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In Vivo Sample Uptake and On-line Measurements of Cobalt in Whole Blood by Microwave-assisted Mineralization and Flow Injection Electrothermal Atomic Absorption Spectrometry

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An on-line automated microwave-assisted mineralization and flow injection system was developed for the determination of cobalt in whole blood with *in vivo* sample uptake by electrothermal atomic absorption spectrometry. The samples were drawn and at the same time pumped directly from the vein of a patient's forearm to a timed injector, which is automatically controlled to inject the sample–nitric acid–anticoagulant mixture into the carrier stream. Volumes (20 µl) of the mineralized samples, collected in a capillary of a sampling arm assembly, were introduced by means of positive displacement with air through a time-based solenoid injector (TBSI) into the graphite tube atomizer. The spectrometer autosampler, used for the introduction of 10 µl of the chemical modifier, magnesium nitrate, and the furnace programme were re-programmed to synchronize with the operation of the flow system. The linear range was from 0 to 50 µg l^{-1} of cobalt, achieving a precision of 2.6 and 3.1% for 10 replicate analyses of a 5 µg l^{-1} cobalt standard solution and a certified sample, respectively, with a detection limit of 0.3 µg l^{-1}. The agreement between the observed and certified values obtained from Seronorm Whole Blood Standard Reference Materials was good. The results obtained by using an in-batch procedure were found to linearly increase by 5% during the time interval between sample collection and analysis for the first hour; after this time the results for cobalt levelled off and were then in close agreement with those obtained by the on-line procedure proposed here.

Keywords: *Cobalt; whole blood; on-line mineralization; microwave-oven mineralization; flow injection; electrothermal atomic absorption spectrometry*

Interest in metals as essential elements for normal biological development or as potential sources of danger to health has resulted in many reports dealing with methods for their determination in biological materials. Cobalt is a vital trace element that is required in the normal diet of man in the form of cyanocobalamin complex (vitamin B_{12}) and in animal nutrition, specially for ruminants, where it is utilized for synthesis of vitamin B_{12} by rumen bacteria.[1] The concentration of cobalt in whole blood is low, usually at part-per-billion levels,[2] and its determination in this matrix is, therefore, difficult. Results differing by several orders of magnitude almost certainly reflect methodological errors rather than true variations of environmental origin. For this reason, diagnosis of cobalt deficiency is usually made indirectly by determination of vitamin B_{12}[3] or methylmalonic acid[4] levels. However, the measurement of the concentration of cobalt in whole blood may provide valuable clinical information in certain circumstances of cobalt therapy[5] and for monitoring persons who are occupationally exposed to this element.[6]

Occupational exposure to cobalt can occur by inhalation in industries that use or manufacture super alloys, hard-facing alloy or cemented and tungsten carbides.[7–9] Owing to the low levels of cobalt involved, its determination by colorimetric methods requires a preconcentration step that also increases the risk of interferences and of sample contamination. Electrothermal atomic absorption spectrometry (ETAAS) offers the required sensitivity, allows direct analysis of the digested sample, avoids the preconcentration step, saves time and reduces contamination risks or loss of analyte. Spectral, physical and chemical interferences can be eliminated or minimized by use of background correction systems, chemical modifiers and a L'vov platform.

Since Burguera *et al.*[10] and Fang *et al.*[11] first described the applications of flow injection (FI) systems for on-line microwave digestions and preconcentration in connection with conventional flame atomic absorption spectrometry (FAAS) and ETAAS, respectively, attempts have been made to provide truly on-line systems.[12–16] The development of time-based injectors,[17,18] which are readily suitable for automated routine operations with a significant reduction of sample and reagent consumption, offered new possibilities in the development of on-line systems. Recently, a time-based injector has offered the possibility of developing *in vivo* sample uptake and on-line measurements of zinc and copper in whole blood by microwave-assisted mineralization and FI-FAAS.[19] In the work presented here, an attempt is made to develop a similar system, but coupled with ETAAS, for the determination of cobalt in whole blood, which exhibits all the basic advantages of an on-line procedure and, additionally, without exposure of the sample to the environment.

