomization of the analyte to form free atoms, which absorb the incident radiation to generate an absorption signal.

The processes are interrelated and are affected by a number of factors upon which these processes are based. Therefore, it is important to understand each of them, especially the atomization process, in order to improve the technique as a metal specific detector for HPLC.

A. Atomization Efficiency in Flame AAS

The sensitivity of FAAS depends upon the atomization efficiency, since the free atom concentration governs atomic absorption. The degree of absorption of radiation by free atoms is determined by

\[ \int_0^\infty K_v \, dv = \frac{\pi e^2}{m c} f_{jk} N \]

where \( K_v \) is the absorption coefficient of the element at a certain wavelength, \( f_{jk} \) is the oscillation frequency, \( e \) is
the charge of an electron, \( m \) \( m \) is the mass of electron, and \( c \) is the speed of light. It should be noticed that the temperature, \( T \), and the energy of transition, \( E \), which equals the optical energy \( h \nu \), are not part of the mathematical relationship relating the degree of absorption with the number of atoms in the optical path. For a given element at a given transition, the total degree of absorption can be approximated by a constant, \( K_{jk} \), the absorption coefficient of the element, times the number of free atoms, \( N \), in the light path:

\[
\text{Total absorption} = K_{jk} \, N
\]  

This equation reveals that the total absorption is directly proportional to the number of free atoms formed in the flame. This is related to the concentration of the analyte by a number of factors, including atomization efficiency.

The efficiency of free atom generation is very poor in the flame. In 1966, Robinson calculated that if 0.10-ml of copper solution were aspirated into the flame per second, the \( 10^{15} \) copper ions were present in the light path but only one ion per \( 10^6 \) were atomized. This poor overall atomization was believed to be caused by incomplete nebulization and to insufficient thermal dissociation. In order to improve the atomization efficiency in the flame, the understanding of free atom formation is necessary.
B. Formation of Free Atoms in the Flame Atomizer

In the FAAS, the formation of free atoms can be described by a relationship established by Winefordner and Vickers (59):

\[ N = 3 \times 10^{21} \times \frac{n_{298}}{n_T} \frac{F \epsilon B}{Q T} \times C \]

where, 
- \( N \) = number of atoms M per ml in the flame
- \( C \) = concentration of element M in the solution
- \( \frac{n_{298}}{n_T} \) = ratio of number of moles of gas in the flame at 298 K and flame temperature (e.g. 2800 K)
- \( F \) = sample feeding rate
- \( \epsilon \) = nebulization and transportation efficiency
- \( B \) = atomization efficiency
- \( Q \) = fuel/oxidant flow rate
- \( T \) = flame temperature(K).

At a certain flame temperature and with a fixed sample concentration, the number of free atoms formed in the flame is directly proportional to the sample feeding rate, nebulization and transportation efficiency, and atomization efficiency; but indirectly proportional to the gas flow rate.

The gas flow rate \( Q \) is usually kept high to maintain the flame burning consumption of fuel/oxidant. This causes a very short residence time for the atoms in the optical
path and reduces the number of atoms in the optical path. This in turn reduces the absorption efficiency. The absorption reaches a maximum at a certain gas flow rate and then decreases at higher gas flow rates because of the reduced nebulization efficiency.

The sample feeding rate, $F$, is controlled by the gas flow rate in the commercial FAAS. Increasing the gas flow through the nebulizer increases the sample feeding rate. However, it decreases the nebulization efficiency and flame stability. In addition, the fuel/oxidant mixture and solvent overloading cause sample dilution and flame temperature drop, reducing the atomization efficiency.

The nebulization efficiency, $e$, is the ratio of the amounts of analyte finally entering the flame to the amounts of sample aspirated, which is the aerosol generation and transportation efficiency. It is a very inefficient process with the commercial system. For the concentric pneumatic nebulizer, it is only 5-10%, depending on the design. Large populations of droplets with large particle sizes are formed. Those greater than 10-um are recondensed and are drained away, never reaching the flame. Studies have showed that only droplets smaller than 10-um can be effectively atomized within the short residence time in the optical path; the larger ones even if they reach the flame will pass through without being atomized. Consequently, the atomization efficiency is dependent partially upon the
droplet size, i.e. nebulization efficiency - so to improve the latter will definitely improve the former, and improve the sensitivity of FAAS as well.

C. Nebulizers used in Flame AAS

The main function of a nebulizer is to convert the liquid sample into fine droplets of vapor/aerosol spray and deliver them into the base of the flame.

[1] Pneumatic Concentric Nebulizer

The pneumatic concentric nebulizer is the most popular system used for sample introduction in FAAS. The nebulization process can be described as follows (60): The high velocity of the oxidant/fuel mixture flow causes a pressure drop at the outlet of the liquid sample aspiration capillary and induces a venturi effect. A high interaction between the gases and liquid stream then produces fine droplets that are further shattered into even smaller droplets by the pneumatic energy.

The efficiency of the pneumatic concentric nebulizer is about 5% (61, 57), and for the commercially available Perkin-Elmer is reported to be 6.6% (55).

In the preliminary speciation study of cadmium in human body fluids, the pneumatic concentric nebulizer was assembled on a commercial Perkin-Elmer FAAS system. This
system was retained to enable comparison with and without the TSP nebulizer.

[2] Ultrasonic Nebulizer

The ultrasonic nebulizer was first reported by Word and Loomis (62), who suggested that it might be superior to the pneumatic nebulizer for atomization. Its nebulization process can be described as follows: The ultrasonic waves created by a piezoelectric transducer are focused onto the sample liquid surface. With sufficient wave amplitude, the wave crests in the liquid surface collapse and small droplets break off from the tip of the nebulizer to form a cloud of vapor aerosol.

Ultrasonic nebulizers have some advantages over pneumatic nebulizers in generating aerosols. The droplet sizes of the aerosol generated from ultrasonic nebulizers are more uniform, and the aerosol production rate is controlled by the energy input and is independent from the fuel/oxidant gases flow. However, the average size of the aerosol is still too large to be effectively totally atomized.

The ultrasonic nebulizer used in the commercial FAAS was placed in the front of the cloud chamber, which was cold enough to recondense the aerosol. As a consequence, the nebulization efficiency, and in turn the atomization efficiency, were largely reduced. This was, for some
applications, overcome by placing an external heating glove around the chamber; and later, by moving the nebulizer from the front to the bottom of the burner.

Previous applications of ultrasonic nebulizers in our group (63) showed a sensitivity increase of 2-fold for Cr, and 7-fold for Cd compared with pneumatic nebulizers. However, the concentrations analyzed in those cases were high (> 1.0-ppm for Cd). This concentration range is not low enough for cadmium speciation because the concentration of cadmium is several orders of magnitude lower in body fluids.


The TSP nebulizer used in HPLC-GFAAS was applied to this study with minor construction changes to fit into the laminar flow burner.

It is believed that, according to our previous observations (64), the major advantage of using TSP nebulizer in flame AAS was that it gave the highest sample transportation rate. It also desolvated sample to form very fine solute aerosol with very uniform and small droplets (0.5-1.5 um). The processes prior to atomization, which take place inside the flame when the pneumatic nebulizer was used, now can all be completed inside the TSP nebulizer. The desolvated analyte can then be transported into the flame and can be atomized in the brief residence time. The atomization efficiency is tremendously increased as the
results of the enhanced nebulization and atomization efficiency.

In HPLC-TSP-flame AAS, the large volume of solvent in HPLC effluents was no longer a problem. The sample effluents from the HPLC column, vaporized and desolvated through the TSP nebulizer, could be completely delivered into the flame and atomized. No skimmer was necessary, and sample loss was reduced to minimum.

Furthermore, the sensitivity of the TSP nebulizer interfaced HPLC-flame AAS was not dependent on the flow rate of air/fuel mixture as is the case of using a pneumatic nebulizer. It would only depend on the characters of the TSP nebulizer which could be optimized to improve the sensitivity of metal detection. This will be discussed in Chapter Four in detail.

3. EXPERIMENTAL

A. Equipment Used


[2] Radiation Source: Demountable cadmium hollow cathode lamp with power supply at 12.5-mA and 12-V.
[3] Solvent Delivery System: Laboratory Data
Control High Performance Liquid Chromatography Constametric
III 6000-psi solvent delivery pump equipped with sinusoidal
dual piston.

pulse dampener with 1/4" to 1/16" fitting convertor.

[5] Sample Injector: Rheodyne 7125 sample injector
with 0.10-ml sample loop and 1.00-ml syringe.

recorder.

[8] Separation Column: 150-mm X 4.6-mm, 5-um
packing, reverse-phase HPLC column (Zorbax C-8-RPC).


[10] Nebulizer: 100-W "Firerod" electric heating
cartridge by Watloo, St. Louis, MO., with 0.005" ID, 1/16"
OD stainless steel capillary tubing by Alltech, and modified
in home.

transformer with 1-100% voltage variac and circuit.


B. Chemicals and Reagents Used

[1] Stock Solutions: 1,000-ppm of CdCl₂ was made
by dissolving 1.0000-g of metal cadmium in just enough 1:1
HCl:Water (v/v); and diluted to 1-L. 0.5-, 0.4-, 0.3-, 0.2-, 0.1-ppm standards were diluted from the 1,000-ppm.
[6] Cleaning Solution: 10% nitric acid for burner and spray chamber, 0.5-mM citric acid for TSP nebulizer.

C. Construction of Thermospray Nebulizer

Figure 20 shows the diagram of the TSP nebulizer used in HPLC-TSP-Flame AAS system for the studies.

A stainless steel ring with the same threads as the safety diaphragm threads of the burner chamber was made. The ring was soldered onto the cartridge shell. The nebulizer was screwed into the bottom of the burner chamber.

The stainless steel, umbrella-shaped disk was abandoned in this case because all of the effluents from the HPLC were completely delivered into the flame without skimming. No effluent loss was expected.

The length of the cartridge was reduced to 1.25" by cutting off 1/4" of the unheated top portion of the heating cartridge without damaging the heating wire compartment. The reason for doing so was to keep the capillary tip of the TSP nebulizer as close to the heated portion as possible to gain
the best heating efficiency. In addition, this reduced the TSP nebulizer size for better interfacing with the burner.

D. Construction of HPLC-TSP-FAAS Interface

A schematic diagram of the HPLC-TSP-FAAS interface is shown in Figure 21.

At the base of the burner stem, the flashback safety diaphragm was removed, and the pressure relief vents were filled up with quartz cement. The TSP nebulizer was screwed into the bottom of the burner stem for direct sample aerosol feeding into the flame. The distance between the tip of the nebulizer and the burner could be adjusted by screwing the nebulizer up and down, and the nebulizer was positioned in the center of the burner stem.

The thermal energy of the hot TSP nebulizer (>500°C, for 100-W cartridge), was directly transferred to the burner stem and kept the stem above 110°C. An extra heating device preventing the sample aerosol condensation was unnecessary in this system.

E. Experimental Conditions

[1] Constant Power Supply Circuit

The power surge and fluctuation in the chemistry building is quite severe during daytime, and a constant power supply was necessary to maintain stable operation
FIGURE 21. THE TSP NEBULIZER INTERFACED HPLC-FLAME AA SYSTEM. THE TSP NEBULIZER WAS SCREWED IN THE SAFETY DIAPHRAGM OF THE BURNER FOR DIRECT SAMPLE AEROSOL INTRODUCTION TO FLAME.
conditions for the system, especially for an electrically heated TSP nebulizer.

Figure 22 shows the circuit diagram of the constant voltage power supply used in the studies. A constant amplifier transformer combined with a 1-100% variac gave a continuously fine voltage adjustment. The voltage range was 102-V to 116-V with the switch at position 1, and 116-V to 130-V at position 2. The voltage was fine tuned using the variac to within 0.14-V and 0.01-A for 1% variation. The TSP nebulizer power consumption was monitored with analog voltmeter and ampere meter.


The atomic absorption spectrometer was checked regularly to maintain optimum operation conditions. In order to avoid the absorption line reversal, the demountable hollow cathode lamp was operated under moderate low current (<5-mA). The energy input of the photomultiplier tube (PMT) was always set to moderate level to avoid its saturation. The metal resonance wavelength of 228.8-nm Cd line was regularly checked. The system was always pre-warmed as the manufacturer suggested.

The 3-dimensional position of the burner head was also adjusted routinely to maintain the maximum absorption signal. The burner head and the spray chamber were cleaned regularly with 10% nitric acid to eliminate possible memory
FIGURE 22. THE CIRCUIT OF THE TSP NEBULIZER CONSTANT ELECTRICAL POWER SUPPLY. THE SWITCH SHIFTS THE VOLTAGE RANGE FROM 102-116 TO 116-130-V WITH 0.14-V AND 0.01-A VARIATIONS.
effects. An air regulator was connected to the air supply line to reduce the flame fluctuation.


A tiny angle deviation of the TSP capillary tip from the actual center inside the burner stem could cause a large spray position change. This would bring severe signal loss, because the sample aerosol would be deposited on the burner stem and not reach the flame.

Figure 23 shows that the precise central position of the spray in the stem could be adjusted experimentally. With the burner head off, an air flow of the total air/fuel volume to imitate the actually operational condition was set. A soft tissue was placed on top of the stem opening to collect the spray vapor, which would condense on it to form a wet spot. The position of the spray was adjusted by twisting the capillary tip with a metal tube according to the position of wet spot on the tissue.

4. EVALUATION OF THERMOSPRAY NEBULIZER

Before the system was applied to the cadmium speciation studies, the TSP nebulizer was evaluated with respect to its function as a sample introduction device for FAAS.
FIGURE 23. THE ADJUSTMENT OF THE PRECISE POSITION OF THE TIP OF TSP NEBULIZER WAS CARRIED OUT WITH THE FUEL/AIR FLOW ON TO IMITATE THE ACTUAL BURNER OPERATING CONDITIONS.
A. Experimental Procedures

After the TSP nebulizer was properly installed, it was heated with low energy input (50-W). The solvent was delivered through by the solvent delivery pump at flow rate of 1.0-ml/min. A heating period of 10-15 minutes was necessary for optimum spray formation. The spray position inside the burner stem was then adjusted. The nebulizer was always preheated for more than 45-min before everyday experiments were started.

Sample introduction was performed using flow injection with a 0.10-ml sample loop. The FAAS signal responses versus different metals of different concentrations were measured. This was systematically repeated for different heating energy inputs, nebulizer equilibrium periods, solvent flow rates, and solvent types. The absorbances (A) were recorded at 228.8-nm of cadmium resonance wavelength on a linear recorder, and the percentage absorbances (A%) were plotted versus different variables.

B. Results and Discussions

[1] Effect of Energy Input to Thermospray Nebulizer on Atomic Absorption Signals

For a given solvent at a given flow rate, the energy input to the nebulizer directly controlled its tip temperature. This in turn controlled the vaporization of the solvent and the nebulization efficiency (51, 58).
Figure 24 shows the hypothetical processes of the aerosol generation occurring inside the TSP nebulizer, which are described as follows:

(a) If the energy input to the nebulizer was too low, most of the liquid sample passing through the capillary was not vaporized. Only a hot liquid stream emerged in which nebulization was not initiated, therefore, no atomization signals were observed.

(b) When the energy input was raised beyond 60-W, the aerosol started to form at the capillary tip. Since the energy input was low, only a small portion of the liquid was nebulized, and a wet aerosol emerged from the capillary tip. The droplets were too large to be effectively atomized.

(c) As the energy input was increased, the aerosol started to form at earlier stage inside the capillary. Enough energy input converted more liquid sample into aerosol, and generated smaller droplets with a more uniform size distribution. The efficiency of nebulization, and in turn, of atomization was increased; resulting in increased signal responses.

(d) Only under the optimum conditions, i.e. when the energy input was correct for a particular solvent flow rate, was the nebulization completed at the capillary tip. A dry aerosol was formed and emerged from the capillary producing the smallest and uniform droplets into the flame. The nebulization and in turn the atomization efficiency then
LOW ENERGY INPUT, ONLY LIQUID STREAM FORMED

INADEQUATE ENERGY INPUT, LARGE DROPLETS FORMED

ENOUGH ENERGY INPUT TO GENERATE MORE AEROSOL WITH SMALLER DROPLET SIZE WHICH UNDERGO DESOLVATION

ADEQUATE ENERGY INPUT TO EFFECTIVELY GENERATE VERY SMALL DROPLETS WHICH UNDERGO COMPLETE DESOLVATION

TOO MUCH ENERGY INPUT CAUSES SUPERHEATED VAPORIZATION THE SOLUTE DEPOSITS INSIDE THE CAPILLARY. NO SIGNAL.

FIGURE 24. THE HYPOTHETICAL PROCESSES OF THE AEROSOL GENERATION OCCURRING INSIDE THE CAPILLARY OF THE TSP NEBULIZER.
reached a maximum and maximum absorption signals were observed.

