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Determination of Metals at the Microgram-per-Liter Level in Blood Serum by Simultaneous Multielement Atomic Absorption Spectrometry with Graphite Furnace Atomization

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A method is described for the simultaneous determination of seven metals—Al, Co, Cr, Mn, Mo, Ni, and V—in blood serum using a simultaneous, multielement atomic absorption spectrometer (SIMAAC) and graphite furnace atomization. Our original intention was to determine two additional metals—Pb and Sn—however, the high ashing temperature needed resulted in premature loss of these volatile metals. Serum samples (2 mL) are dry-ashed with 20 μL of $Mg(NO_3)_2 \cdot 6H_2O$ (20 g/L) as an ashing aid/matrix modifier. The organic-free residue is reconstituted with 0.5 mL of nitric acid (50 mL/L), allowing for a fourfold concentration step. The addition of $Mg(NO_3)_2$ permits a graphite furnace ashing temperature of 1300 °C. Atomization from a L'vov platform with a fast heating rate and the use of peak area measurements permit calibration with acidified aqueous standards. A well-characterized bovine serum pool is used to establish the accuracy and precision of the method. Serum samples from 30 fasting adult volunteers, drawn under controlled conditions, are analyzed to assess the efficacy of this method to determine these seven metals over the ranges normally encountered in human sera.

The SIMAAC system has been used successfully to determine Na, K, Ca, Mg, Cu, Fe, and Zn in 0.5 mL of blood serum using flame atomization (*1*) and Cu, Fe, and Zn in 25 μL of blood serum using graphite furnace atomization (*2*). However, the nine metals under consideration at microgram-per-liter levels in serum—Al, Co, Cr, Mn, Mo, Ni, Pb, Sn, and V—are present at concentrations close to the detection limits of line-source graphite furnace atomic absorption spectrometry (GFAAS). Detection limits for the SIMAAC system are generally similar to line-source AAS above 280 nm but poorer below 280 nm. Direct analysis of samples with complex matrices with high levels of both organic and inorganic constituents, such as serum, for metals close to detection limits is difficult even for single element determination. A variety of techniques has been used to solve the problems arising from such complex matrices (*3*).

Physical and chemical interferences arising from the serum matrix can be solved by dilution and the use of wetting agents (*2*). However, this is not a feasible approach for metals present at very low concentrations. In fact, a concentration step is indicated. Some form of sample pretreatment is therefore necessary. Wet ashing, dry ashing, or a combination approach has been used. Any form of sample pretreatment increases the risk of contamination for the metals of interest. Therefore, for trace metal analysis, a minimum amount of sample handling and pretreatment is preferable.

If a concentration step is used, not only are the analytes of interest concentrated but so are the matrix constituents. Organic constituents can be removed by wet or dry ashing. High chloride matrices, such as serum, are especially troublesome in GFAAS, and many chloride interferences have been documented (*4*). A L'vov platform, a fast heating rate, and peak area measurements are partial remedies to use. However, matrix modification is also necessary. A variety of matrix modifiers have been used in the analysis of high chloride matrices such as seawater and serum. Organic acids (*5*), NH_4NO_3 with a low-temperature ashing step in the graphite furnace (*6*), and $Mg(NO_3)_2$ with a high-temperature ashing step in the graphite furnace (*7*) have been utilized. A low-temperature ashing step is indicated for the metals under consideration as Pb and Sn are quite volatile.

There are additional problems in multielement as opposed to single-element work. A set of compromise furnace conditions must be established (*8*). *But*, this does have an advantage of normalizing conditions for multielement work, whereas conditions for the single-element analysis of serum are generally quite specialized.

A problem common to all researchers concerned with the determination of trace metals in serum is the absence of suitable reference materials. Very few established methods report confirmation of the accuracy of the method by the use of reference materials or report the routine use of quality control materials.

A specially collected, well-characterized bovine serum pool (*9*) was used to develop this method and was also used as a quality control material and to assess the precision of this method. Accuracy of this method was confirmed by comparison to values obtained on this bovine serum pool by other researchers. This was an integral part of the project initiated by Veillon of the USDA to characterize this bovine serum pool for use as a reference material for the determination of trace metals in serum.