EXPERIMENTAL

The FI-ETAAS system is shown schematically in Fig. 1. The instrumentation, tubing (l_1–l_7) and reagents were as previously described,[19,20] unless otherwise stated. The light source was a 10 mA conventional cobalt hollow cathode lamp and a deuterium-arc background corrector was used throughout. The wavelength used was 324.8 nm with a spectral bandpass of 0.7 nm. The carrier stream was of 0.01 mol l^{-1} nitric acid. A 1000 mg l^{-1} stock standard solution of cobalt was prepared by dissolving 4.036 g of cobalt chloride ($CoCl_2 \cdot 6H_2O$, Analar reagent grade from Merck) in a minimum volume of nitric acid (1 + 1) and diluting to 100 ml to give a 0.5 mol l^{-1} nitric acid and 3% by volume of glycerol final concentration solution. Working cobalt standards were freshly prepared as required

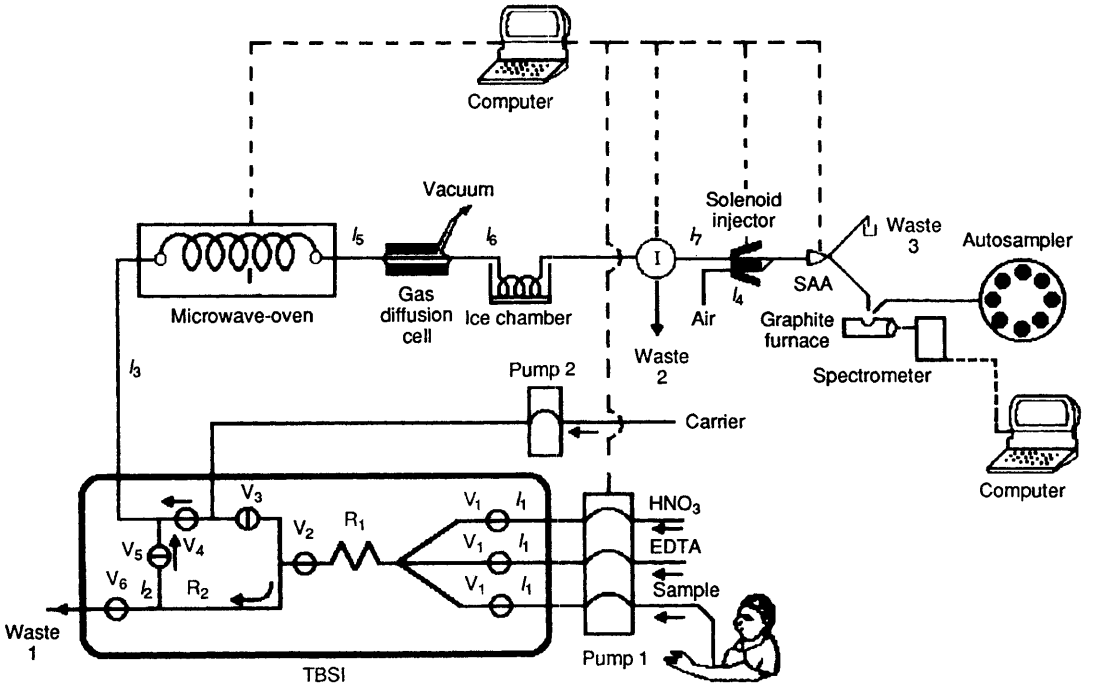

Fig. 1 Schematic diagram of the FI–MWO–ETAAS manifold: l_1–l_7, tubing length; V_1–V_4, valves of the time-based solenoid injector; R_1, mixing tube; R_2, sample/reagent entrapment tubing; I, valve injector; SAA, sampling arm assembly. For further details and operating procedure, see text

by suitable dilution of the stock standard with 1% v/v nitric acid. All glassware was soaked for 1 h in 20% nitric acid and then rinsed with water before use. The 5 m of 0.8 mm i.d. poly(tetrafluoroethylene) (PTFE) tubing inside the oven was wrapped around a 3 cm glass holder.