(e) However, if the energy input exceeded a certain too high level, the nebulization was completed inside the capillary at very early stage; the total vaporization of the liquid sample occurred and only a superheated vapor jet of solvent emerged. Sample analyte was deposited on the internal wall of the capillary. At this stage, the absorption signals decreased or dropped to zero.

Figure 25 shows the results of absorption signals of Cd, As, and Cu versus TSP energy input using TSP nebulizer interfaced HPLC-FAAS system. At low energy inputs, the absorption signals of these metal are low, but the signals are increasing with the energy inputs increases. After the energy inputs to the TSP nebulizer exceed certain levels for different metals, the signals start to drop.


The capillary in the TSP nebulizer was heated indirectly by an electric heating wire through cartridge filler materials around it. Preheating was therefore necessary for the maximum absorption signals.

Figure 26 shows that 30-45 minutes of preheating period, depending on solvent flow rates, were needed for reproducible signal responses at 105-W energy input. Only
FIGURE 25. THE EFFECTS OF ENERGY INPUT TO THE TSP NEBULIZER ON THE ABSORPTION SIGNALS OF As, Cu, AND Cd. THE SIGNALS INCREASED WITH THE ENERGY INPUT INCREASE TO A CERTAIN POINT.
SOLVENT: WATER, FLOW RATE: 1.00-ML/MIN,
SAMPLES INJECTED: ALL 100-uL OF 0.50-PPM
FIGURE 26. THE PREHEATING PERIOD FOR THE TSP NEBULIZER.
NOTE THAT A 30-45 MINUTES OF THE EQUILIBRIUM PERIOD IS NEEDED FOR REPRODUCIBLE RESPONSE.
after 30 minutes of the nebulizer preheating, could the absorption signals reach a reproducible maximum for all three solvent flow rates, 1.00-ml/min, 1.25-ml/min, and 1.50-ml/min. At 112-W, the energy input increase caused a preheating period shift towards a longer time ( >75-min) with different solvent flow rates, but the thermal equilibrium was still reached eventually with higher maximum signal responses.

Accordingly, the TSP nebulizer was always preheated for at least 45 minutes with the solvent flow at the optimum operating condition throughout the entire study.

[3] Effect of Solvent Flow Rate on AA Signals

The solvent flow rate through the nebulizer affected the absorption signal response as much as that of the energy input. Similar observations were reported by Mayar (65).

With 1.0-ppm cadmium standard and using a 0.004/0.006" ID capillary TSP nebulizer, the solvent flow rate dependence of absorption signals with different energy inputs was studied and results are shown in Figure 27.

For a given solvent at a given energy input to the TSP nebulizer, the solvent flow rate affected the nebulization efficiency, which in turn affected the FAAS absorption signal. At a lower energy input (98-W), the signals were all very low at any flow rates. This was contributed to the low
FIGURE 27. THE EFFECTS OF SOLVENT FLOW RATE ON THE ABSORPTION SIGNALS OF Cd AT DIFFERENT TSP NEBULIZER ENERGY INPUT VALUES:
(1) o ----- 98-W,
(2) O ------ 112-W,
(3) X ------ 119-W.
thermal energy input being insufficient to convert the liquid sample into fine droplets. The atomization efficiency was therefore poor.

At a higher energy input (112-W), the signals decreased with the flow rate increase. This was because the higher flow rate quickly carried the thermal energy out of capillary, and there was less time to convert liquid sample into fine droplets. This caused reduced atomization efficiency.

At an energy input greater than 119-W, however, the signals disappeared at low flow rate (<0.75-ml/min) but increased with the flow rate increase (>0.75-ml/min), and near-linear responses were observed.

The interpretation was that at low flow rate, superheated vaporization of the solvent occurred inside the capillary. With higher energy input, the analyte deposited inside the capillary and the signal disappeared. When the flow rate increased, the solvent cooled the capillary. The capillary then operated at a proper temperature. The nebulization efficiency of the liquid sample was increased, and so did the atomization efficiency. As the energy input was kept above a certain level to maintain effective nebulization, increased flow rate increased the analyte transportation rate in unit time into the flame and, therefore, the absorption signal responses were enhanced.
Effect of Solvent on AA Signals

Commercial FAAS with pneumatic nebulizer usually suffer from severe solvent interference. This is because the efficiencies of its vaporization and desolvation in the flame are dependent significantly upon the solvent properties such as viscosity, density, surface tension, heat of vaporization, boiling point, volatility, and specific heat, etc. In general, organic solvents give rise to higher signals than aqueous solvents because of increased nebulization efficiency. For quantitative work, if several different solvents are used, a calibration curve for each solvent is necessary.

The TSP nebulizer acts as a preliminary desolvation device in which the vaporization and desolvation processes are expected to go to completion and, in practice, this was shown to eliminate the solvent interferences.

The solvent effect on the flame atomic absorption signal with the TSP nebulizer was studied. The sample solution was prepared by dissolving metal magnesium and cadmium in different solvents, pure water, 50% methanol in water, and 50% acetonitrile in water. A 0.10-ml of sample was flow injected with different HPLC solvent flow rate, and FAAS signals were measured.

Figure 28 shows the absorption signals obtained using TSP nebulizer. With 110-W energy input, 0.50-ppm of
FIGURE 28. THE EFFECTS OF SOLVENTS ON THE ABSORPTION SIGNALS OF 0.50-PPM COPPER. ALL SOLVENTS GAVE THE SAME MAXIMUM SIGNAL AT A CERTAIN HIGHER TSP ENERGY INPUT.
magnesium in the different solvents mentioned above gave almost identical signal responses at any HPLC solvent flow rates from 0.75- to 2.50-ml/min. Only very slight absorption signal differences existed in between different solvents. The pure water solvent gave slightly lower signals and organics in water gave slightly higher signals.

The results indicate that the TSP nebulizer eliminated the major solvent effect by desolvating solvents inside the capillary before the dried analyte aerosol entering the flame. The results demonstrate the ability of the TSP nebulizer to be a perfect interface for HPLC-FAAS system. It will give reproducible signals even when different solvents are used as the mobile phase.


A further comparison of the sensitivity of metal cadmium detection was conducted between commercial FAAS system and the TSP nebulizer interfaced HPLC-FAAS system. Figure 29 shows a tremendous sensitivity increase in cadmium detection with the 0.005" ID capillary TSP nebulizer interfaced HPLC-FAAS system over that of the commercial system using pneumatic nebulizer. Calculation showed an approximate 100 fold increase in cadmium detection sensitivity.
FIGURE 29. THE COMPARISON OF DETECTION SENSITIVITY BETWEEN TSP AND PNEUMATIC (PN) NEBULIZERS. (A) CADMIUM SIGNALS FROM USING TSP (B) CADMIUM SIGNALS FROM USING PN.
[6] Reproducibility of the System

Figure 3.0 shows the typical absorption signals of cadmium in the concentration range of 0.10 to 0.50 ppm. The absorption signals were measured at 228.8-nm resonance wavelength of cadmium. The capillary ID of the TSP used was 0.005" and the energy input to the TSP nebulizer was 140-W. The solvent flow rate used was 2.25-ml/min. The test was repeated and the signals of 20 successive injections were averaged.

The absorption signals of 0.1-ppm to 0.5-ppm cadmium concentration range showed a linear calibration curve. The calculation showed a standard deviation of $1.0 \times 10^{-3}$ absorbance at 0.20-ppm cadmium and $\pm 0.9\%$ relative standard deviation.

5. PRELIMINARY STUDIES ON CADMIUM SPECIATION IN BODY FLUIDS

A. Introduction to Cadmium Toxicity

[1] Non-occupational Exposure to Cadmium

Cadmium is widely dispersed in the environment as either the free metal or as metal compounds. The non-occupational human exposure to the metal mainly stems from three sources: ambient air (0.01-0.05-ug/m), drinking water (10-ug/L), and diet (50-ug/day), which give a total intake of the metal upto 100-200-ug daily (66). Smokers may absorb
FIGURE 30. THE ABSORPTION SIGNALS OF CADMIUM CHLORIDE. NOTE THAT THE REPRODUCIBILITY AND LINEARITY OF SIGNALS OF FIVE DIFFERENT CONCENTRATIONS.
an extra of 0.1-ug cadmium into lung tissue by smoking one cigarette (67).

[2] Biological Fate of Cadmium

The major routes of cadmium absorption into the body are dietary intake and respiratory inhalation. In animals and humans, following either acute or chronic exposure to the metal, the kidneys, liver, spleen, pancreas and testes will contain the highest cadmium concentrations. The metal will then redistribute and accumulate in the liver and kidneys, and is retained there (2).

Based on the studies of total concentration in human excretions (urine: 20-ppb and feces: variable), the half-life of the metal in the body was believed to be 9-30 years (8, 11). However, previous studies in our group on the metal excretion in human perspiration revealed a large amount of cadmium (90-ppb). This is much higher than that found in urine (12, 68). The total daily volume of perspiration loss is on average 2.5-3.0-L. From this it can be calculated that the half-life of the metal is less than 1.5 years, indicating that the chronic toxicity of the metal is considerably less than previously believed.


Cadmium is apparently a non-essential metal to humans and animals alike, but is an accumulative toxin (8). Its
toxicity has both chronic and acute biological effects (10). The former are associated with pulmonary and cardiovascular diseases, proximal renal tubular malfunctions, and hypertensions (2, 8, 10, 11); whilst the latter may involve nausea, vomiting and abdominal pain. The carcinogenicity of cadmium in mice and humans has also been reported (12, 69). High concentrations of either the free metal or metal compounds can cause high blood pressure, heart disease and in extreme cases lead to painful death.

[4] Reasons of the Studies

Cadmium is listed by EPA as a toxic metal which can cause severe toxic effects on patients who are heavily exposed to it. Clinically, the attention was paid to the total concentration of the metal in urine. No previous work had been done on cadmium excreted by perspiration. Nor had any studies on cadmium speciation been performed in either body fluids. In order to reveal the real causes of the toxicity of the metal, speciation studies are necessary.

On the other hand, the extremely low total concentrations of the metal in body fluids presents an analytical challenge to the speciation studies. For example, an average total cadmium concentration of 20-ppb in urine may be distributed in a dozen cadmium compounds. Each may have less than 2-ppb of cadmium. The sensitivity for cadmium using the commercial FAAS system is 26-ppb. This is too poor
for the speciation. However, using a TSP nebulizer interfaced HPLC-FAAS system with enhanced nebulization and atomization efficiencies, the speciation of cadmium compounds in body fluids might be performed.

B. Preliminary Speciation Studies of Cadmium Compounds in Human Body Fluids

[1] Experimental Procedures

The perspiration samples were usually collected in the sauna from individuals after their exercises. Chemical pretreatment of the sample was avoided in order to eliminate possible contamination from reagents used in wet ashing or loss during decomposition by heating involved using dry ashing. The sample was always analyzed immediately after arrival at the lab to minimize any possible decomposition reactions.

For HPLC separation, the mobile phase selected was pure, distilled, deionized and degassed water for the biological samples which were analyzed through reverse-phase chromatography. The column used was C-8 bonded-phase column. The flow rate was 1.00-ml/min.

As soon as the sample was delivered, the energy input to the nebulizer was raised to 110-W. Then, a 0.10-ml of filtered (0.45-um filter) fresh sample was injected into the column, and the chromatograms of absorption signals of the separated cadmium compounds were recorded.
Results and Discussions

(a) Organic Cadmium Compounds Detected

Cadmium compounds in perspiration were successfully separated using a C-8 bonded reverse-phase analytical chromatographic column. Figure 31 shows, during a 20-min scan, the separation of a dozen different cadmium compounds in human perspiration, which displayed very strong peaks in the chromatogram.

The parallel test on cadmium speciation in human urine was also conducted under the same analytical conditions. The purpose of this was to compare the cadmium compounds excreted from these two different body fluids. Figure 32 shows, during a 15-min scan, a group of thirteen cadmium peaks were detected in urine with less intensities than in those detected in perspiration.

Comparing these two chromatograms however, the retention times of the peaks in the two chromatograms showed that the cadmium compounds excreted in the two body fluids were quite different from each other.

The implications are that perspiration and urine both eliminate cadmium compounds, but that the compounds responsible are different. Hence, urine may be effective in removing some species but not others. Conversely, perspiration is effective in removing a different set of species. Thus, two modes of excretion are probably
FIGURE 31. THE CADMIUM COMPOUNDS EXCRETED IN SWEAT. NOTE A DOZEN PEAKS OF CADMIUM COMPOUNDS WERE SEPARATED USING THE HPLC-TSP-FAAS.
FIGURE 32. THE CADMIUM COMPOUNDS EXCRETED IN URINE. NOTE THAT MORE THAN A DOZEN PEAKS, WITH LESS INTENSITY, OF CADMIUM COMPOUNDS WERE SEPARATED USING HPLC-TSP-FAAS.
complementary, and suggest that profuse sweating may be very beneficial in removing some forms of cadmium from the system. This observation may pertain to other toxic metals.

(b) Signal vs. Time—Sample Stability

Figure 3 illustrates the successive tests for cadmium species in the same perspiration sample. An expected degradation reaction of the metal compounds with time inside the sample was observed. The sizes of the peaks showed in the fresh sample analysis, some peaks decreased with time, while some increased with time; and also some new peaks appeared at longer retention time, indicating that possible decomposition and recomposition reactions were taking place in the sample with time.

These peaks were not identified. Such information would be interesting but the inavailability of HPLC-MS equipment prevented such a study.

C. Conclusions

[1] The detection sensitivity of the TSP nebulizer interfaced HPLC-FAAS system was enhanced by about 100 fold compared with that of the commercial system.

[2] The system can be used for speciation studies with low metal concentrations, such as cadmium in human body fluids. The sensitivity increase achieved with aqueous solvent also reveals its potential for handling non-aqueous
FIGURE 33. THE INSTABILITY OF THE CADMIUM COMPOUNDS IN HUMAN PERSPIRATION SAMPLE. NOTE THE INTENSITY OF PEAKS CHANGED WITHIN TWO HOURS AFTER SAMPLE COLLECTION.
samples with environmental, pharmaceutical, industrial and agricultural interest.

[3] The system simplified the complicated speciation procedures in conventional methods. The method is faster, direct and accurate with high separation resolution, which was preserved in direct interfacing HPLC to FAAS.

[4] The cadmium speciation studies confirmed that the concentration of cadmium was higher in perspiration than in urine. The combined results confirmed that the half-life of cadmium in human body was less than 1.5 years.

[5] The speciation study of cadmium also confirmed that the cadmium compounds were different in perspiration and urine. It also confirmed that the cadmium compounds in the biological sample were not stable. Decomposition and recomposition reactions take place and the samples must be analyzed immediately.

[6] The identification of the cadmium compounds separated by the system cannot be further studied because the TSP nebulizer interfaced HPLC-MS system was not sensitive enough to handle such a low metal concentration in body fluids, and other methods were not available.

Figure 34 is the photographs of the complete HPLC-TSP-Flame AAS system, and the thermospray nebulizer built in house for this study.
FIGURE 34. THE PHOTOGRAPH OF THE HPLC-TSP-FAAS SYSTEM.
CHAPTER FOUR

OPTIMIZATION OF THERMOSPRAy NEBULIZER DESIGN:
STUDY OF THERMOSPRAy DESOLVATION MECHANISM
1. INTRODUCTION

The thermospray (TSP) phenomenon was first observed in late 16th century (70). Blakley and Vestal first explored its application as a part of an analytical tool in 1977 (71, 72). Since then, TSP has become a standard interfacing device for liquid chromatography/mass spectrometry (LC/MS) (71, 51).

Since 1984, the number of TSP users in LC/MS coupling has rapidly increased (72, 73). Recently, it has been used as a sample introduction device in ICP (58, 74), and as an interface of LC with ICP and AAS (75, 54, 76, 77). The concentrations of metal analyzed in those cases were quite high (ug/ml), and the TSP merely functioned as a thermal nebulizer to convert the liquid samples into vapors.

However, sensitivity enhancements in metal analyses were observed in our group and by other researchers (75, 77, 74), when the TSP nebulizer was used to replace the conventional nebulizers in flame AAS. Furthermore, interferences from solvent effects could be eliminated. However, flow rate remained an important variable (65, 78). An optimized TSP nebulizer was applied to trace metal speciation analysis, and moderate successes were achieved (75, 79).
The reason why the application of TSP nebulizer improved analytical sensitivity was not well understood, nor the real mechanism of the thermospray process. This chapter will discuss the research work done in this respect, and will try to interpret the possible desolvation mechanism inside the capillary of the thermospray based upon our experimental observations.

2. REVIEW OF PROPOSED DESOLVATION MECHANISM OF TSP

A. Historical Reviews

The TSP interface could be considered as the simplified final version of direct liquid introduction (DLI) probe, which was originally developed by McLafferty in 1973 for LC/MS coupling (80). However, from this author's point of view, the embryonic form of the TSP was actually the modification of the simple capillary vaporizer (SCV), which was first explored by the Russian chemist Talroze in 1968 (81, 82).