Serum samples, drawn under contamination-controlled conditions from 30 "normal" fasting adult volunteers, were analyzed by this method to show the applicability for analysis of human serum as well as bovine serum. It should be noted that these volunteers were not chosen statistically. The primary objective of this research was method development and not the establishment of reference ranges.

EXPERIMENTAL SECTION

Instrumentation. The SIMAAC system (*10*) is a prototype instrument not currently available commercially. It consists of a continuum source, an atomizer (either flame or furnace), a 20-channel Echelle polychromator which produces a two-dimensional spectra array, a quartz refractor plate for wavelength modulation, and a dedicated minicomputer. Wavelength mod-

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Table I. Elements of Interest and Corresponding Wavelengths Used for the Analysis of Serum Using SIMAAC

channel	element	wavelength, nm
1	Mn	279.48
5	Pb	283.31
6	Cr	357.87
7	Ni	352.45
8	V	318.54
9	Co	240.73
10	Sn	286.32
11	Mo	313.26
12	Al	396.15

Table II. Furnace Conditions[a]

	ramp, s	hold, s	temp, °C
dry	30	10	200
ash	10	40	1300
cool down	1	14	20
atomize	0	10	2700[b]
clean out	1	5	2700
cool down	1	14	20

[a] L'vov platform and pyrolytically coated graphite tube. [b] Internal flow = 20 mL/min (argon).

ulation provides for double-beam performance with background correction at all wavelengths. The wavelengths used (*11*) for the elements of interest are shown in Table I. A sample volume of 20 μL of the reconstituted fourfold concentration of ashed serum was used for each determination. This was delivered by using an AS1 autosampler (Perkin-Elmer, Norwalk, CT) from an acid-washed, autoanalyzer cup (Elkay Products, Shrewsbury, MA). Nitric acid (50 mL/L) was used in the rinse cycle to reduce carry-over contamination. A Perkin-Elmer HGA 500 graphite furnace, with a pyrolytically coated graphite tube, and a L'vov platform made from the same material (*12*) were employed. The furnace program is shown in Table II. Argon was used as a sweep gas. The atomization time was 10 s. Data for Al, Cr, Mo, and V were taken for the entire atomization time; data were taken over integration intervals of the first 4.65 s for Co, 6.4 s for Mn, and 7.1 s for Ni. These integration intervals were established from absorbance-time tracings of a 100 μg/L standard.

Apparatus. A corrosion-resistant freeze dryer with no exposed metal parts (FTS Systems, Inc., Stone Ridge, NY) and a muffle furnace (Lindberg, Watertown, WI), again with no exposed metal parts, were used for the lyophilization and dry ashing, respectively, of the serum samples.

Reagents and Supplies. Single-element standards, containing 1000 mg/L each of the metals of interest (Fisher Scientific, Silver Spring, MD), were combined to give a 10 mg/L mixed stock standard in nitric acid (50 mL/L) (Ultrex, J. T. Baker Chemical Co., Phillipsburg, NJ). A 1 mg/L mixed stock standard in nitric acid (Ultrex, 50 mL/L) was prepared fresh daily and used to make working standards of 1, 5, 10, 25, 50, and 100 μg/L, again in Ultrex nitric acid (50 mL/L). These working standards also contained 10 μL/mL of a $Mg(NO_3)_2 \cdot 6H_2O$ solution (20 g/L) (Johnson Mathey Chemicals, Ltd., obtained from Alfa Chemicals, Danvers, MA). Acetic acid and ammonium hydroxide were prepared by isothermal distillation (*13*). NH_4NO_3 was prepared by combining nitric acid (Ultrex) and the isothermally distilled NH_4OH. Eighteen megohm water (Millipore Corp., Bedford, MA) was used throughout. Quartz tubing, 10 mm i.d. × 100 mm (Vitrosil, Thermal American Fused Quartz Co., Montville, NJ) was rigorously cleaned (*14*) and then made into test tubes (J. Trembly, Glass Shop, University of Maryland, College Park, MD). Before use, these test tubes were cleaned by boiling with 5% nitric acid, soaking overnight in 5% HNO_3, and rinsing 10 times with 18-MΩ water. Dry "clean" tubes were silanized using dichlorodimethylsilane (50 mL/L) (Pierce Chemical Co., Rockford, IL) in toluene (Fisher Scientific) followed by a methanol rinse (Fisher Scientific) and then rinsing 10 times with 18-MΩ water. Blood was collected from 30 fasting adult volunteers by using minicatheters which have a very short (1.5 cm) siliconized needle attached to small bore poly(vinyl chloride) tubing (Minicath, Deseret Medical, Inc., Sandy, UT) and sterile plastic syringes (Sarstedt, Princeton, NJ), which permit centrifugation in the barrel of the syringe. Centrifugation and separation of the serum samples were carried out under Class 100 filtration units. Samples were aliquoted into polypropylene tubes (Falcon, Fisher Scientific), tightly capped, and stored at −20 °C until analysis.