For 'in batch' measurements, 10 ml of blood were drawn into plastic syringes. Each syringe was first washed with 10% v/v nitric acid and rinsed with water to ensure that it was 'metal free'. Then, 5 ml of the blood sample were diluted with 5 ml of 0.01% v/v Triton X-100 and partially digested with 5 ml of 0.2% v/v of nitric acid. This mixture was subjected to ultrasonic shaking for a period of 10 min before analysis by ETAAS using the same procedure described elsewhere.[21] Sample (20 µl) and $Mg(NO_3)_2$ chemical modifier solution (10 µl) were dispensed onto the platform using the programme recorded in Table 1.

For the present work, the time-based solenoid injector (TBSI)[17] (Fig. 1) has two sets of tubing (set 1: V_1, V_2, V_4 and V_6; set 2: V_3 and V_5), which are used for regulating the introduction and mixing of sample and reagents, and to carry this mixture downstream through the FI system. The duration and function of each sequence of the FI-microwave oven (MWO) programme are described as follows.

In sequence 1, pump 1 was activated, the tubing, set 1, was opened and the tubing, set 2, was closed to allow the carrier solution to flow to waste 2 through valve I, and to mix sample, ethylenediamine tetraacetic acid (EDTA) and nitric acid solutions inside R_1 and to fill R_2. The duration of this sequence was 60 s.

In sequence 2, as the solenoid valve of the TBSI is switched to the alternate position (injection cycle), the carrier solution is diverted to displace the sample/reagent mixture from R_2 toward the MWO, which was turned on. After the passage of the sample/reagent mixture through the coiled PTFE tubing located inside the MWO (l_4), the power of the oven was turned off. Thereafter, the sample plug passed through the coil, immersed in the ice trap. The earlier section of the mineralized sample–reagent mixture plug went to waste 2 through valve I, which was turned to divert the flowing stream to fill the tubing of the solenoid injector l_4 and of the sampling arm assembly (SAA) in the rest position. When this tubing was filled with the intermediate section of the sample–reagent plug, it was

Table 1 Recommended tube atomizer programme settings using in-batch and on-line measurements; integrated absorbance was measured with deuterium background correction

		Assay step				
Mode of measurement	Parameter	Dry	Pyrolysis	Atomize	Cool	Clean
In-batch	Temperature/°C	160	1400	2400	20	2800
	Ramp/s	10	5	0	0	2
	Hold/s	20	10	5	20	2
	Internal gas flow rate/ml min^{-1}	300	300	0	300	300
	Integration tme = 4 s					
On-line	Temperature/°C	90/150	500/1400	2500	20	2600
	Ramp/s	5/10	40/20	0	2	1
	Hold/s	10/20	10/20	5	20	2
	Internal gas flow rate/ml min^{-1}	300	300	0	300	300
	Integration time = 5 s					

turned back to its initial position to discard the later section of this plug. The duration of this sequence was 80 s.

In sequence 3, the synchronous activation of the solenoid injector allowed the sequential deposition of 20 μl of mineralized sample onto the graphite tube platform by means of the SAA, alternatively operated in the waste 3 and injection mode. This sequence was timed to synchronize with the spectrometer computer, which had been pre-programmed to introduce the chemical modifier to each aliquot of the sample and to run the furnace temperature (Table 1). The duration of this sequence was 200 s per measurement.

In sequence 4, the washing procedure was performed. The tubing sets were activated as specified in sequence 1. In this way, while the carrier solution flows to waste 2 through I, a solution of nitric and hydrochloric acids (0.5 mol l^{-1} each) was pumped through the sampling tube to waste 1 and then the sequences 2 and 3 were followed. This operation was repeated three times between the analysis of different samples.

A calibration graph was obtained by the introduction of working standard solutions through the sampling tube and following the sequences 1–4.