[1] Direct Liquid Introduction Interface in LC/MS

Figure 35 shows the picture and diagram of a DLC device. The capillary tubings of different DLI devices were all moderately heated, either directly by the probe assembly or indirectly by an ion source block (80, 83).
A. PHOTOGRAPH OF THE END OF A DLI INTERFACE, SHOWING THE LIQUID JET ISSUING FROM A 2-μm DIAPHRAGM

B. Dual purpose DLI/thermospray LC/MS probe interface: (A) central microbore (0.004 in. i.d.) through-put tube; (B) two 50-W 120-V ac cartridge heaters; (C) heated copper vaporizer; (D) thermocouple; (E) removable end cap; (F) stainless steel pinhole diaphragm.

FIGURE 35. A DIAGRAM OF THE DLI DEVICE USED IN LC-MS AND A PHOTOGRAPH OF A DLI SPRAY PATTERN
The evaporated liquid vapor inside the capillary passed through a 2-5 um pinhole or restriction at the tip of the probe, and entered a vacuum chamber. The large pressure drop between the vapor and the vacuum caused the formation of fine droplets (84). An extra desolvation chamber selectively removed most of solvent from solute by the molecular weight, volatility, and vapor pressure differences between them.

[2] Simple Capillary Vaporization Interface in LC/MS

The SCV operated at temperature lower than 200°C. The linear velocity of the effluent inside the capillary was low because of the low flow rate of microbore LC (83). Figure 36 illustrates the vaporization processes occurring inside the capillary. (a) At low temperature, the solvent flowed out of the capillary as liquid. (b) As the temperature increased above a certain point, some solvent evaporated at the tip of the capillary. (c) At higher temperatures the evaporation rate increases and the solvent front retracted inside the capillary. Complete evaporation of eluate occurred inside the capillary and the vapor was ejected into the vacuum.


The TSP also utilized a heated capillary, but operated in different regimes from SCV and DLI. With the TSP, the
LIQUID FLOW RATE < EVAPORATION RATE

LIQUID FLOW RATE = EVAPORATION RATE

LIQUID FLOW RATE > EVAPORATION RATE

FIGURE 36. EVAPORATION OF A LIQUID INTO VACUUM, AT THE END OF A SIMPLE CAPILLARY VAPORIZER. THREE POSSIBLE PROCESSES MAY BE INVOLVED WITH THE RATIO OF THE LIQUID FLOW RATE TO THE EVAPORATION RATE.
linear velocity of the effluent inside the capillary was high. The 0.005" ID capillary was directly heated to a much higher temperature (>700°C). The length of the capillary heated varied between 1" to 10".

Complete evaporation inside the capillary could be avoided with higher effluent velocity and controllable energy input. The liquid front retracted and disintegrated inside the capillary with appropriate heating energy input. A fine mist of droplets or aerosol/vapor mixture was ejected with very high velocity (>200-m/sec) at the capillary tip.

The TSP interface used in LC/MS was modified to achieve nebulization using (a) a high power laser (85), (b) an oxy-hydrogen torch (86), (c) several electrical heaters (87), and, finally, (d) by a capillary directly heated electrically (88). Vestal claimed that the latter gave the best analytical results (51).

B. Proposed Desolvation Mechanism Review

Several researchers claimed that desolvation processes occur inside the TSP capillary (89, 74, 65, 89, 90).

Koropchak et al. reported that, at high solvent flow rate and controlled heating, the aerosols appeared dense with narrower distribution of particle sizes. The heated droplets rapidly began to desolivate and concentrate the less volatile analytes, providing higher analyte transport rate and yield enhanced detection (74).
Mayar et al. also observed the desolation phenomenon (65). Droplet size related aerosol transporting efficiency, the desolation rate, and the vaporization rate of the dried analyte particles after desolation were studied. The results showed that the TSP nebulizer aerosol generated a smaller mean particle diameter compared to that of the pneumatic nebulizer. The numbers of small particles increased as functions of the elevations of TSP temperature and sample solution introduction rate.

Vestal proposed a "soft-ionization" mechanism inside the TSP capillary (89). The first step of the mechanism involved a nearly complete vaporization of solvent at a given flow rate and heating energy input, with which a superheated mist containing a supersonic vapor jet was ejected. The jet consisted of a solute concentrated central cone and a solvent concentrated edge cone, which resulted from the desolation process. This is described in Section 6E [2] of Chapter 2, and is illustrated in Figure 10.

Vestal's desolation assumption was supported by the Leidenfrost phenomenon noticed in 1756 (91). Figure 37 shows that, when a drop of liquid is dropped on a hot plate, it evaporated rapidly by efficient heating of the plate. Above a certain temperature, the Leidenfrost temperature, the liquid could no longer wet the plate, but danced and bounced and isolated from the hot plate by a thin layer of vapor.
LEIDENFROST TEMPERATURE
(1756)

below above
HOT PLATE
A B

FIGURE 37. BOILING AND EVAPORATION OF A LIQUID ON A HOT PLATE.
(A) NUCLEATE BOILING BELOW THE LEIDENFROST TEMPERATURE.
(B) FILM BOILING ABOVE THE LEIDENFROST TEMPERATURE.
The Leidenfrost temperature of water is 185-325°C, depending on the material and surface structure of the plate.

3. REASONS OF THE STUDY

According to the desolvation mechanism proposed by Vestal, the solute should be concentrated in the spray center, while the solvent vapor layer around the jet cone could be eliminated by skimming. However, this did not appear to be the case in the TSP interfaced HPLC-GFAAS. With the skimming and solvent removal system, the graphite furnace still burned rapidly because of considerable amounts of water solvent in the spray center. The presumed, dried, solute concentrated, central aerosol cone was not detected.

A simple experiment was then conducted. Two test tubes were coaxially arranged one in another. The ID's of the two tubes were chosen to give the same cross-sectional entrance areas for equal vapor collection in unit time. After the TSP reached its optimum operating condition, 1.00-ml of 20-ppm cadmium chloride was flow-injected into the TSP. The spray from the TSP was collected and measured with TSP interfaced HPLC-Flame AAS. The results showed the same concentrations of cadmium in the two tubes, indicating that the solvent and the solute were not separated upon emerging.
This phenomenon was observed by several different researchers (92, 93, 94). The sensitivity losses due to the use of splitter or skimmer to divert 99% of LC column effluent were reported. The compensation for this was to remove the solvent remaining in the 1% effluent using an extra desolvation chamber after the TSP interface (94).

Without an extra desolvation chamber, however, analyte enrichment seemed to have actually happened inside the TSP capillary before entering the atomizer in Flame AAS. A 100 fold sensitivity increase for speciation studies of nonvolatile cadmium compounds was achieved in our group. This seems to indicate that a different desolvation process is taking place inside the TSP nebulizer and that the analyte enrichment improved the detection sensitivity.

Through the studies of cadmium speciation, for the detection of trace concentration of cadmium in human body fluids, we realized the necessity of optimizing the TSP nebulizer in order to achieve the highest sensitivity of our TSP interfaced HPLC-flame AAS system. If the potential TSP desolvation mechanism could be revealed, it would suggest the correct direction of improving the performance of the TSP nebulizer, and in turn, it would further improve the detection sensitivity of our system for even tougher analytical tasks. These became the main reasons of this work. Therefore, a series of systematic experiments on researching the direction of optimizing the performance of
the TSP nebulizer were conducted, and numerous tests were performed on several variables, hoping to find the potential desolvation mechanism.

4. EXPERIMENTAL

A. Equipment Used


[2] Radiation source: Perkin-Elmer cadmium hollow cathode lamp was used at 228.8-nm, 5-mA and 12-V.


[5] Sample Injector: Rheodyne 7125 injector with 100-ul sample loop except specified (20-ul or 1-ml).


[9] Nebulizer: 150-W electric heating cartridge by Watloo (Firerod), with 1/16" OD, 0.005", 0.007", 0.01" 0.02", and 0.03" ID stainless steel capillary tubings.


[12] TSP Power Supply: Constant voltage transformer with 0-100% variac providing 0.14-V interval variation.

B. Reagents and Chemicals

[1] Stock solution: 500, 400, 300, 200, 100, and 50 ppb CdCl₂ freshly prepared from 1,000-ppm stock solution which was prepared from 1.00-g of metal cadmium dissolved in HCl acid and diluted to 1.00-liter.


[3] Cleaning solutions: 10% nitric acid for spray chamber and burner; 0.5-mM citric acid for capillary.


C. Experimental Conditions

The same TSP nebulizer interfaced HPLC-flame AAS system used in cadmium speciation studies was used for these studies. It is described in Chapter 3, Sections 3C and 3D with the schematic diagrams in Figure 20 and Figure 21.
The operating conditions of the TSP nebulizer interfaced HPLC-FAAS system were optimized using the same procedures described in Chapter 3, Section 3E. Additional preparation procedures for the experiment are discussed below.

[1] Solvent Purification Process

The lab deionized, distilled water from the chemistry building contained trace impurities, probably arising from pipes and deionizing columns. These inorganic or organic materials were potential causes of capillary orifice clogging, and higher metal background levels. The water was therefore re-distilled to further remove any impurities. It was then degassed, and its pH value was adjusted routinely to the optimum value for the experiment to be performed.

[2] Solvent Delivery Pump Pulse-Dampening

The ultra microbore (0.004/0.006" ID) capillary of the TSP nebulizer operated under high temperature and pressure. This caused very high pressure fluctuations, which influenced the solvent delivery system. This in turn sometimes resulted in a very noisy background oscillation that had to be eliminated.

A free flow pulse dampener was connected to the outlet of the solvent delivery pump. The pulsing was reduced slightly. Then, a 4.6-mm HPLC column was connected after
the dampener. The pulsing was effectively eliminated and a linear background was obtained.

[3] Preparation of Standards

Because of their instability, standard solutions with metal concentrations lower than 1.0-ppm were freshly prepared daily from 1000-ppm. A period of homogenizing the low solute standards was applied. Their pH values were adjusted routinely because of pH changes in the 'pure' water solvent. The different pipet uncertainties were also taken into consideration to ensure precise data acquisition.

5. MODIFICATIONS OF TSP NEBULIZER

A. The Replaceable Capillary Configuration

In the cadmium speciation, the capillary of the TSP nebulizer was completely silver soldered into the stainless steel hollow rod of the cartridge. However, if the capillary was clogged by residues, the nebulizer no longer worked. Regenerating it by replacing the capillary was very difficult. Further, a new nebulizer would probably give somewhat different analytical sensitivity compared to the old one.

Figure 38 shows a new, reusable TSP heating cartridge that was constructed in house. This was much simpler to
FIGURE 38. DIAGRAM OF THE FINAL DESIGN OF A REUSABLE TSP NEBULIZER. NOTE THAT A HOLLOW COPPER ROD WITH A 1/16" ID THROUGH-HOLE WAS FIT TIGHTLY INTO THE HOLLOW ROD OF THE HEATING CARTRIDGE. THE 1/16" OD CAPILLARY WAS THEN INSERTED THROUGH THE COPPER ROD TO FORM A TSP NEBULIZER. THE CAPILLARY CAN BE CHANGED VERY EASILY WHENEVER IT IS CLOGGED.
operate than the version previously reported (95). The 1/4" ID hollow rod of the "firerod" was filled up with a 1/4" OD brass rod in which a 1/16" ID through-hole was machine drilled. Then, the 1/16" OD capillary was inserted into the through-hole of the brass rod. The ID's and OD's of the three units were so precisely machined that optimum thermal contact was assured.

This new TSP nebulizer was then put to use. Whenever the capillary tubing was clogged, it was pulled out of the brass rod, and replaced with a new one in minutes. This renewable nebulizer only changed the capillary, thus, preserving the operational conditions, thereby maintaining reproducible results.

In addition, this design enabled us to conveniently change capillaries with different ID's at will. This was a freedom necessary for the study.

B. Modified Capillary Tips of Thermospray

The 2-5 um pinhole diaphragms used in the DLI device (80, 83, 84), and the 25-50 um pinhole nickel diaphragm to the TSP capillary tip (77) were reported to improve nebulization. An indirect proportional sensitivity increase with the pinhole diameter was observed.

In our metal analysis, a three fold sensitivity enhancement was noticed when the diameter of the capillary orifice was decreased three fold by residue deposition. This
accidental discovery led to the strategy of sensitivity improvement by TSP capillary tip modification.

[1] Mechanically Modified Capillary Tip

When the capillary tip was cut using a tubing cutter, the ID around the cut was gradually reduced until a pinhole formed after the tubing was cut apart. Based on previous observation, this tiny pinhole should increase sensitivity.

However, the stainless steel tubing became very fragile as the cut depth increased. This caused a very rough surface, and an irregular orifice at the tip of the capillary. This kind of tip on the TSP capillary caused an irregular spray. Besides, the pinhole diameter of each capillary could not be made reproducibly. Therefore, the comparison of analytical data using capillaries with different ID's was meaningless.

Therefore, other approaches for making narrower tip orifice with smooth tip surface and perfectly round orifice edge were explored.

[2] Electrochemically Modified Tip

Figure 39 shows that a new approach was explored for making a pinhole with smooth interior and exterior surfaces, an even orifice diameter, and coaxially straight guiding tunnel.
Figure 39. The processes of electrochemically making a narrowed capillary tip orifice for TSP.

(A) Inserted a 80-um copper wire into the capillary.
(B) Cut the capillary with the wire in
(C) Electrolyzed the copper to open the narrowed capillary tip.
(a) First, a 80-um OD copper wire was inserted through the 250-um ID capillary. (b) Then, with a trapezoid-edged cutting wheel, the capillary was gradually squeezed into a conical cut, inside which a coaxially straight guiding tunnel was made. At this point, the copper wire was firmly stuck in the capillary, and a conical-shaped gap was made. (c) Finally, the capillary was cut apart at the narrower side of the gap with sharp wedge-edged cutting wheel, and filed smooth.

A mini electrolyser was assembled using 15% sulphuric acid solution (v/v) in a 50-ml beaker. The copper wire inside the capillary was then electrolyzed to CuSO₄ at the cathode. The process was quick since only a little copper trapped at the tubing tip when the wire was pulled out. A tiny pinhole was found at the tip of the capillary with a diameter slightly smaller than that of the wire.

However, the cold spray from this tip deviated from the linear axis of the capillary, and shot only a short distance indicating that the pinhole diameter was not small enough. A much smaller diameter tip orifice was needed.

A thinner copper wire (50-um OD) was tried using the same procedures. However, the copper and stainless steel became a bimetal at the cut. The copper at the cut did not dissolve even in the electrolyser. The capillary was clogged with the copper wire and the method was abandoned.

Finally, the tip was modified manually. The capillary was first cut by the same trapezoid-shaped cutting wheel, but without copper wire being inserted. This was done very carefully and slowly. An even force was applied to avoid any bending or twisting to prevent a sudden breaking of the tubing producing an irregular cut. A co-axially oriented, straight guiding tunnel with a smooth internal surface was expected to form inside the capillary tip.

Figure 40 shows how the conical tip of the capillary was manually made, and shaped into a dome-shaped end by slightly tapping with a small wrench while the capillary was slowly rotated by hand. The shiny, chrome coated wrench could make the tip surface smooth. The orifice ID was controlled under a 50 X magnifier while tapping. Under the microscope, the picture in Figure 40 shows, a 75-um ID orifice was observed in the center of a very smooth, dome-shaped tip.

Finally, the same dome-shaped tips with 75-um ID orifice were made for capillaries of 0.005", 0.007", 0.01", 0.02" and 0.03" ID. Examination under a 3-dimensional microscope showed that all these orifices had an even edge and smooth surface. Spray testings under hot and cold conditions demonstrated excellent performances in reproducibility and similarity.
FIGURE 40. A DOME-SHAPED CAPILLARY TIP WITH A 75-UM ORIFICE WAS MADE BY CUTTING THE CAPILLARY USING A WHEEL-CUTTER, AND TAPPING INTO A DOME SHAPE TIP. THEN, USING A SHARP NEEDLE TO SMOOTH THE ORIFICE INTERNAL TUNNEL.
6. EVALUATION OF THE MODIFIED TSP NEBULIZER

The maximum analytical signal can only be obtained when the system is run under optimum conditions. A number of variables affect the running conditions of the system. These must each be optimized to get maximum analytical sensitivity and reproducibility.

The variables examined include the TSP nebulizer performance at room temperature, the effects of electrical energy input to TSP, capillary tip orifice, solvent flow rate, capillary ID size, and capillary heating length. These were assessed by their effects on the AAS signals. Results are discussed below.

A. Behaviors of Capillary with Narrowed Tip Orifice at Room Temperature

At a given volume flow rate, according to hydrodynamics, the linear velocity of a liquid flowing through a pipe is inversely proportional to the pipe diameter (96). If the tip of the TSP capillary is narrowed, the solvent should undergo a linear acceleration, and form a very fast-flowing jet. At room temperature, a straight, thin liquid thread should shoot out to a considerable distance. At high temperature, a coaxially conical shape thermospray jet can be expected. This type of spray is highly desirable for efficient nebulization.
[1] Experimental Procedures

The TSP capillary with a narrow orifice was directly connected to the HPLC solvent delivery pump. The effects of solvent flow rate, orifice diameter and tip microstructure on the pattern, position, and height of the thin thread jet were studied to optimize the microstructure of tip orifice made on capillaries with different ID.