Table III. Muffle Furnace Program

temp, °C	time, h	temp, °C	time, h
100	1	250	1
150	1	480	(overnight)
200	1		

Procedure. Two milliliters of serum specimens and controls is pipetted into the silanized quartz test tubes. Twenty microliters of the magnesium nitrate solution is added, and the contents are gently mixed on a vortex mixer. The addition of the $Mg(NO_3)_2$ at this stage acts as an ashing aid, and the silanizing helps prevent the serum from adhering to the test-tube walls during ashing. The specimens are then frozen in a −20 °C freezer and then placed overnight in the freeze dryer. The freeze-dried samples are then ashed in the muffle furnace program according to the program shown in Table III. Although this sample preparation procedure is lengthy (2 days), it is not very labor intensive. The ashed sera are then dissolved in 0.5 mL of Ultrex nitric acid (50 mL/L) immediately prior to analysis. Sample blanks are processed in the same manner. Samples, controls, and appropriate blanks are analyzed in triplicate against aqueous, acidified standards with added $Mg(NO_3)_2$. All work was carried out under Class 100 air filtration units or in a Class 100 clean room.

RESULTS AND DISCUSSION

Matrix Effects. Initially, it was hoped that all nine of the metals under consideration at the microgram-per-liter level in serum could be simultaneously determined, by use of the appropriate furnace conditions, sample pretreatment to remove organics, and matrix modification. Several matrix modifiers were tried.

The most readily available organic acid (*5*) that was sufficiently contamination-free was isothermally distilled acetic acid. It was found, however, that over the period of time between sample and standard preparation and analysis, the absorbance signal for Mo, Sn, and V decreased, suggesting a precipitation of these metals. It was also found—by observation of the absorbance profile on the SIMAAC system's oscilloscope—that the background, due mostly to the molecular absorption of sodium chloride, was not decreased by the use of acetic acid.

Ediger (*6*) proposed the use of a large excess of ammonium nitrate as a matrix modifier for trace metals in seawater, and we had successfully used ammonium nitrate for our furnace dilution method for Fe, Cu, and Zn. The basis of its use is that the sodium chloride matrix would be eliminated as NH_4Cl at 335 °C and as $NaNO_3$ at 380 °C. We found that when NH_4NO_3 was used with the reconstituted ashed serum concentrate, a great deal of smoke emerged at these temperatures. It was also impossible to dry the sample containing this high concentration of NH_4NO_3 on a L'vov platform, without splattering on to the walls of the graphite tube. This had the effect of producing two analyte peaks—one atomized from the wall and one atomized from the platform. Even the use of a very slow drying step (up to 6 min) did not allow for smooth drying.

We decided to use magnesium nitrate as a matrix modifier/ashing aid/bulking agent and to ash at a graphite furnace temperature sufficiently high to volatilize the inorganic serum matrix and reduce the background absorbance and chloride interferences. Plots of peak area (absorbance-s) vs. ashing temperature of 100 μg/L standards containing $Mg(NO_3)_2$ show

Table IV. Run-to-Run Blank Values (μg/L)

metal	low	high	metal	low	high
Al	0.8	14.2	Mo	2.9	13.5
Co	<0.6	2.9	Ni	<0.4	1.2
Cr	0.20	1.22	V	<0.7	3.2
Mn	<0.1	0.9			

Table V. Comparison of Method Limits[a] with Published Ranges[b] (μg/L)

metal	detection limit	mean method limit	published range
Al	0.80 ± 0.36	0.20	2.1–6.2
Co	2.31 ± 0.57	0.57	0.04–0.30
Cr	0.37 ± 0.25	0.09	0.04–0.40
Mn	0.43 ± 0.16	0.11	0.38–1.04
Mo	1.85 ± 0.66	0.47	0.29–1.70
Ni	1.66 ± 0.71	0.42	0.8–5.2
V	2.72 ± 1.20	0.68	0.04–0.90

[a] Detection limit divided by four. [b] Reference 3.