RESULTS AND DISCUSSION

Preliminary Experiments

When blood and acid (nitric, sulfuric, hydrochloric or perchloric) were introduced through two independent channels, continual obstruction resulted inside the tubing due to the formation of blood clots, which resulted from blood deproteinization. Therefore, the procedure for analysis involved the use of a third channel with the anticoagulant EDTA. The introduction of EDTA satisfactorily prevented blood coagulation because, unlike water, it causes complete lysis of the red cells and minimizes frothing with an improvement of the sample plug flowing capacity.

Standard Solution

An investigation of the effect of viscosity on the absorbance signals was carried out by adding different proportions of glycerol to the aqueous standard solutions to match the viscosity of the blood samples, because the sample uptake rate changes with viscosity. This effect is specially observed for glycerol : water ratios of $<3:97$ and $>10:90$, by volume. With a glycerol : water ratio of 5 : 95, there was no apparent carbonaceous residue build-up, but the dry and pyrolysis steps were critical (see below) and the volume uptake of samples and standard solutions was almost the same, with a difference of $<5\%$. These steps gave background absorbance values well within the deuterium background corrector capability and about 400 firings could be obtained per platform and tube atomizer before the precision was noticeably degraded.

Optimization of Sample Mineralization Conditions

The sample–reagent mixture was continuously passed through the MWO, operating at a power level of 300 W, which ensures the appropriate conditions to mineralize the sample. An additional load of 500 ml of water was located inside the oven to avoid damage of the magnetron.

The four acids, hydrochloric, nitric, sulfuric and perchloric, tested for protein mineralization were compared in terms of the slopes of the graphs obtained from standard solutions. The slopes of the four graphs for 0.5 mol l^{-1} hydrochloric, sulfuric, perchloric and nitric acids were 0.010 ($r=0.9996$), 0.009 ($r=0.9999$), 0.008 ($r=0.9999$) and 0.007 ($r=0.9999$), respectively. In view of these results, ten pooled whole blood sample specimens were selected and analysed by the method of the standard additions with the different acids, and using aqueous standard solutions prepared in 0.5 mol l^{-1} of each acid and 5% by volume of glycerol. The slopes of the graphs obtained with hydrochloric and sulfuric acids were statistically different from those obtained without the addition of acid. The slopes of the eleven graphs obtained with nitric and perchloric acids did not vary by more than 3%. Hence, in principle, the method of standard additions did not have to be used if 0.5 mol l^{-1} nitric or perchloric acids were used for sample mineralization. These results could be attributed to an incomplete mineralization process when hydrochloric and sulfuric acids were used. Even with long ramp times and varied pyrolysis cycles, it was impossible to reduce the non-atomic absorption caused by the organic matrix when these acids were used. Although the addition of perchloric acid mineralized the bulk of the organic matter, thereby minimizing the formation of carbonaceous residues and the 'smoke' signal, it generated too much gaseous end products which could not be successfully removed by the gas diffusion cell (Fig. 1). The generation of these gaseous products greatly influenced the precision of the results, which varied between 15 and 25% relative standard deviation (RSD). When nitric acid was used, a maximum absorbance with acceptable repeatability (RSD $<4.0\%$ for 5 μg l^{-1} Co, $n=10$) was obtained. In view of these results, nitric acid was chosen for further studies. However, higher concentrations of nitric acid were avoided as they might cause severe oxidation of the pyrolytic coating of the graphite tube, a shortened tube life, a degraded precision and sensitivity, and an increased risk of contamination.[22]

Optimization of Flow Injection Conditions

In the manifold employed, the FI conditions that significantly affected the accuracy and precision of measurements were those related to the degree of sample dissolution in the microwave cavity. These conditions were the tubing length inside the oven and the carrier flow rate. By varying both parameters the residence time of the sample–reagent mixture in the oven can be adjusted. Table 2 summarizes the recovery, precision and residence time of the sample–reagent mixture in the oven[13] found for the determination of cobalt in whole blood using different coil lengths and carrier flow rates.