The solvent (water) flow rate was increased stepwise from 0.50- to 3.00-ml/min at 0.25-ml/min intervals. The vertical height of the thin thread jet against each flow rate was measured along with the pressure on the HPLC pump. The microstructure of the orifice was examined under a microscope whenever jet angle deviation, a split spray and droplet formations were observed.

Finally, five capillaries with identical orifices but different ID's were tested.

[2] Results and Discussion

(a) Effect of Capillary ID on Jet formation

The commercial capillaries (by Alltech) have smooth, electrically cut tips. However, without a narrowed orifice, they would not generate any kind of spray at any flow rates with ID larger than 0.005". The 0.005" ID capillary did not spray until the flow rate was higher than 2.25-ml/min. This indicated that the spray could only be formed with a very
narrow capillary, and was the reason why 0.005" ID capillary was chosen for the commercial TSP nebulizer.

(b) Generation of Liquid Jet from the Capillary

The formation of straight, thin thread of liquid jet from unheated but pressurized capillary with perfectly narrowed orifice (75-um) is described as follows.

i] At a solvent flow rate below 0.45-ml/min, a droplet emerged from the capillary tip and then dripped off the end. The pressure on the HPLC pump was below 250-psi.

ii] As the flow rate exceeded 0.45-ml/min, a 3-4" thin liquid thread jet was shot out from the tip at 300-psi.

iii] At flow rate of 0.50-ml/min, a 6" straight thread jet was formed, and shot up from the tip coaxially.

iv] From 0.50-ml/min and up, every 0.25-ml/min flow rate increase would cause 250-300 psi pump pressure rise; and 5-7" of jet thread height increase upto 36-45" at 2.00-ml/min.

v] From 2.25-ml/min and up, every 0.25-ml/min flow rate increase would only bring a 2-3" increase to the jet height.

vi] At 3.00-ml/min flow rate and 2250-2500-psi pump pressure, five capillaries with identical 75-um orifices but different IDs' (0.005" to 0.03") all produced a thin thread jet up to 52" maximum height. This indicated that, at the
same volume flow rate, the capillaries with different IDs' and identical orifices behaved identically.

The relationships between the flow rate, pump pressure, and the height of thin thread jet are listed in Table 3.

Table 3. Relation of Jet Height, Pressure and Flow Rate

<table>
<thead>
<tr>
<th>Flow Rate (ml/min)</th>
<th>Pump Pressure (psi)</th>
<th>Jet Height (inch)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.40</td>
<td>200</td>
<td>0</td>
</tr>
<tr>
<td>0.45</td>
<td>250</td>
<td>4</td>
</tr>
<tr>
<td>0.50</td>
<td>350</td>
<td>6</td>
</tr>
<tr>
<td>0.75</td>
<td>550</td>
<td>15</td>
</tr>
<tr>
<td>1.00</td>
<td>750</td>
<td>23</td>
</tr>
<tr>
<td>1.25</td>
<td>1050</td>
<td>30</td>
</tr>
<tr>
<td>1.50</td>
<td>1250</td>
<td>35</td>
</tr>
<tr>
<td>1.75</td>
<td>1500</td>
<td>40</td>
</tr>
<tr>
<td>2.00</td>
<td>1700</td>
<td>42</td>
</tr>
<tr>
<td>2.25</td>
<td>1900</td>
<td>44</td>
</tr>
<tr>
<td>2.50</td>
<td>2100</td>
<td>46</td>
</tr>
<tr>
<td>2.75</td>
<td>2300</td>
<td>48</td>
</tr>
<tr>
<td>3.00</td>
<td>2500</td>
<td>52</td>
</tr>
</tbody>
</table>

(c) Uneven Spray formation

If the internal surface of the tip was rough, and the orifice opening was irregular, an uneven spray formed at a flow rate above 0.50-ml/min. Usually, it was not coaxially
oriented and split into several branches. The maximum height reached was only 6" at 3.00-ml/min. It subtended a solid angle which was always wider than 90 degree. It was always accompanied with a small droplet dripping off the tip. This caused sample loss inside the burner stem. Whenever this kind of spray was formed, it indicated that the microstructure of the orifice was irregular, and the tip had to be redone.

[3] Summary

The behavior of capillaries with a narrowed orifice at room temperature showed that a straight, coaxial, thin, thread jet could be generated if the microstructure of the tip was smooth and the orifice diameter was narrow enough. The capillaries with different ID sizes but the same orifice diameter would produce identical jet patterns. This ensured the comparison study on TSP nebulizer performances responding to different parameters under heated operating conditions.

B. Effect of Energy Input to TSP on AA Signal

In previous studies, the relationships between the energy input to the TSP nebulizer and the flame AAS signal were extensively studied with different metals and solvents (12). However, tests were conducted with high metal concentrations (>2.0-ppm), low energy inputs (<150-W), low
solvent flow rates (<2.50-ml/min), and the same capillary ID size (0.005") without tip orifice modification (i. e. narrowed orifice).

This present study focuses on the relationships between the flame AAS signal and higher energy input to the TSP nebulizer, with much lower metal concentration, wider flow rate range, on different capillaries with different ID sizes but the same tip orifice diameter.

For a given solvent at a given flow rate, the temperature of the capillary was directly proportional to the heating energy put into the TSP nebulizer. The temperature controlled the degree of vaporization, desolvation and other properties of the aerosol. These factors influenced the signal responses in LC/MS (51), as well as in TSP interfaced ICP (58, 74).

[1] Experimental Procedures

The nebulizer was directly connected to a solvent delivery pump outlet without the HPLC column. Flow injection of the sample into the HPLC system was used throughout. The nebulizer was always equilibrated by preheating at 110-w for 45-min with water being pumped through.

The sample concentration range tested was from 50.0- to 500-ppb standard cadmium chloride. In most cases, 200-ppb was chosen guided by the Ringbom Plot theory (56).
The energy input range studied was from 110- to 180-W, with 4.0-W increase interval. The solvent flow rate range was from 1.00- to 3.00-ml/min with 0.25-ml/min interval increase.

After the nebulizer reached its optimum condition. A 0.10-ml of 200-ppb cadmium chloride was injected. The absorption signal was recorded at 228.8-nm Cd resonance wavelength. The absorbances of 20 injections were averaged as one datum point for a given flow rate at 110-W. The same procedure was repeated for each flow rate increment up to 3.00-ml/min for this energy input; and then repeated for each increment of energy input up to 180-w.

Capillaries with different ID sizes, narrowed tip orifices, and regular cut tips were all tested in the same way. The data collected were averaged, plotted and analyzed. The results are discussed in the following sections.

[2] Results and Discussion

(a) Energy Input and Temperature Distribution

The temperature distribution of the 150-w heating "Firerod" against electrical energy input was indirectly measured, without capillary insertion, through the hollow rod in the cartridge with thermal couple. The data are listed in Table 4:
Table 4. Temperature Distribution inside 150-W TSP

<table>
<thead>
<tr>
<th>Energy Input (W)</th>
<th>Bottom</th>
<th>1.0-cm</th>
<th>Center</th>
<th>3.0-cm</th>
<th>Tip</th>
</tr>
</thead>
<tbody>
<tr>
<td>52.5</td>
<td>345</td>
<td>405</td>
<td>465*</td>
<td>410</td>
<td>355</td>
</tr>
<tr>
<td>63.1</td>
<td>365</td>
<td>425</td>
<td>505*</td>
<td>455</td>
<td>390</td>
</tr>
<tr>
<td>77.3</td>
<td>415</td>
<td>475</td>
<td>575*</td>
<td>520</td>
<td>425</td>
</tr>
<tr>
<td>91.0</td>
<td>435</td>
<td>510</td>
<td>620*</td>
<td>580</td>
<td>480</td>
</tr>
<tr>
<td>117.6</td>
<td>440</td>
<td>545</td>
<td>665*</td>
<td>620</td>
<td>505</td>
</tr>
<tr>
<td>130.0</td>
<td>520</td>
<td>625</td>
<td>715*</td>
<td>640</td>
<td>555</td>
</tr>
</tbody>
</table>

Placing the thermal couple on the external surface of an operating TSP nebulizer, the temperature distribution of 100-W cartridge, with 0.005" ID capillary, under 1.00-ml/min flow rate was also indirectly measured. The data obtained are listed in Table 5 below:

Table 5. Temperature Distribution outside 100-W TSP

<table>
<thead>
<tr>
<th>Energy Input (W)</th>
<th>Bottom</th>
<th>Center</th>
<th>Tip Base</th>
<th>Tip</th>
</tr>
</thead>
<tbody>
<tr>
<td>68.5</td>
<td>135</td>
<td>275*</td>
<td>255</td>
<td>135</td>
</tr>
<tr>
<td>78.8</td>
<td>140</td>
<td>305*</td>
<td>270</td>
<td>155</td>
</tr>
<tr>
<td>89.6</td>
<td>150</td>
<td>315*</td>
<td>280</td>
<td>160</td>
</tr>
<tr>
<td>103.5</td>
<td>155</td>
<td>375*</td>
<td>300</td>
<td>165</td>
</tr>
<tr>
<td>115.9</td>
<td>165</td>
<td>385*</td>
<td>310</td>
<td>200</td>
</tr>
<tr>
<td>133.0</td>
<td>175</td>
<td>440*</td>
<td>325</td>
<td>200</td>
</tr>
</tbody>
</table>
The data show that the temperature distribution of both cartridges were similar. The temperatures were higher in the middle portion of the cartridges, and lower on both ends with all energy input values. The temperature of the 100-W heating cartridge was usually 300°C lower than that of the 150-W at any energy input.

Both cartridges displayed the temperature distribution patterns that were similar to that which Vestal observed (51). This is shown in Figure 10, except the temperature at the capillary tip was reversed.

(b) Effect of Energy Input to TSP on AA Signals

Eight capillaries with different ID sizes, with orifices, and without narrowed orifices were systematically examined.

i] Capillaries without Narrowed Orifices—

Signal Dependence on Energy Input

The nebulizer using 0.005" ID capillary showed, from 120-to 170-W, that the signal increased directly proportional to the energy input. After reaching the maximum, the signals dropped to zero rapidly. This phenomenon was observed at all flow rates.

The 0.007" ID capillary showed the same trends when the solvent flow rates were below 1.75-ml/min. However, there
was no signal drop off at flow rates greater than 2.00-ml/min. There was a direct proportional increase of signals to the energy input at those higher flow rates.

The 0.01" ID capillary acted almost the same as that of the 0.007" ID; and the signal drop only occurred at 1.25-ml/min flow rate. But, the signal response using 0.01" ID capillary was worse than using 0.007" ID capillary.

The worst AA signals observed were those using 0.02" ID capillary without narrowed orifice. Compared with that of 0.005" ID capillary, the 0.02" ID capillary gave only 7.0% absorption to 0.20-ppm cadmium detection signal, at 1.75-ml/min flow rate and 140-W energy input; while the 0.005" ID capillary gave 19.0% of absorption. Not only were the signals the lowest, but the reproducibility was the poorest. The relative standard deviations of the signals were the greatest, which calculated about 15%. This compared with 1.40% for that of 0.005" ID capillary, it was ten fold worse. In addition, the noise levels were the highest that the flame was sometimes almost extinguished by the pulsing of the spray.

The behavior of capillaries without narrowed orifices at room temperature might explain the above observations. The capillary with large diameter and straight outlet did not produce a large pressure drop at the tip. The aerosol/liquid mixture was not shattered to fine droplets. The desolvation process was far from complete. These large
liquid droplets caused the slowest sample transportation rate, worst desolvation, highest sample loss, least atomization efficiency and lowest AA signals.

Figure 41 shows that, at 2.25-ml/min solvent flow rate, the absorbance of 0.20-ppm cadmium versus TSP nebulizer energy input compared with 0.005" ID and 0.02" ID capillaries without narrowed orifices. The remarkable difference in the nebulizer performance between these two capillaries are obvious.

ii] Capillaries with Narrowed Orifices---
Signal Independence on Energy Input

A very interesting phenomenon was noticed when the same experiment was conducted with different capillaries having the same 75-um orifices (=0.0025" ID).

Over a wide range of energy inputs, from 110- to 160-W, the signal dependence on energy input disappeared with capillaries having narrowed orifices. This occurred at flow rates up to 3.0-ml/min with few exceptions. The range of signals independent of energy input varied with capillary ID sizes.

The 0.007" ID capillary showed a signal independent of energy input range from 120-w to 155-w, with flow rate range from 1.00-ml/min to 2.50-ml/min. However, the signals at lower energy inputs were unusual. A signal decrease occurred with energy input increase at flow rates lower than 2.25-
FIGURE 41. ABSORPTION SIGNALS VERSUS TSP NEBULIZER ENERGY INPUT WITH CAPILLARIES BY ALLTECH
(A) 0.005" ID WITHOUT ORIFICE
(B) 0.020" ID WITHOUT ORIFICE
Cd CONC.: 0.20-PPM. FLOW RATE: 2.25-ML/MIN
ml/min, and reversed beyond 2.50-ml/min.

The 0.01" ID capillary behaved totally different. At flow rate below 1.75-ml/min, the signal independent region on energy input started below 110-W; and ended above 150-W. At 2.00-ml/min, the region stretched out towards low and high energy input limits, from 100-W to 180-W. Above 2.25-ml/min, the region started to decreased from both sides, and disappeared beyond 2.75-ml/min.

With the capillary of 0.02" ID, a similar trend was observed. Below 2.25-ml/min, the independent regions started below 110-W and ended at 165-W, and the signals started to drop. At 2.50-ml/min, the region started to shrink from both sides but the absorbance signals were 3% larger than that at 2.25-ml/min. Beyond 2.50-ml/min, the regions were narrower but still kept a portion for each flow rate increment at which the signals increased as well. The maximum signals were obtained at much lower energy input values than those that were required for 0.01" ID capillary.

Figure 42 shows that, at 2.50-ml/min solvent flow rate, the absorbance of 0.20-ppm cadmium versus TSP nebulizer energy input compared with 0.007", 0.01", and 0.02" ID capillaries with narrowed orifices. Obviously, the TSP nebulizer with 0.02" ID capillary and 75-um orifice gave the best performance.
FIGURE 42. ABSORPTION SIGNALS VERSUS TSP NEBULIZER ENERGY INPUT WITH THREE CAPILLARIES WITH THE SAME 75-UM ORIFICE BUT DIFFERENT ID'S
iii) Conclusions

The signal dependency of energy input to the TSP nebulizer existed for the capillaries without narrowed orifices, but was much less important for those with narrowed orifices. Within a certain range of energy input and solvent flow rates, an independent range of signal to the energy input existed in TSP nebulizers with capillaries having narrowed tip orifices.

The maximum signals appeared within the independent range for the capillary with the smooth orifice. This allowed choosing different energy inputs to the TSP nebulizer without critical restrictions and sensitivity loss.

For 0.02" ID capillary with 75-um orifice, the signal independence region started at lower energy input value with higher responses compared to that of 0.01" ID. This enabled the TSP nebulizer to be operated at lower temperatures for longer lifetime.

[3] Summary

The TSP nebulizer of 0.02" ID capillary with 75-um outlet orifice appeared to have the best signal responses with lowest energy input, and least relative standard deviation. The wide range of signal independence of energy input made it easy to operate with reliable analytical results.
C. Effect of Capillary Tip Orifice on AAS Signal

[1] Experimental Procedure

Two 0.02" ID capillaries, without and with narrowed orifice, were applied into the 150-W TSP cartridge to compare the AAS signals. 0.10-ml of 200-ppb CdCl₂ standards were flow-injected, and absorption signals with different flow rates and energy inputs were measured.

[2] Results and Discussion

The results indicated that a narrower outlet orifice of the TSP capillary increased the detection sensitivity in a relation that was indirectly proportional to the diameter of the orifice.

Figure 43 shows a about 10-fold AAS signal increase, at 2.25-ml/min solvent flow rate and 140-W TSP energy input, with the capillary equipped with a 75-um tip orifice (1/7 of 508-um, i.e. 0.02") gave a 31% of absorption signal with a 0.0009 absorbance standard deviation; while the 0.02" ID capillary without 75-um orifice, only gave a 2.9% absorption with a 0.002 absorbance standard deviation. In addition, the signal reproducibility of the 0.02" ID capillary with 75-um orifice was absolutely much better than that of the capillary without tip modification.

Figure 44 shows a similar result observed on the 0.01" capillary with and without a narrowed tip orifice. A triple
FIGURE 43. THE ABSORPTION SIGNAL COMPARISON BETWEEN TWO 0.02" ID CAPILLARIES WITH AND WITHOUT 75-UM ORIFICE. A 10-11 FOLD INCREASE IN CADMIUM DETECTION SENSITIVITY IS SEEN.
Figure 44. The absorption signal comparison between two 0.01" ID capillaries with and without 75-µm orifice. A three fold increase in cadmium detection sensitivity is seen.
A signal increase was obtained with reducing the diameter of the TSP capillary tip three times.