Table VI. Analysis of BS 7292 (Bovine Serum Pool) Using SIMAAC

metal	RSD % within run[a]	RSD % day to day[b]	mean μg/L
Al	10	24	12
Co	29	31	1.2[c]
Cr	23	32	0.34
Mn	13	17	2.2
Mo	16	23	12
Ni	15	18	2.1
V	30	50	1.4[c]

[a] $n = 8$. [b] $n = 10$. [c] Semiquantitative value.

that at an 800 °C ashing temperature there is no premature loss of analyte signal for any of the metals under consideration. However, at 800 °C the background absorbance was significant and poor signal-to-noise ratios were obtained. At an ashing temperature of 1300 °C, however, the background signal was very much reduced, but the absorbance signals for Pb and Sn showed a decrease due to the premature loss of these analytes. It was, therefore, decided to determine only Al, Co, Cr, Mn, Mo, Ni, and V.

Blanks. Blanks were found to vary from run to run by different amounts according to the analyte (Table IV). Aluminum proved to be particularly troublesome. Sporadically, erroneously high blanks were observed that far exceeded the highest standard (100 μg/L) and could be estimated at 600–1000 μg/L, whereas the samples were being measured at about 10–20 μg/L. To date no satisfactory explanation has been reached to explain this phenomenon. Coated Al is the only metal present in our clean rooms, and unfortunately the high efficiency particulate (HEPA) filters have Al separators (this will hopefully be rectified at a later date). This very high Al contamination was never encountered in the control serum or samples, only in the reagent blanks. Within-run blanks were very consistent, varying by less than 6% from each other.

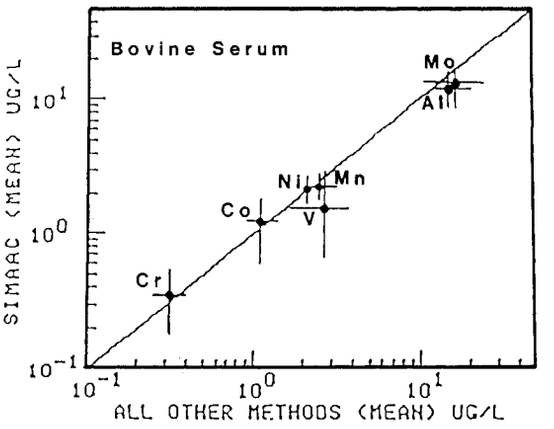

Figure 1. Correlation plot comparing mean values and ranges obtained by using SIMAAC to mean values and ranges of all other methods for Al, Co, Cr, Mn, Mo, Ni, and V in a bovine serum pool.

Detection Limits. The detection limits and method limits for 12 experiments are shown in Table V. The detection limit for the SIMAAC system is defined as three times the standard deviation of the base-line signal. The method limit is defined as the mean detection limit divided by four (because of the fourfold concentration factor). It can be seen that "normal" levels in serum of Al, Cr, Mn, and Ni should be detectable; however, Co, Mo, and V will be only just detectable.

Instrument Stability. One of the very real problems with the present SIMAAC system when used with graphite furnace atomization for the determination of very low level trace metals was long-term wavelength and order drift when the room temperature varied. This problem was partially rectified by repeatedly running base-line measurements throughout the experiment to compensate for this drift.

Precision Studies. The means, within-run, and day-to-day percent RSD's for the bovine serum pool are shown in Table VI. The day-to-day percent RSD for Al is 2.5 times that of the within-run precision, probably due to the wide blank variation. The values for Co and V were all semiquantitative, i.e., they fall between the detection limit (3σ) and our laboratory's defined quantitation limit (15σ) for these metals.