[3] Summary

The performance of TSP nebulizer can be improved by reducing the diameter of its tip. A linear, inverse relationship existed between the analytical sensitivity and the diameter size. It revealed the benefit of using a narrowed, aperture-based capillary TSP nebulizer.

D. Effect of Solvent Flow Rate on AAS Signal

As the solvent flow rate increased, the transportation rate of the sample into the flame rate was increased, which in turn increased the sensitivity. The nebulization efficiency was controlled by the energy input to the TSP nebulizer, not by the fuel gas flow rate, which was the case with the present day commercial pneumatic nebulization.

[1] Experimental Procedures

With each energy input increment interval (4.0-W from 110- to 180-W), the absorbances of 50-ppb and 200-ppb cadmium chloride were measured with every 0.25-ml/min solvent flow rate increase. Whenever the flow rate was changed, a five minutes equilibrium period was applied to avoid any flow surge in the system. The results were compared within capillaries with different IDs'.
[2] Results and Discussion

(a) Relationship between Signal and Flow Rate

Without a narrowed orifice on the tip of TSP capillary, in our previous study, a relatively linear relationship between the signal and flow rate was observed only up to 1.75-ml/min. With a narrowed orifice, however, this linear range increased with all the capillaries of different IDs', and wider energy input range.

From the results of measuring 200-ppb cadmium chloride, similar signal to flow rate linearities were observed for 0.007" and 0.01" ID capillaries with 75-um orifices up to 2.0-ml/min. At energy input below 130-W, the linearity reached 2.00-ml/min flow rates. The signal decreased with flow rate increase. The linearity range extended towards higher flow rates as the energy input increased. Above 132-W, linearity was good from 1.00-ml/min up to 3.00-ml/min.

For TSP nebulizer with 0.02" ID capillary, much better linearity was observed. Below 117-W, good linearity extended from 1.00-ml/min to 2.50-ml/min. Within the range 120-W to 156-W, excellent linearity was obtained from 1.00-ml/min up to 3.00-ml/min. However, the signals decreased with flow rate increase above 160-W and below 120-W were observed.

Figure 45 illustrates the above observations. The signal decrease at high energy input was probably due to solute preconcentration inside the capillary at that flow rate. The decreased signal at high flow rate could probably
FIGURE 45. AT TSP ENERGY INPUT 110-, 120-, AND 150-W, Cd ABSORPTION SIGNAL VS. SOLVENT FLOW RATE HAS A LINEAR RELATIONSHIP ONLY TO 2.5-ML/MIN. BEYOND THAT FLOW RATE, THE SIGNALS DROPPED.
be explained by the fact that an adequate energy was not available at that particular flow rate of the liquid sample. Thermal energy was not transferred into the fast-moving sample quickly enough for complete evaporation. This resulted in incomplete vaporization and desolvation, producing larger sample droplets, which in turn reduced the atomization efficiency.

(b) Relationship between Linear Signal and the Region of Flow Rate

Figure 46 shows the linearity of signal to flow rate of TSP nebulizer with 0.02" ID capillary and 75-um tip orifice. Excellent linear relationships between the signal and flow rate were observed for other TSP nebulizer as well:

<table>
<thead>
<tr>
<th>Capillary ID, Tip Orifice</th>
<th>Energy input W (ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.02&quot;, 75-um</td>
<td>120-156</td>
</tr>
<tr>
<td>0.01&quot;, 75-um</td>
<td>135-160</td>
</tr>
<tr>
<td>0.007&quot;, 75-um</td>
<td>144-165</td>
</tr>
</tbody>
</table>

TSP of 0.02" ID, 75-um orifice, had the widest linear signal to flow rate range, TSP of 0.01" ID had the moderate, while TSP of 0.007" had the narrowest.
Signal vs. Solvent Flow Rate

Figure 46. The linear relationship between absorption and solvent flow rate with a 0.02" ID TSP capillary with 75-μm tip orifice. Note the perfect linearity within 128-140 W input.
[3] Summary

The TSP with 0.02" ID capillary, 75-μm orifice showed an excellent linear relationship between signals and flow rates. It covered the range of all flow rates and almost 40-W energy input value. It demonstrated its best conditions for making and operating the capillary for the TSP nebulizer used in the study.

E. Effect of TSP Capillary ID Size on AAS Signal

It was believed that the narrower the capillary ID size, the better the signal. The TSP nebulizers made elsewhere were composed of 0.005" ID (150-μm) capillaries.

[1] Experimental Procedures

The data from the above cadmium analysis, using different ID sizes of TSP capillary, with and without narrowed orifices, are compared and discussed below.

[2] Results and Discussion

(a) Capillary without Narrowed Tip Orifice

Without tip modification, the 0.005" ID capillary gave the best AA signals, while the 0.02" ID gave the worst. The narrower the capillary ID, the better the sensitivity. The reason for this could be that the narrower the ID, the faster the sample linear velocity. Simultaneously there was a bigger aerosol pressure drop between the capillary and
atmosphere, causing a higher transportation rate of the finer droplets of sample into the flame.

The performance of these TSP nebulizers are compared at 2.25-ml/min flow rate, 140-W energy input, with 0.20-ppm cadmium sample detection signals, and data are listed in Table 7:

<table>
<thead>
<tr>
<th>Capillary ID (in)</th>
<th>Orifice (0.2-ppm Cd)</th>
<th>Absorbance</th>
<th>Standard Deviation</th>
<th>Relative Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.02</td>
<td>No</td>
<td>1.3 x 10^{-2}</td>
<td>2.0 x 10^{-3}</td>
<td>15 %</td>
</tr>
<tr>
<td>0.01</td>
<td>No</td>
<td>4.8 x 10^{-3}</td>
<td>1.8 x 10^{-3}</td>
<td>4.0%</td>
</tr>
<tr>
<td>0.007</td>
<td>No</td>
<td>6.7 x 10^{-3}</td>
<td>1.4 x 10^{-3}</td>
<td>2.0%</td>
</tr>
<tr>
<td>0.005</td>
<td>No</td>
<td>1.0 x 10^{-1}</td>
<td>1.4 x 10^{-3}</td>
<td>1.4%</td>
</tr>
<tr>
<td>0.005</td>
<td>Yes</td>
<td>1.1 x 10^{-1}</td>
<td>1.0 x 10^{-3}</td>
<td>0.9%</td>
</tr>
<tr>
<td>0.007</td>
<td>Yes</td>
<td>1.2 x 10^{-1}</td>
<td>1.4 x 10^{-3}</td>
<td>1.2%</td>
</tr>
<tr>
<td>0.01</td>
<td>Yes</td>
<td>1.5 x 10^{-1}</td>
<td>2.4 x 10^{-3}</td>
<td>1.5%</td>
</tr>
<tr>
<td>0.02</td>
<td>Yes</td>
<td>1.6 x 10^{-1}</td>
<td>9.0 x 10^{-4}</td>
<td>0.6%</td>
</tr>
</tbody>
</table>

The data showed that the performance of those TSP nebulizers without capillary tip orifices are as follows:

0.005", the best > 0.007" > 0.01" > 0.02", the worst.

b) Capillary with Narrowed Tip Orifice

With tip modification, the totally opposite results were observed. With the same 75-um orifices at the capillary tips, the 0.02" ID capillary gave the best signals, while
the 0.005" ID gave the worst. The sensitivity decreased as the ID size decrease as shown in Table 7.

This phenomenon could possibly be explained as follows. At the same volume flow rate, with large ID, the solvent linear velocity inside the capillary was slower. The sample solution took a longer time passing through the thicker tubing than through the thinner one. This resulted in better thermal reactions, a better sample vaporization, better desolvation, better thermal solute decomposition; and then better sensitivity.

The performance of these TSP nebulizers are as follows: 0.02", the best > 0.01" > 0.007" > 0.005", the worst.

[3] Summary

The capillary of the TSP nebulizer with narrowed outlet tip orifice demonstrated better sensitivity with thicker ID size, which was totally opposite with that of the capillary without modified tip orifice.

F. Effect of Heating Length of Capillary on AAS Signal

The TSP nebulizers used by most researchers in the past utilized a 10" heated capillary length (12, 28, 36, 41). The heating length was considered to be not particularly critical (2, 39), and a shorter heating length was reported, and used in our group. However, if the heated length could
be reduced, the nebulizer could be made smaller and have wider applications.

[1] Experimental Procedures

In this experiment, two electrical heating cartridges, one was 150-W and another was 100-W power, were slid onto the same capillary in series, one after another. The 150-W cartridge was equipped with the thread ring and screwed into the safety diaphragm of the burner stem. The 100-W cartridge was slid onto the capillary immediate to the bottom of the 150-W cartridge. Both cartridges had the same 1.50" heating lengths, but their power inputs were separately controlled. With the first cartridge being kept at 123.6-W, the power input of the second one was varied from 0.0-W to 135-W.

The capillary used was 0.01" ID with a 85-um tip orifice. Two different solvent flow rates, 1.00-ml/min and 2.00-ml/min, were tested, and 0.10-ml of 200-ppb CdCl₂ was injected. The absorption signals against the power inputs to the 100-W cartridge, with the two flow rates, were measured.

[2] Results and Discussion

(a) Signal at Lower Solvent Flow Rate

It can be seen that, at 1.00-ml/min flow rate, the signal did not increase until 70-W energy was put into the secondary cartridge. The signals reached a steady maximum after 100-W, up to 140-W. The increase, however, from 7% of
absorption to 8.3% absorption, was very little. This could not prove that the increase in heating length was beneficial to the detection sensitivity.

(b) Signal at higher Solvent Flow Rate

Figure 47 shows that, at 2.00-ml/min, a rapid decrease in signal was observed. The sensitivity started to drop at very low energy input to the second cartridge about 30-W, from 25% of absorption to 18% of absorption, and dropped nearly 7% of absorbance at 100-w energy input. This indicated that the increase in heating length of the TSP nebulizer actually decreased the sensitivity of detection.

[3] Summary

The observed results were not well understood. However, they indicated that long heating length was not necessary, and actually decreased the detection limits. Therefore, the optimum heating length of the TSP nebulizer used in this experiment was chosen as 1.0". On the other hand, results revealed the possibility of further reducing the heating length to make the TSP nebulizer even smaller.

G. Sensitivity and Reliability of AAS Signal

The cadmium detection sensitivity of the TSP with 0.02" ID capillary and 75-um tip orifice was measured, and the relative standard deviations (RSD) of the signals were
Figure 47. The absorption signal versus TSP heating length. Note that longer heating length decreases the absorption signal in AAS.
calculated. The signal to noise ratio and the reliability of the signal were also discussed. The statistical analysis on the linearity of responses was also conducted.

[1] Experimental Procedures

Each datum point was averaged from about 20 injections, and the RSD's were calculated. The resulted sensitivities were compared with those using the pneumatic nebulizer, as well as with the TSP nebulizer without any mentioned modifications.

In order to confirm the sensitivity improvement in using modified TSP nebulizer, the cadmium speciation study was again conducted.

0.10-ml of perspiration sample from the same source was injected into a 3-um, C-8 reverse-phase HPLC column. The 2.00-ml/min solvent flow rate was used. The energy input to the TSP nebulizer was 140-w, which was within the region of maximum response independent to energy input.

The response linearity of the TSP nebulizer with low solute concentrations was studied with 50-200 ppb CdCl₂.

[2] Results and Discussions

The comparison of RSD was made between two modified TSP nebulizers. At 130-W energy input and 2.75-ml/min solvent flow rate, the calculation showed that the standard deviation of TSP nebulizer with 0.01" ID capillary and 75-um
orifice was 0.01 absorbance. For the TSP nebulizer with
0.02" ID and 75-um orifice, the standard deviation was 0.001
absorbance, which was ten times better than that of the TSP
with 0.01" ID capillary. Those calculations are shown in
Figure 48.

The value of the relative standard deviation of the
0.02" ID capillary TSP nebulizer was 0.6%.

Figure 49 shows the separation of cadmium compounds in
human urine with a TSP nebulizer of 0.02" ID and 75-um tip
orifice. A remarkable sensitivity increase was observed. A
doublet peak of absorbance of 0.265 and 0.227 was observed
at retention times of 28 seconds and 38 seconds. This
doublet peak revealed that there were two different cadmium
compounds eluted from the column at an earlier retention
rather than an unresolved broadened single peak shown in the
previous speciation. Also, the intensity of the signals were
almost six times stronger compared with the result obtained
in previous cadmium speciation in human urine from the same
person, in which the absorbance of the single peak was less
than 0.04 absorbance (Figure 31).

The calculation showed that the concentration
sensitivity of cadmium detection was approximate 3.0-5.0
ppb, and the limit of detection of cadmium was 0.2-ppb. This
gave a 0.50-ng absolute concentration sensitivity, and a
0.02-ng absolute limit of detection for cadmium. This
STANDARD DEVIATION CALCULATIONS

\[ s = \sqrt{\frac{\sum_{i=1}^{N} x_i^2 - \left( \sum_{i=1}^{N} x_i \right)^2}{N - 1}} \]

(A) THERMOSPRAY CAPILLARY: 0.01" i. d. W/0 75-um ORIFICE:
Cd CONC. 0.5-ppm,
INJECTION: 0.10-ml,
TN POWER: 112-W,
FLOW RATE: 1.50-ml/min

ABSORPTION READINGS:
.206, .211, .239, .238, .238, .228, .245, .230, .218, .220, .208, .215.

\[ N = 12, \sum x_i = 2.696, \sum (x_i)^2 = 0.6057 \]
\[ s = 0.0135 = 0.01 \text{ (abs.)} \]

(B) THERMOSPRAY CAPILLARY: 0.02 " i. d. W/ 75-um ORIFICE:
Cd CONC.: 0.2-ppm,
INJECTION: 0.10-ml,
TN POWER: 130-W,
FLOW RATE: 2.75-ml/min

ABSORPTION READINGS:
.199, .197, .198, .201, .200, .196, .198, .197, .200, .202, .200, .197.

\[ N = 12, \sum x_i = 2.385, \sum (x_i)^2 = 0.474 \]
\[ s = 0.00131 = 0.001 \text{ (abs.)} \]

FIGURE 48. THE CALCULATION OF STANDARD DEVIATIONS OF
ABSORPTION OF 0.20-PPM CADMIUM WITH TSP
NEBULIZERS WITH AND WITHOUT NARROW TIPS
USED IN FLAME AAS.
FIGURE 49. THE HPLC CHROMATOGRAM OF CADMIUM COMPOUNDS SPECIATED USING TSP NEBULIZER INTERFACED HPLC-FLAME AAS SYSTEM. NOTE THE SIMILARITY TO THE CHROMATOGRAM OF CADMIUM COMPOUNDS IN METALLOTHIONEIN COMPLEX. (CHAPTER FIVE)
demonstrated an approximate 300-fold increase in sensitivity compared with pneumatic nebulizer sample introduction.

The linearity test with cadmium analysis also showed very good relationship between the signal responses and very low sample concentrations. Figure 50 shows the perfect linear calibration curve obtained from the cadmium standards from 0.10-ppm to 0.50-ppm detections.

[3] Summary

The TSP nebulizer composed of 150-W heating cartridge and 0.02" ID, 75-um tip orifice capillary demonstrated excellent analytical power as an advanced sample introduction device for flame AAS, and as an improved interface device for HPLC-Flame AAS system for metal speciation studies.

6. PROPOSED DESOLVATION MECHANISM OF THERMOSPRAY

A. Introduction

The particle size, linear velocity, and shape of thermospray generated from a fixed tip orifice size of a TSP capillary should be constant if the sample volume flow rate and TSP nebulizer energy input are constant; and independent of the capillary ID's. In addition, the sample
FIGURE 50. THE CALIBRATION CURVE OF ABSORPTION SIGNAL VERSUS CADMIUM CONCENTRATIONS. NOTE THE PERFECT LINEARITY OF THE SYSTEM WITH TSP Cd: 0.1-, 0.2-, 0.3-, 0.4-, AND 0.5-PPM.
transportation rate into the flame should be the same if the burner and the nebulizer arrangement is kept unchanged. Then, consistent atomization efficiency should be achieved because the analyte residence time in a constant flame is constant. This may vary, however, if the sample desolvation processes are different inside the capillaries with different ID sizes.

Results from the evaluation of the TSP nebulizers with different capillary ID and tip modification showed that, under the same operating conditions, 0.02" ID capillary with 75-um tip orifice gave the best performance among TSP nebulizers with other combinations. It demonstrated the highest sensitivity with lowest heating energy, the best signal to concentration linearity, and the lowest RSD in flame AAS trace metal detection.