Accuracy. The mean values obtained by using SIMAAC were plotted against the values obtained by all other methods (Figure 1). Regression statistics were $y = 0.08 + 0.80x$, $r = 0.994$, SE = 0.36. One laboratory's results were consistently high for the metals (Mo and V) that they determined; however, these values could not be statistically discarded (Chauvenet's criterion). All values obtained by SIMAAC and other methods

Table VII. Comparison of Values (μg/L) Obtained by SIMAAC with Other Methods for a Bovine Serum Pool

method	Al 12 ± 3	Co 1.2 ± 0.4[a]	Cr 0.34 ± 0.11	Mn 2.2 ± 0.4	Mo 12 ± 3	Ni 2.1 ± 0.4	V 1.4 ± 0.7[a]
SIMAAC, furnace							
ICP-AES	12			2.5	7.6	<4.0	1.2[a]
					20		
ICP-AFS				2.4			
GFAAS	20	0.9	0.28	2.9	25	2.2	4.0
	9.9		0.2	2.5	17.6		
				3.3			
IDMS			0.27				
NAA		1.3	0.33	2.5	15		

[a] Semiquantitative.

Table VIII. Comparison of Values Obtained by Using SIMAAC with Published Values

	SIMAAC			published
	mean[c]	std dev[c]	95% range[c]	range[a]
Al, μg/L	3.4	+2.9 −1.6	0.9–12.3	2.1–6.2
Cr, μg/L	0.24	+0.21 −0.12	0.07–0.89	0.04–0.4
Mn, μg/L	0.48	+0.22 −0.19	0.15–1.49	0.38–1.04
Ni, μg/L	1.7	+1.3 −0.7	0.50–6.0	0.80–5.2
Co, μg/L	0.28[b] 0.27[c]			0.04–0.30
Mo, μg/L	0.80[b] 0.58[c]			0.28–1.17
V, μg/L	0.70[b] 0.52[c]			0.04–0.9

[a] Reference 3. [b] Gaussian. [c] Logarithmic.

are shown in Table VII. It should be noted that the Mo concentration of the bovine serum pool is an order of magnitude higher than the expected levels in human serum (*3*).

Analysis of Adult Human Serum Samples. Frequency distribution histograms of the results obtained on the 30 adult human sera were plotted for all of the metals using normal and log-normal concentrations. For Al, Cr, Mn, and Ni, it was found that a log-normal distribution best fit these data. The means and 95% ranges calculated by using a log-normal distribution are shown in Table VIII compared to the most accepted published ranges.

Goodness-of-fit of the frequency distribution histograms was tested using a method recommended by Petitclerc (*15*), in which test statistics utilizing the coefficients of skewness and kurtosis and their standard deviations are used. This is a two-tailed test at a 0.01 significance level. This method is very convenient to use, and the type of deviation from Gaussian behavior can be predicted by the sign of these coefficients. Some indicators of a log-normal distribution are that (1) the standard deviation is large when compared to the mean (calculated by using Gaussian statistics), (2) the frequency distribution histogram is positively skewed, and (3) the calculation of a 95 percentile reference range (the most commonly used percentile for reference ranges in clinical chemistry) gives a negative lower limit. log-normal distributions are not uncommon in environmental and food samples. log-normal distribution of many analytes in serum has been shown (*15*) especially in a pediatric population (*16*).

Co, Mo, and V were at best semiquantitatively determined for these 30 samples. In fact some of the values fell below the method limits for the SIMAAC system for these metals, resulting in concentrations that were zero or negative—both of which are physiologically impossible. Obviously, this is an artifact introduced because of the large relative standard deviation for these metals at very low levels. Distributions of these data were ambiguous, so standard deviations and ranges are not given; however the means—calculated using *all* data points—are shown in Table VIII. The logarithmic means were calculated using grouped data and the zero and negative values placed in the lowest distribution interval—again introducing an artifact.

CONCLUSION

The SIMAAC system with graphite furnace atomization can determine Al, Cr, Mn, and Ni in blood serum at about 20–30% RSD. Co, Mo, and V cannot be successfully determined at the normal levels found in human serum. An improvement of detection limits twofold for Mo and tenfold for Co and V is required.

Although this method was developed specifically for the SIMAAC system, the sample preparation and analytical conditions should be applicable to single-element AAS determinations.

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Registry No. Al, 7429-90-5; Co, 7440-48-4; Cr, 7440-47-3; Mn, 7439-96-5; Mo, 7439-98-7; Ni, 7440-02-0; V, 7440-62-2; $Mg(NO_3)_2$, 10377-60-3.

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