In addition, the AAS signal comparison between different capillaries applied to the TSP nebulizer showed that, with the same 75-um tip orifice, the larger the capillary ID size, the better the flame AAS signal response. This result indicated that, before the sample solution passed the same tip orifices, some different sample desolvation mechanisms existed inside the capillaries with different IDs'. Understanding these processes was the objective of this thermospray desolvation mechanism study.
B. Observations Conflict with Vestal's Mechanism

According to the Vestal (2) proposed desolvation mechanism, solvent vaporized around the capillary wall which was heated above the Leidenfrost temperature of the solvent (30), while nonvolatile analytes were supposed to concentrate in the very center of the liquid stream inside the capillary from which an analyte-enriched central jet cone was ejected upon emerging. However, the experiment described in section 3 indicated that such an analyte-enriched central cone did not exist, and the same analyte concentrations were found throughout the whole spray.

Furthermore, if only the solvent vapor was formed around the internal wall of the capillary, analyte deposition on the wall was impossible. However, in fact, large amounts of analyte deposit were found on the wall in our investigation on superheated TSP nebulizer applications. In fact, this was proposed as a preconcentration method for using the TSP nebulizer to increase analytical detection sensitivity (40).

C. Analyte Adsorption/Desorption inside Capillary

The Leidenfrost phenomenon was applied to pure solvent, not solution. Yet, what would happen to a solution? Suppose a NaCl solution is dropped on a heated pancake pan. Above the Leidenfrost temperature of water, a steam of condensed water vapor forms with salt liquid beads dancing above the
hot pan surface. However, if the pan is too hot, the residence time of the liquid beads is so short that the steam can only be seen in instantaneously. A white spot of salt residue dried on the pan surface would indicate the solute deposition. This may happen to the sample analyte passing through a very hot TSP capillary (>700°C).

Inside the TSP capillary, with the fast-moving solution, the situation might be a little different. At lower capillary temperatures, an equilibrium between adsorption and desorption of the analytes may exist between the solution and capillary internal surface. At higher capillary temperatures, adequate thermal energy would complete the sample desolvation. Then, the desolvated analytes would have a higher tendency to be adsorbed onto the deposition sites of the capillary internal surface. This would probably slow down the analyte migration speed, causing it to be concentrated towards the capillary internal surface. The analytes would then be ejected out of the capillary altogether in an instant by on-coming HPLC solvent flow. This would increase the signal peak height with the reduction of the peak width.

This assumption can be confirmed by collecting the analytes eluted from an operating TSP nebulizer. An experiment was then designed for this purpose.
D. Experimental Procedures

A fractional collector with 5.5-tubes/sec collecting rate was used. The TSP nebulizer was composed of a 150-W heating cartridge and 0.02" ID capillary with 75-um tip orifice, and heated at 110-W. It was installed face-down on the collector sampling arm to spray into the test tubes, and was directly connected to a HPLC pump. The water solvent flow rate was 2.0-ml/min, and the sample was 0.10-ml of 20.0-ppm cadmium chloride. The tubes were rinsed thoroughly with the purified water. The nebulizer tip was precisely aligned to the center of each rotating test tube, and was kept minimum distance to the tubes so that the sample effluent loss could be minor.

With the nebulizer operating at its optimum conditions, the sample was injected into the nebulizer at the same time as the collector was turned on. The effluent collected in test tubes were then diluted with 1.00-ml of purified water, and the cadmium contents were measured with the HPLC-TSP-flame AAS system.

E. Results and Discussion

A 0.10-ml of sample should take 3 seconds to pass through an unheated TSP capillary at a 2.00-ml/min solvent flow rate. During this period, 0.10-ml of sample solution should be equally distributed into 17 or more (at 5.5-tubes/sec rate) test tubes of the collector, because the
possible sample diffusion in the solvent. 0.10-ml acid eluted from an unheated TSP nebulizer at 2.00-ml/min flow rate showed red color on pH test paper for 6 seconds, and confirmed the acid diffusion in the solvent.

Interestingly, however, with 0.10-ml of cadmium chloride passing through the heated TSP nebulizer at 2.00-ml/min flow rate, the results showed very little cadmium collected in the first 16 tubes; but significant cadmium were detected from the collections of tube No. 17 to tube No. 21. After tube No. 22, the cadmium signals dropped almost to zero again. Similar results were observed with six repeated tests.

Figure 51 shows the results from the collections. The signals of cadmium concentration increased only after the No. 15, and reached a maximum at No. 17 test tube, indicated that the most cadmium solute was eluted at that point.

The cadmium only collected in five tubes indicated that the entire analyte eluted from a heated TSP capillary within one second, rather than the 3 seconds expected for an unheated capillary. This seemingly indicated that the analyte was somehow being preconcentrated inside the heated capillary before emerging. Little analyte found in the first 16 tubes could account for the possible migration delay of the majority of analyte inside the hot capillary. The delay could be upto 2 seconds if one second for sample injection
FIGURE 51. THE ABSORPTION OF Cadmium COLLECTED IN TEST TUBES. NOTE THAT ONLY 7 TUBES CONTAIN Cadmium, INDICATING THAT 0.1-ML SOLUTE ELUTED FROM TSP ONLY IN 1.5-sec.
was required (i.e., rotating the injector from load to inject). Within these two seconds, the solvent vaporized and separated from the solute, passed the capillary and emerged first.

F. Proposed Desolvation Mechanism in TSP Nebulizer

Based on the above-observed facts, a new sample desolvation mechanism inside the heated TSP nebulizer can be proposed. The processes of the mechanism are discussed and illustrated in Figure 52 as follows.

[1] The Analyte Desolvation Processes

Upon entering the hot zone of the TSP capillary, the very front of the sample first started to vaporize. Its solvent was separated from its solute via vaporization, and trying to stay in the center of the capillary; while the solute was trying to deposit onto the capillary internal wall.

At a certain TSP heating power and solvent flow rate, the equilibrium of solute adsorption and desorption to capillary favored adsorption. With the sample progressing, the solute spread into a layer which was "semi-floating" above the entire heated internal surface. The newly desolvated solute, which also favored deposition onto the
FIGURE 52. PROPOSED DESOLVATION MECHANISM INSIDE THE TSP CAPILLARY. (A) SOLUTE FRONT DEPOSITION. (B) SOLUTE DEPOSIT FORMING A "SOLUTE-TUBE". (C) SOLUTES ARE PUSHED TOGETHER IN A "PLUG"
surface, would be spread onto the top of the previous solute layer. A hollow "solute tube" was temporarily formed and increasing in thickness by oncoming new solute layer.

Meanwhile, through the "solute tube", the sample solvent vapor was being pushed by new vapor to form a sample solvent vapor front. At the capillary tip, the vapor front tried to pass the narrowed tip orifice, and caused tremendous vapor pressure which was exerted back to the "solute tube". The length of the "solute tube" behind its solvent front became shorter and shorter until all the solutes were pushed together by its own solvent vapor pressure and the on-coming solvent flow. At this point, the hollow "solute tube" closed its end to form an instant "solute plug", which was then ejected from the tip orifice.

This was an effective desolvation process. The "solute plug" could account for the reasons why the entire analyte was detected within one second from the heated TSP nebulizer, and why the TSP nebulizer resulted in intensified signal peaks compared to other nebulizers.

[2] Desolvation with Different Capillary ID

For the TSP nebulizers, with the same 75-um tip orifices, the best signals were obtained using 0.02" ID TSP capillaries, while the worst were observed using 0.005" ID capillary.
Figure 53 may explain the different desolvation process between using the two capillaries. With the same volume flow rate, the linear velocity \( V_2 \) of solvent passing through the 0.005" ID capillary was 16 time faster than that of 0.02" ID. The sample residence time inside the 0.005" ID capillary was, therefore, 16 time shorter than that of 0.02" ID. At a certain power input, the thermal energy was removed by the fast-moving solution, and the desolvation process was far from completion inside the 0.005" ID capillary compared with that of 0.02" ID.

On the other hand, the sample movement \( V \) inside the capillary was the sum of its linear velocity \( V_2 \) and its solvent radial vaporization rate \( V_1 \). The radial vaporization rate inside the 0.005" ID capillary was much suppressed by its narrow diameter. The desolvated solute would spread and remixed with its solvent inside the capillary by much faster linear velocity of the sample solution. The "solute tube" and the "solute plug" could hardly be formed, and incompletely desolvated sample would hardly produce enhanced signals.

With 0.02" ID capillary, however, the radial vaporization was not so suppressed and the solution moved slower. The thermal reaction in the sample was better, the sample solvent separation from the solute was better, and the solute concentrated "solute plug" resulted from better desolvation would bring in enhanced detection sensitivity.
THERMOSPRAY DESOLVATION MECHANISM

FIGURE 53. THE DESOLVATION PROCESSES IN CAPILLARY OF DIFFERENT ID'S. INCOMPLETE DESOLVATION IN THINNER CAPILLARY, BUT MORE COMPLETE IN THICKER CAPILLARY DUE TO SLOWER VELOCITY.
There were different observations that may support the proposed mechanism from different points of view, and will be discussed and illustrated with photographs and figures.

(a) Effective Desolvation Zone in Capillary

With the same heating length, the thermal contact area between the capillary internal surface and the sample solution was four time larger with 0.02" ID than with 0.005" ID. Taking the 16 time slower linear velocity of sample solution into consideration, the desolvation efficiency of the sample in 0.02" ID capillary should be much higher than in 0.005" ID capillary.

If adequate thermal energy could effectively convert all the liquid sample into solvent vapor and dried solute aerosol inside the capillary, then the entire capillary heated region would be constantly kept at high temperature by excessive thermal energy. If the linear velocity of solution was high and the thermal contact area was small, as in 0.005" ID capillary, then the heated region would be small. This may be confirmed by examining the color change on the external surface of used capillaries.

Figure 54 shows that the examinations on used 0.02" and 0.005" ID capillaries. It shows the different external appearance of capillaries with different ID's but the same orifice size. The entire 1.5" of 0.02" ID capillary was
FIGURE 54. THE EXTERNAL APPEARANCES OF FOUR USED TSP CAPILLARIES. NOTE THAT THE 0.02" ID WITH 75-UM ORIFICE HAS COMPLETE DARKENED SURFACE, WHILE THE 0.007" ID WITH 75-UM ORIFICE ONLY SHOWS LESS THAN 1/4" OF SLIGHTLY BROWNISH COLOR AT THE END OF THE TIP, INDICATING THAT THE SOLVENT QUICKLY REMOVES HEAT FROM IT AND RESULTS IN INCOMPLETE SAMPLE DESOLVATION.
completely heated to dark-black color, indicating that it was heated to a high temperature (750°C) after complete desolvation. For the 0.005" ID capillary, however, less than 1/4" of the end tip of the capillary was light-brown color. This indicated that the fast-moving solvent continuously removed the heat from the first 1-1/4" of capillary. In this region, the thermal energy was inadequate to completely vaporize the sample or solvent, and the sample desolvation only took place at the final 1/4" capillary. This caused an incomplete desolvation of sample in the 0.005" ID capillary, and explained why the detection sensitivity and RSD were the worst with the 0.005" ID capillary compared to the best with the 0.02" ID.

(b) Signal Peak Appearances

The direct measurements of the signal peak width could be another criterion to confirm that solute enrichment occurred inside the capillary before it was ejected from the tip orifice.

Figure 55 shows a group of very sharp cadmium absorption peaks with about one second widths. This actually confirmed that the TSP nebulizer did effectively desolvate the sample solution, enriched the analytes, delayed the analyte migration inside the capillary, and therefore enhanced the signal peak height to increase the detection sensitivity.
FIGURE 55. THE SHAPE OF THE ABSORPTION SIGNAL PEAKS OF CAPILLARIES WITH THE SAME 75-μM ORIFICE BUT DIFFERENT ID'S. NOTE THAT THE LARGER ID CAPILLARY YIELDS NARROWER AND SHARPER PEAKS.
(c) Solvent Effect in TSP Nebulizer

Our previous investigations on the solvent effect on TSP nebulizer application showed that, at appropriate TSP energy input and solvent flow rate, the absorption signals were independent of the solvents used (39). However, examining the results of the study, we noticed that there were solvent dependent regions when inadequate power was supplied to the nebulizer. Figure 31 and Figure 32 in Chapter 3 have shown that the lowest signals were obtained with water, the best with acetonitrile/water, and moderate with methanol/water.

Thermodynamics indicate that the enthalpy of water is higher than methanol, and that of methanol is higher than acetonitrile. The desolvation process occurring inside the capillary is an endothermic process, and higher thermal energy is needed to vaporize the solvent with higher enthalpy. Besides, the hydrogen bonding also account for the phenomenon observed with different solvents. Therefore, more energy was needed to desolvate the aqueous sample than the nonaqueous sample.

Consequently, at lower energy input to the TSP nebulizer, the desolvation of solvent from solute depended upon the thermal energy adequacy for breaking the hydrogen bonding, and resulted in minor solvent effects in detection. At higher energy input to the TSP nebulizer, more than adequate thermal energy could break all intermolecular
bonding to desolvate the sample effectively, resulting in the absorption signals independence on the solvents used.

Recalling the discussion on the region of signal independence on energy input to the TSP nebulizer (Section B [2] (b) ii)), as the energy input to the TSP nebulizer maintained above a certain high level, desolvation of the sample was completed. Certain excessive thermal energy would not change the signal responses unless it exceeded a maximum point, then the signals dropped at superheated condition.

G. Conclusions

This entire study on the TSP desolvation mechanism now can be concluded as follows:

At the appropriate energy input to the TSP nebulizer and solvent flow rate with a TSP capillary of certain ID size and narrowed tip orifice, the sample solution entering the heated capillary region and transported with moderate linear velocity. The thermal energy transferred to the liquid sample was adequate to vaporize the sample solvent, and the solute may tend to semi-adsorb towards the internal surface of the hot capillary and form a "solute tube". The solvent vapor would be pushed by on-coming solvent vapor through the "solute tube", and form a solvent front. The "solute tube" would increase its thickness by the new solute layers deposited upon, until the thickness reached the maximum and closed its tubular end to form an instant
"solute plug". The solvent front would then be separated from its solute and emerge from the capillary first. Then the solute-enriched "solute plug" would be ejected out of the capillary tip orifice to enter the flame.

This effective sample desolvation processes would result in a solute-enhanced aerosol and high atomization efficiency. The detection sensitivity was then increased by increasing the peak height and reducing the peak width.
FIGURE 56. THE PHOTOGRAPH OF A REUSABLE TSP NEBULIZER. THE THREAD RING AND THE HOLLOW COPPER ROD SEEN. THE TSP DIMENSIONS ARE COMPARED WITH pH TEST PAPER TO GIVE THE IDEA OF ITS SIZE.
CHAPTER FIVE

PRELIMINARY STUDIES ON INTERACTIONS BETWEEN METALLOTHIONEIN CADMIUM, METALLOTHIONEIN ZINC AND INORGANIC CADMIUM AND ZINC COMPOUNDS
1. INTRODUCTION

Metallothionein, a cysteine-rich, low molecular weight protein was first recognized in equine renal cortex by Margoshes and Vallee in 1957 (100, 101). It complexes strongly with divalent ions such as Cd, Zn, and Cu. The same protein occurs in large quantity in human kidney, and in equine, rabbit and human liver (102, 103). It accumulates in various parenchymatous tissues of laboratory animals after administration of salts of cadmium, zinc, or certain other heavy metals, implicating a role in metal metabolism and detoxification (104). The protein usually contains between 6 and 11% metal and between 30 and 35% cysteine when calculated on the basis of the weight of the polypeptide chain, but is completely devoid of aromatic amino acids, histidine, leucine, and isoleucine.

For example, metallothionein-IB, one of the two main variants of the protein separated from horse kidney cortex (MT-IB-HK), showed an abundance of cysteine which comprised 33% of all residues. Figure 57 shows that this single-chain protein contains 20 cysteinyl residues, which are distributed along the entire chain of 61 amino acids, but are somewhat more crowded in the center portion. Fourteen out of 20 cysteinyl residues form part of seven Cys-X-Cys tripeptide sequences, where X is an amino-acid residue other than cysteine. In addition to these sequences, there are
FIGURE 57. THE SINGLE LONG CHAIN OF A METALLOTHIONEIN MOLECULE.
NOTE THAT 20 CYSTEINE FORM 7 CYS-X-CYS TRIPEPTIDE.
three Cys-X-X-Cys and three Cys-Cys sequences.

The metallothioneins all have structurally defined metal-binding sites, most of which contain three cysteinyl residues as the principal metal-binding ligands, a complex $[\text{Metal}^{2+} (\text{Cys}^-)_3]$ (105). It is believed that the metal ions are bound through the trimercaptide linkages (102) and that all cysteine residues of the protein participate in metal coordination. The ratio of cysteinyl residues to the sum of cadmium and zinc is close to 3, which is 3 cysteines to 1 zinc or 1 cadmium ion, in all forms of metallothionein. The abundance of Cys-X-Cys sequences in MT-IB-HK and their stoichiometric correspondence to the number of metal ions bound suggest that these sequences constitute such primary chelation sites for cadmium or zinc ions (106).

It has been reported that some binding sites in the metallothioneins favor zinc ions over cadmium ions (107). In other words, the zinc ions have higher affinity towards the binding sites of the protein than the cadmium ions. In human biological system, zinc ions are considered an essential element, important to human health, while cadmium ions are toxic. When the cadmium ions are bound to the protein, they are detoxified because the free cadmium ions form complexes with the protein. However, if the concentration of zinc ion exceeds a certain level in the human body, the equilibrium of the complexation between these two ions would be shifted towards zinc-complexation side. Free ionic cadmium would be
released from the protein, which might impose a toxic effect towards human health.

Clinically, physicians are prescribing zinc tablets to patients who are considered as zinc deficient in order to re-establish the zinc balance in patients' bodies. However, if the zinc concentration absorbed by the patient exceeds a certain level, the excess zinc ions would replace the cadmium ions in the metallothionein, and release cadmium as free ions in the body, which could cause serious toxic effects to the patients.

In vivo, this quantitative experiment is impossible to perform in human beings; in vitro, however, it is possible to establish an experimental condition that imitates the condition for cadmium and zinc complexation reactions with metallothionein. Using our advanced TSP nebulizer interfaced HPLC-Flame AAS system along with the metal speciation method, the cadmium or zinc complexation reactions with the protein may provide qualitative information to confirm the replacement of cadmium by zinc, and reveal the possible inorganic forms of cadmium compounds formed in the process.

In addition, if the ionic cadmium concentration does increase with the additions of zinc compounds to the protein, it might indicate that the excess zinc uptake into the human body is harmful. Furthermore, if the minimum concentration of zinc ion uptake which would cause the replacement of cadmium in the protein can be determined, it
would provide important clinical information. This also is our main purpose of this study.

In short, the purposes of this study were:

(a) to qualitatively confirm the reaction of zinc ions replacing cadmium from the binding sites on metallothionein to release cadmium ions,

(b) to study the difference in zinc replacing cadmium reaction in different types of metallothioneins commercially available,

(c) to study the capacity of the protein binding sites towards cadmium or zinc ions to form metal complexes,

(d) to quantitatively determine the minimum zinc ion concentration range which initiates the reaction of replacing cadmium from the protein complexes to form ionic cadmium,

(e) to determine the possible forms of the ionic cadmium compounds which was formed after the cadmium are replaced by zinc ions.

2. EXPERIMENTAL

A. Equipment Used

[2] Radiation Source: Perkin-Elmer cadmium hollow cathode lamp was used at 228.8-nm, 5-mA and 12-V.


[6] **Table 8: Reverse-Phase HPLC/Guard Columns:**

<table>
<thead>
<tr>
<th>Column Brand, Maker</th>
<th>Packing</th>
<th>Size (mm)</th>
<th>Guard*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Dynanmax Shortone, Rainin</td>
<td>C-8, 3-um</td>
<td>50x4.6</td>
<td>Yes</td>
</tr>
<tr>
<td>2) Econosphere, Alltech</td>
<td>C-8, 3-um</td>
<td>100x4.6</td>
<td>Yes</td>
</tr>
<tr>
<td>3) Econosphere, Alltech</td>
<td>C-18, 3-um</td>
<td>100x4.6</td>
<td>Yes</td>
</tr>
<tr>
<td>4) Lichrome, Alltech</td>
<td>C-2, 5-um</td>
<td>250x4.6</td>
<td>Yes</td>
</tr>
</tbody>
</table>

*Guard columns were 10x4.6mm filled or 20x4.6mm refillable.

[7] Interface: TSP nebulizer, fabricated in the lab and described in Chapter four, with 0.02" and 0.03" ID capillaries, equipped with 75-um and 50-um orifices, 150-W "Firerod" heating cartridge operated at 110-W.

[8] pH Meter: Chemcadet digital pH meter at 28°C.


[11] TSP Constant Power Supply: The same TSP constant power supply used in Chapter Three and Chapter Four, gave a 102-130 V range and 0.01-A current variation.
B. Reagents Used

[1] Table 9: Metallothionein Samples

<table>
<thead>
<tr>
<th>Sample Sources</th>
<th>Metal Conc. as Received</th>
</tr>
</thead>
<tbody>
<tr>
<td>Separated from</td>
<td>Cd (ng/mg)</td>
</tr>
<tr>
<td>(a) Horse kidney (MT-IB-HK):</td>
<td>52,000</td>
</tr>
<tr>
<td>(b) Rabbit liver (MT-RL):</td>
<td>67,400</td>
</tr>
<tr>
<td>(c) Rabbit liver (MT-RL-I):</td>
<td>57,300</td>
</tr>
<tr>
<td>(d) Rabbit liver (MT-RL-II):</td>
<td>69,000</td>
</tr>
</tbody>
</table>

[2] Solvents: (a) 100% purified water, (b) 99.5% purified water and 0.5% Cl⁻ (in NaCl), (c) 20/80, 40/60, 60/40, 80/20 of methanol/water and 0.5%, 0.1% Cl⁻ (in NaCl). Methanol used was HPLC grade, NaCl used was high analytical grade. The pH values of all the solvents were adjusted to 5.0 ± 0.2 with 1% HCl (v/v).

[3] Standard Solutions: 100-ppm cadmium chloride; 1,000-, 100-, 10-, 5.0-, 1.0-ppm zinc sulfate; 100-ppm zinc chloride; 100-ppm copper chloride. The zinc sulfate solution of 1,000-ppm was prepared from high analytical grade salt with 1.0000-g of metal zinc content, and then diluted to different concentrations; the cadmium chloride was diluted from the 1,000-ppm standard prepared in previous cadmium speciation studies.

[4] Washing Solutions: 10% nitric acid, 10% HCl for burner assembly; 0.5-mM citric acid for thermospray nebulizer; methanol, THF, and DCM were used for column regeneration. 20% nitric acid for frits and filters.
C. Experimental Procedure

[1] Preparation of Metallothionein Standards

In the first experiment, one milligram of MT-IB-HK was dissolved in 10-ml purified water. The cadmium signal of the solution indicated that it was too concentrated, and then all other samples were dissolved in 25-ml water. According to the data listed in Table 9, the concentrations of cadmium and zinc in 25-ml of solutions would be in the range from 2.1- to 2.8-ppm and 0.30- to 0.70-ppm, respectively.

Several aliquots of 2.00-ml of each protein were taken and known additions of cadmium chloride, zinc chloride, zinc sulfate, and with different concentrations and volumes were made up to make standard solutions. Three 2.00-ml of pure protein solutions were taken as controls. The mixtures and the controls were well shaken. They were allow to sit for 24 hours, at room temperature (28°C), to allow the completion of the possible, slow reaction of replacement of cadmium in the metallothionein complexes by ionic zinc.

The limited protein samples, i.e. only 1-mg of each, were washed into 25-ml flasks with successive 1.0-ml of purified water in the original bottles to ensure the proteins were

[2] Solvent Composition Determination

In order to imitate the real biological conditions of the human blood stream, our initial choice of solvent was
limited to pure water with the pH value adjusted near 6.5.

Neither the application of buffer, nor the ion-pairing method was used, to avoid any possible error from unexpected reactions between the buffer reagents, counter ions, and metallothioneins. It should be noted that these methods have been widely used for separating ionic and molecular compounds in reverse-phase liquid chromatography (108, 109).

Because the inorganic metal compounds of our choice to be added in the protein were cadmium chloride, zinc chloride, and $3\text{CdSO}_4\cdot8\text{H}_2\text{O}$, our initial tests were focused on the chromatographic separation of these metal compounds and measure their retention times. Unfortunately, however, after hundreds of trials of eluting the $\text{CdCl}_2$ from different HPLC columns, none of the columns listed in Table 8 could elute the cadmium chloride using 100% purified water alone as solvent, no matter what pH value was. When using buffer or higher methanol/$\text{H}_2\text{O}$ ratio in the solvent, the columns used did elute cadmium chloride. However, this was not our original choice of the solvent compositions because it was not similar to the human blood serum.

The reason that the reverse-phase column was not able to elute cadmium chloride when pure water was used as solvent was then studied. Cadmium ion hydrolysis was impossible under the pH value lower than 7.2. A possible reaction might be the cadmium chloride dissociation inside the column packings,
that formed cadmium cations, which could be possibly retained in the bed of the column packings. If a solvent was rich in chloride anions, it would shift the equilibrium of the dissociation of the cadmium chloride back to CdCl₂ molecular forms which might be eluted with water. The concentration of chloride in human blood stream was used as a guide(110). A solvent composed of 99.5% of water and 0.5% of Cl⁻ was made (in NaCl by weight), and its pH was adjusted to 5.00-5.20 with 1% HCl to imitate the acidity of human renal conditions.

With the TSP nebulizer of 0.02" ID capillary, 75-um tip orifice, heated at 110-W, solvent flow rate at 1.25-ml/min, 100-ul of 0.50-ppm cadmium chloride standard resulted in 20 reproducible absorbance values of an average 0.230. This value was 4.0% higher than the value obtained under the same conditions without the analytical column being connected, and was equal to that of using buffer solvent. The standard deviation calculated was 0.001 absorbance with this solvent, which was as good as the value obtained without the column. This indicated that the entire 0.10-ml of 0.50-ppm cadmium chloride sample injected was completely eluted from the C-8 HPLC column. Figure 58 shows the signal comparison under these conditions and high methanol/H₂O ratio solvent.
FIGURE 58. THE EFFECT OF DIFFERENT SOLVENTS ON THE Cd ABSORPTION SIGNAL: (A) BUFFER (B) 80/20 OF METHANOL/WATER (C) 0.5% Cl− IN WATER, pH=5.5 (D) NO COLUMN. NOTE THAT (C) YIELDS SLIGHTLY HIGHER SIGNAL THAN (D)
For future work, a solvent composition was selected as 0.5% of NaCl in pure water at pH 5.0. Further studies on the interactions between the metallothionein cadmium and inorganic cadmium, zinc, and copper could then be pursued.

[3] Optimum Chromatographic conditions

It was necessary that the TSP nebulizer interfaced HPLC-flame AAS system operated at its optimum conditions, in order to resolve the composition change between the ionic cadmium and the metallothionein cadmium compounds. For the best limit of detection (LOD) of the system, the TSP nebulizer was operated at the highest solvent flow rate possible. Hence, high-speed HPLC separation mode (111) was chosen. Consequently, the columns with 3-um packing material and short cartridge lengths were required for the entire experiment, as well as the high quality, pulse free solvent delivery system.

The length of the alkyl chain of the bonded phases on the reverse-phase column packings could alter the elution times between the ionic and the molecular compounds. Therefore, C-2, C-8, and C-18 bonded-phase analytical columns were all investigated.

The solvent flow rate was first set at 1.25-ml/min, as a compromise between higher solvent flow rate for better detection sensitivity and slow flow rate for better separation. Several flow rates were investigated. However,
only one large cadmium peak appeared. After trial and error, a flow rate of 2.0-ml/min was chosen to ensure high sensitivity. The results are illustrated in Figure 59.

[4] In Vitro Testings of Metal Interactions with Metallothionein

The in vitro testings of metal interactions between ionic metal and metallothionein metal compounds were quantified using the standard addition method (112). The changes in the concentrations of cadmium compounds in the metallothionein solution before and after the additions of standard zinc compounds solutions were measured.

The chromatographic column separated the ionic cadmium and the metallothionein cadmium in the protein complex. The ionic cadmium peak could be identified by adding cadmium chloride to the protein solution, and noting the retention time of the peaks.

After the addition of zinc, however, cadmium bonded in the protein might be replaced from the complex by the zinc complexation with the binding sites of the protein. This could result in an increase with ionic cadmium peak, and a decrease in the complex cadmium peak.

Each protein solutions were divided into 2.0-ml sample cuvettes. Different quantities of inorganic metal compounds were added to each. 2.0-ml of each protein solution was kept as control for each addition testing. The compositions of
FIGURE 59. Cd ABSORPTION SIGNAL IN MT-IB-HK ELUTED FROM HPLC COLUMN WITH DIFFERENT SOLVENT FLOW RATES. NOTE THAT AT 1.75-ML/MIN FLOW RATE THE HIGHEST SIGNAL WAS ACHIEVED WITH LESS TAILING.
these sample are listed in Table 10-12 below.

**Table 10: Sample Compositions of 2.0-ml MT-IB-HK**

<table>
<thead>
<tr>
<th>Group</th>
<th>CdCl₂</th>
<th>ZnSO₄</th>
<th>Final Zn Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>4-ml, 0.5-ppm</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>60-ul, 100-ppm</td>
<td>4.5X10⁻⁵-M</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>40-ul, 100-ppm</td>
<td>3.0X10⁻⁵-M</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>20-ul, 100-ppm</td>
<td>1.5X10⁻⁵-M</td>
</tr>
</tbody>
</table>

**Table 11: Sample Composition of 2.0-ml MT-RL**

<table>
<thead>
<tr>
<th>Group</th>
<th>CdCl₂</th>
<th>ZnSO₄</th>
<th>Final Zn Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>20-ul, 25-ppm</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>20-ul, 100-ppm</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>20-ul, 100-ppm</td>
<td>40-ul, 1,000-ppm</td>
<td>3.0X10⁻⁴-M</td>
</tr>
<tr>
<td>5</td>
<td>20-ul, 100-ppm</td>
<td>120-ul, 100-ppm</td>
<td>9.0X10⁻⁴-M</td>
</tr>
<tr>
<td>6</td>
<td>20-ul, 100-ppm</td>
<td>80-ul, 100-ppm</td>
<td>6.0X10⁻⁵-M</td>
</tr>
<tr>
<td>7</td>
<td>20-ul, 100-ppm</td>
<td>40-ul, 100-ppm</td>
<td>3.0X10⁻⁵-M</td>
</tr>
<tr>
<td>8</td>
<td>20-ul, 100-ppm</td>
<td>200-ul, 10-ppm</td>
<td>1.4X10⁻⁵-M</td>
</tr>
<tr>
<td>9</td>
<td>20-ul, 100-ppm</td>
<td>160-ul, 10-ppm</td>
<td>1.1X10⁻⁵-M</td>
</tr>
<tr>
<td>10</td>
<td>20-ul, 100-ppm</td>
<td>120-ul, 10-ppm</td>
<td>9.0X10⁻⁶-M</td>
</tr>
<tr>
<td>11</td>
<td>20-ul, 100-ppm</td>
<td>80-ul, 10-ppm</td>
<td>6.0X10⁻⁶-M</td>
</tr>
<tr>
<td>12</td>
<td>20-ul, 100-ppm</td>
<td>40-ul, 10-ppm</td>
<td>3.0X10⁻⁶-M</td>
</tr>
</tbody>
</table>
Table 12: Sample composition of 2.0-ml MT-RL-I

<table>
<thead>
<tr>
<th>Group</th>
<th>CdCl₂</th>
<th>ZnSO₄</th>
<th>Final Zn Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>20-ul, 100-ppm</td>
<td>200-ul, 5-ppm</td>
<td>7.0X10⁻⁶-M</td>
</tr>
<tr>
<td>3</td>
<td>20-ul, 100-ppm</td>
<td>160-ul, 5-ppm</td>
<td>6.0X10⁻⁶-M</td>
</tr>
<tr>
<td>4</td>
<td>20-ul, 100-ppm</td>
<td>120-ul, 5-ppm</td>
<td>4.0X10⁻⁶-M</td>
</tr>
<tr>
<td>5</td>
<td>20-ul, 100-ppm</td>
<td>80-ul, 5-ppm</td>
<td>3.0X10⁻⁶-M</td>
</tr>
<tr>
<td>6</td>
<td>20-ul, 100-ppm</td>
<td>40-ul, 5-ppm</td>
<td>1.5X10⁻⁶-M</td>
</tr>
<tr>
<td>7</td>
<td>20-ul, 100-ppm</td>
<td>60-ul, 1-ppm</td>
<td>4.5X10⁻⁷-M</td>
</tr>
<tr>
<td>8</td>
<td>20-ul, 100-ppm</td>
<td>30-ul, 1-ppm</td>
<td>2.3X10⁻⁷-M</td>
</tr>
</tbody>
</table>

Zinc sulfate was used rather than zinc chloride, because it did not contain cadmium impurity.

[5] Experimental Conditions

The final conditions for the experiment were:

(a) Column: Econosphere C-18, 3-um, 100 X 4.6-mm,
(b) Solvent*: 80:20 of methanol:water with 1% NaCl,
    pH was adjusted to 5.0 with 1% HCl,
(c) Flow Rate: 2.0-ml/min,
(d) TSP Heating: 110-Watt,
(e) Radiation Source: Cd HCL lamp at 228.8-nm,
(f) Flame: Acetylene/air oxidizing,
(g) Recorder: 1.0-cm/min chart rate.

*The reason of using 80:20 of methanol:water as solvent was it resulted in the same chromatographic separation as
pure water solvent. The chromatograms using these two solvents always showed one ionic cadmium peak, which was confirmed as cadmium chloride, with a large tailing followed, which was considered as cadmium compounds in the protein complexes. However, high methanol ratio resulted high absorption signals and the same elution as pure water for the protein alone. Therefore, it was chosen as the solvent for the experiment.

3. RESULTS AND DISCUSSION

A. Qualitative Studies of Zinc and Cadmium Interaction

Separation of the possible cadmium compounds in the metallothionein solutions was not achieved after numerous trials with different reversed-phase columns, different solvent compositions, flow rates, pH values. Only one cadmium peak with large tailing was eluted from each of the columns used with all three proteins, i.e. MT-IB-HK, MT-RL, and MT-RL-I.

Figure 60 illustrates the chromatograms of cadmium compounds, which were in the three proteins, eluted from the column. Comparison of the retention time of the initial peak with that of pure cadmium chloride revealed that the peak was cadmium chloride for all three proteins. The large tailings were believed to be the signals of metallothionein
FIGURE 60. CADMIUM IN THREE DIFFERENT TYPES OF METALLOTHIONEIN.
NOTE ONLY ONE CADMIUM PEAK ELUTED WITH LARGE TAILING
THE SHARP PEAK WAS CONFIRMED AS CADMIUM CHLORIDE.
cadmium complexes that were eluted immediately after the ionic cadmium. This was confirmed, with the addition of 4.0-ml of 0.50-ppm cadmium chloride to 1.0-ml of MT-IB-HK solution, since the height of the initial peak increased; but the peaks in the tailing were still present, as can be seen in Figure 61.

20-ul of 100-ppm zinc sulfate was added into 1.0-ml of MT-IB-HK solution to observe any possible change of cadmium species in the protein, from metallothionein cadmium to ionic cadmium. A small increase of cadmium chloride peak was observed with a slight decrease of the tailing of the metallothionein cadmium, which is shown in Figure 62.

It is believed that the metallothionein has a high affinity for cadmium. However, not all binding sites on the protein may be filled, and the protein may still have capacity to complex more cadmium. Therefore, cadmium was added in vitro to check if this increased the concentration of the cadmium complex.

Figure 63 shows that, a 20-ul of 25-ppm of cadmium chloride was added to 1.0-ml of MT-RL, but the elution patterns were almost the same as that of 4.0-ml of 0.50-ppm addition into 1.0-ml of the protein. There was not much change of the chromatogram by the addition of cadmium chloride to the protein. the tailing still remained, but the peak height of cadmium chloride was increased.
FIGURE 61. 0.5-PPM IONIC CADMIUM ADDITION (1:4, V/V) ONLY INCREASES THE CADMIUM CHLORIDE PEAK, BUT DID NOT CHANGE THE TAILING WHICH WAS BELIEVED TO BE METALLOTHIONEIN Cd PEAKS
FIGURE 62. ADDITION OF 20-µL, 100-PPM ZnSO₄ INCREASED CdCl₂ PEAK AND REDUCED THE PEAKS IN THE LARGE TAILING (A), INDICATING THAT HIGH ZINC CONCENTRATION DOES RELEASE COMPLEXED Cd FROM METALLOTHIONEIN
FIGURE 63. ADDITION OF 20-μL, 25-PPM CdCl₂ TO MT-RL INCREASED THE CdCl₂ PEAK, AND PARTIALLY REDUCED THE PEAKS IN THE TAILING, INDICATING THAT HIGHER IONIC CADMIUM CONCENTRATION ALSO CHANGES CADMIUM FORMS IN PROTEIN.
Next, 20-ul of 100-ppm of cadmium chloride was added to 1.0-ml of MT-RL. This time, some significant change in the chromatogram of the elution was observed. After the sharp cadmium chloride peak was eluted with no tailing, a group consisting of many cadmium compounds was eluted from the column from retention 2.2-min to 4.5-min. Figure 64 shows the chromatograms of two successive injections.

Figure 65 shows the similar chromatogram resulted from the addition of 20-ul of 1,000-ppm of \(3\text{CdSO}_4\cdot8\text{H}_2\text{O}\) to 1.0-ml of MT-RL-I. After two sharp and strong peaks eluted, which were confirmed to be the peaks of cadmium chloride and cadmium sulfate by standard spiking, a group of numerous cadmium compounds eluted from the column in the retention range from 1.8-min to 5.5-min. The tailing, which belonged to the cadmium complex compounds originally bonded on the protein binding sites, was almost disappeared.

These results confirmed:

(a) The addition of inorganic cadmium compounds, in vitro, to the protein formed numerous cadmium compounds on the binding sites of the protein.

(b) These new formed cadmium compounds in the protein complexes were different from the cadmium complex compounds originally bonded on the protein.

(c) The results showed that the binding sites in the proteins were not saturated with the in vivo feeding of the inorganic cadmium to the animals.
FIGURE 64. TWO SUCCESSIVE INJECTIONS OF MT-RL WITH 20-uL OF 100-PPM CdCl₂ SHOWED A GROUP OF Cd PEAKS REPRESENTING NEWLY FORMED Cd COMPOUNDS IN METALLOTHIONEIN COMPLEXES.
FIGURE 65. ADDITION OF 20-uL OF 1000-PPM 3CdSO₄·8H₂O FORMED MORE COMPLEXED CADMIUM COMPOUNDS IN THE PROTEIN, AND RELEASED IONIC CADMIUM TO FORM CdCl₂ AND CdSO₄.
(d) The reverse-phase HPLC method (columns) used in this experiment was able to separate these newly formed cadmium compounds from the inorganic cadmium compounds in the protein.

From the above results, therefore, the peaks of these new cadmium compounds could be used to identify the composition change of cadmium compound in the protein before and after the addition of inorganic zinc compound.

Thereafter, all the protein in the cuvettes were first treated with 20-ul of 100-ppm of cadmium chloride. The mixed proteins were let set aside for more than 24 hours to allow the new cadmium compounds complexation with the protein.

B. Quantitative Studies of Zinc and Cadmium Interaction

In order to study the replacement of cadmium by zinc in the metallothionein complex, different concentrations of zinc sulfate with different additional volumes were added to each 2.0-ml of proteins mixture that had been already pretreated with 20-ul of 100-ppm cadmium chloride. The minimum concentration of inorganic zinc which could initiate the completion of cadmium replacement reaction in the protein was studied.

Figure 66 shows that when 20-ul of 1,000-ppm of zinc sulfate was added into 1.0-ml of MT-RL solution, the zinc completely replaced the cadmium from the binding sites of
FIGURE 66. ADDITION OF 20-μL, 1000-PPM ZnSO₄ RELEASED ALL COMPLEXED CADMIUM IN THE PROTEIN (A) TO FORM TWO INORGANIC CADMIUMS (B: CdCl₂ AND CdSO₄). NOTE THAT THE NEW PEAKS WERE GONE.
the protein. The metallothionein cadmium peaks and the large
tailings all completely disappeared. They were replaced by
two large cadmium peaks representing the cadmium chloride
and the cadmium sulfate. It was concluded that the 1,000-ppm
zinc concentration replaced the cadmium in the protein, and
transformed all the complex cadmium into CdCl₂ and CdSO₄.

Figure 67 and Figure 68 show the results obtained from
the additions of 100-, 10-ppm of zinc sulfates into 1.0-ml
of MT-RL and MT-RL-I with 100-, 80-, 60-, 40-, and 20-ul
volumes. The results indicated that each of those zinc
concentrations was high enough to completely replace the
metallothionein cadmium to form inorganic cadmium compounds.
The metallothionein cadmium peaks and the tailings all
disappeared, and large cadmium chloride and cadmium sulfate
peaks were formed. It was noted that, with decreased zinc
sulfate concentrations, the cadmium sulfate peaks were
decreased.

Figure 69 showed, however, a slightly different
results. The new complex cadmium compounds formed with 20-ul
100-ppm CdCl₂ addition to the protein were not completely
replaced by the addition of 20-ul 5.0-ppm of zinc sulfate.
The retention times of these compounds were shifted towards
the ionic cadmium compound side, i.e. CdCl₂ and CdSO₄, but
still remained. When 40-ul and 60-ul of zinc sulfate added
to the protein, those complexed cadmium compound in the
protein disappeared. These results indicated that, at these
FIGURE 67. ADDITION OF 20-uL, 100-PPM ZnSO₄ RELEASED ALL COMPLEXED CADMIUM IN THE PROTEIN, AND INCREASED THE CADMIUM CHLORIDE PEAK
FIGURE 68. ADDITIONS OF 40-, 80-, 120-, 160-, 200-uL OF 10-PPM ZnSO₄ TO THE PROTEIN WITH 20-uL OF 100-PPM CdCl₂. IT SHOWED THAT ALL THE ZINC ADDITIONS COULD COMPLETELY REPLACE THE COMPLEXED CADMIUM TO RELEASE IONIC Cd.
FIGURE 69. ADDITIONS OF 40-, 80-, 120-μL OF 5.0-PPM ZnSO₄ TO 2.0-ML PROTEIN WITH 20-μL OF 100-PPM CdCl₂. NOTE THAT ADDITION OF 80-μL OF 5.0-PPM ZnSO₄ INITIATED THE REPLACEMENT OF COMPLEXED Cd FROM THE PROTEIN.
zinc concentrations, the complexed cadmium compounds in the protein were somewhat replaced by the zinc, but not completely. In other words, these ionic zinc concentrations were the initiation zinc concentrations of the replacement reaction of cadmium from the protein binding sites. This actual zinc concentration was calculated as $3.0 \times 10^{-6}$-M.

Finally, 20-ul of 1.0-ppm of zinc sulfate was added to 1.0-ml of MT-RL-I. The chromatogram shown in Figure 70 indicates that the cadmium bonded onto the binding sites of the protein was not completely replaced by the zinc ions. The metallothionein cadmium peaks between retention 2.0-min and 5.5-min were still there, but the sizes of the peaks were a little smaller.

When, 40-ul of 1.0-ppm of zinc sulfate was added to 1.0-ml of the protein solution, a similar chromatogram resulted indicating that the quantity of zinc in the solution was still too low to completely replace all the cadmium bonded onto the binding sites of the protein. Those new metallothionein cadmium peaks still appeared in the chromatogram.

In these chromatograms, minor increase in cadmium chloride peak was observed. This indicated that the complexed cadmium compounds were slightly replaced by the additions of 1.0-ppm zinc ions. In other words, at these ionic zinc concentrations, the zinc replacing cadmium from the protein complexes had already started. Although the
FIGURE 70. ADDITION OF 30-, 60-uL OF 1.0-PPM ZnSO₄ TO 2.0-ML OF PROTEIN WITH 20-uL OF 100-PPM CdCl₂. SOME Cd WAS RELEASED AS INDICATED BY THE INCREASE IN THE Cd ION PEAKS IN B AND C COMPARED TO PEAK A.
cadmium compounds in the complexed forms were still remained, the increase of the ionic cadmium peaks revealed the initiation of the replacement reaction.

C. Calculation of Zinc Concentration Initiating Cadmium Replacement in Metallothionein Complex

It was concluded that, from the above results, the concentration range of zinc ion necessary to initiate the replacement of complexed cadmium bonded onto the binding sites of the proteins was 30-ul of 1.0-ppm added into 2.0-ml of protein solution. This result was equivalent to the stoichiometric concentrations of the zinc ions in a range of $2.3 \times 10^{-7}$-M, which was added to the 2.0-ml of protein MT-RL-I.

Further, the calculation of the results showed that the ionic zinc concentration which completely replaced the cadmium bonded onto the binding sites of the metallothionein molecules was $6.0 \times 10^{-6}$-M, which was equivalent to addition of 80-ul of 10-ppm zinc sulfate to 2.0-ml of protein with 20-ul of 100-ppm of cadmium chloride added.

The concentration composition of the ionic zinc and cadmium, molecular zinc and cadmium originally in the protein, and the concentration of the protein in the final mixture, i.e. 2.0-ml of protein added with 20-ul of 100-ppm of CdCl$_2$, 80-ul of 10-ppm ZnSO$_4$, and with the originally
contained zinc and cadmium concentration in the protein, are listed in Table 13 below:

Table 13: Composition of Zinc, Cadmium, and Protein

<table>
<thead>
<tr>
<th>Compds.</th>
<th>MWt (g/L)</th>
<th>Ions Conc.</th>
<th>Molecular Conc.</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name</td>
<td>g/L</td>
<td>M</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>MT-RL-I</td>
<td>6,800</td>
<td>0</td>
<td>6.0x10^-6</td>
<td>6.0x10^-6</td>
</tr>
<tr>
<td>Zn</td>
<td>65.38</td>
<td>6.0x10^-6</td>
<td>5.9x10^-5</td>
<td>5.5x10^-5</td>
</tr>
<tr>
<td>Cd</td>
<td>112.41</td>
<td>9.0x10^-6</td>
<td>2.0x10^-5</td>
<td>2.1x10^-5</td>
</tr>
</tbody>
</table>

These data show that the final ratio of ionic zinc and cadmium concentrations is about 5:2.

These calculations are based on the cadmium and zinc concentration data provided by the manufacturer of the protein (Sigma) which are listed in the metallothionein sample list in Section B, and the actual additions of ionic zinc concentrations listed in Table 12. The molecular weight of the protein is based on the data in Reference 106, which is around 6,800-g per mole.

Recalling the measurement of cadmium in MT-RL-I using our system, however, showed an ionic cadmium absorption peak about 0.350 absorbance in Figure 68. With that operating conditions, i.e. at flow rate of 2.0-ml/min and 100-W TSP energy input with 0.02" ID capillary and 75-um orifice, this result was equal to the absorbance of 0.50-ppm of cadmium detected. This is equivalent to 3.0x10^-4-moles of cadmium originally in the protein, which was two orders of magnitude
higher than that which the manufacturer suggested. Based upon this datum, the concentration of the total cadmium in the mixture should also be two orders of magnitude higher.

If ignoring the zinc and cadmium contents originally contained in the protein samples, calculation also showed that, the mole ratio of Zn:Cd:metallothionein at which the replacement reaction started was 1:100:1,000 in those zinc and cadmium additions. This ratio was equivalent to the stoichiometric concentrations of the three species, i.e. zinc:cadmium:metallothionein, as 0.015:1.0:40 ppm in the final sample with 30-μl of 1.0-ppm zinc added to 2.0-ml of protein with 20-μl of 100-ppm cadmium chloride.

5. CONCLUSIONS

There are several conclusions which can be drawn from these preliminary studies of the interactions between inorganic zinc ions and metallothionein cadmium:

A. The experimental results confirmed that the uptake of inorganic zinc compounds into the human body does cause metallothionein bonded cadmium to be released from the binding sites of the protein, and form ionic cadmium compounds. If this occurs inside the human body, it could have toxic effects on human health.
B. The mole ratio at which ionic zinc initiate the replacement of cadmium from the protein complexes was about 1.0:100:1,000 of Zn:Cd:metallothionein. The actual concentrations studied at that stage were 0.015:1.0:40 ppm of Zn:Cd:metallothionein, respectively.

C. Zinc and cadmium compete for the binding sites in the metallothionein complexes.

D. The reaction of zinc replacing cadmium in the protein complexes increases with the zinc concentration increase.

E. The binding sites in metallothionein complexes were not saturated with the in vivo cadmium or zinc feeding to the experimental animals. In vitro addition of cadmium or zinc ionic compounds still could form some new metal compounds in the protein complexes.

F. Figure 49 reveals that the cadmium compounds in human urine were mostly complexed with metallothionein. The similarity of this chromatogram of cadmium compared with that of in Figure 65 supports this conclusion.

G. This is a preliminary study of the interaction between cadmium and zinc compounds in the protein. Further
investigations are needed to complete this research on the inter-relationship of these metals in the metallothionein, in order to finally conclude the mechanism of the effect of inorganic zinc on human health.


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VITA

Peter Ping Chang was born August 5, 1951 in Shanghai, People's Republic of China. He graduated with honors from the second elementary school of Huai-hai-zhong Road in June, 1964, Shanghai, China.

He entered Xu-huei secondary school in August, 1964, but he could not finish his secondary school education due to the Cultural Revolution broken in May, 1966.

He was sent to remote countryside in 1970, and worked for next eight years in the field without getting paid.

In 1977, he self-studied all the secondary and high school courses to prepare the National Entrance Examination. He passed the exam and was accepted by the Shanghai University of Science and Technology in February, 1978.

He graduated in February, 1982 with a Bachelor of Engineering of Materials Science. He was then assigned a job working in the Shanghai Refractory Factory for two and a half years as an assistant engineer.

He self-studied English from February, 1982 to August, 1984 before he came to the States. He entered graduate school at Louisiana State University in the fall of 1984 to pursue the Ph. D. degree.

Having completed a major in analytical chemistry and a minor in environmental studies, he is currently a graduate assistant in the Department of Chemistry and a candidate for the degree of Doctor of Philosophy.
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Candidate: Peter Ping Chang

Major Field: Chemistry


Approved:

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Major Professor and Chairman

[Signature]
Dean of the Graduate School

EXAMINING COMMITTEE:

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Date of Examination:

July 11, 1991