The following general prevailing observations were made: (i) the residence time of the sample–reagent mixture in the oven must be optimal to achieve satisfactory mineralization, and, therefore, to obtain accurate results; (ii) higher carrier flow rates provided more precise results; (iii) longer tubing lengths tended to provide less precise results; and (iv) too short a coil length produced less accurate results. Generally, too

Table 2 Recovery of cobalt from a pool of blood samples, precision of the analysis and sample residence time inside the MWO as a function of digestion coil length and different flow rates of the carrier solution

Coil length/m	Carrier flow rate /ml min^{-1}	Sample residence time in MWO/s	Recovery* (%)	RSD (%)
0.5	1	15	92	3.2
	3	5	87	3.0
	5	3	85	2.9
2.5	1	75	97	3.6
	3	25	96	3.2
	5	15	92	3.0
5	1	150	100	4.5
	3	50	101	3.4
	5	30	96	3.0
10	1	300	101	10.4
	3	100	99	4.3
	5	60	100	3.2

* Endogenous and added cobalt concentrations were 1.7 and 2.0 μg l^{-1}, respectively.

large a coil length increased the sample dispersion/dilution processes[10] and higher flow rates made the transport of standards and samples more comparable when treated in the MWO.[23] However, the most important FI parameter to be optimized was residence time of the sample–reagent mixture in the oven. When low residence times were used (<30 s), poor recovery values were obtained owing to an incomplete mineralization of sample. Whereas, at residence times of >75 s, the precision deteriorated owing to a constant changing of the flow rate because of gas evolution generated during the mineralization process, which could not be removed efficiently by the gas–liquid phase separator used in this work.[20] A coil length of 5 m with a carrier flow rate of 3 ml min^{-1} (which gave a sample residence time of 50 s) were found to be adequate for good sampling rate, accuracy and precision and were selected for further work.

Chemical Modification and Optimization of ETAAS Conditions

Several authors[22,24–26] have recently used $Mg(NO_3)_2$ as a chemical modifier in conjunction with a L'vov platform to eliminate interferences when determining cobalt in biological materials. In this study the integrated absorbances obtained with different amounts of magnesium nitrate (10, 20, 50 and 100 μg) enabled a higher pyrolysis temperature to be used (1400 instead of 1200 °C) without loss of cobalt. Above a pyrolysis temperature of 1450 °C, there was a dramatic decrease in the amount of cobalt present at atomization. A pyrolysis temperature of 1400 °C was chosen as optimum. The concentration selected (5 g l^{-1}) was the minimum amount of $Mg(NO_3)_2$ necessary to ensure no loss of cobalt in the mineralized blood matrix.

One of the reasons for difficulties met by other analysts in determining cobalt in biological matrices may be that the parameters for drying, pyrolysis and atomization have not been optimized. Therefore, in this work, various drying and pyrolysis temperatures were used with various heating rates. Firstly, irreproducible losses of analyte due to splattering would result if the sample is incompletely dried. Secondly, a rapid ramp rate could cause covolatilization of the cobalt species with the matrix constituents. When a single drying and charring step were performed, even with long ramp times and varied temperatures, the uneven drying of the sample and the buildup of ash from incomplete sample ashing made it impossible to reduce the background caused by the organic matrix sufficiently for adequate background correction. After only 30 firings of the graphite furnace, the buildup of ash on the platform noticeably degraded the precision, preventing accurate analysis. Therefore, various drying and pyrolysis temperatures with various heating rates were tried. The drying and pyrolysis of each sample with a series of gradually elevated temperatures and ramp times set between 10 and 40 s proved to be more effective in preventing splattering than rapid ramping to the highest temperature of each cycle and holding for a longer time period. When the furnace programme given in Table 1 was used, the background levels produced by the matrix were within the capability of the deuterium-arc background correction system. The results of a cobalt atomization study showed that the integrated absorbance increased very slowly with increasing atomization temperature between 2400 and 3000 °C; 2500 °C was selected for the atomization temperature. It is interesting to note that in this work the mixing of blood samples with Triton X-100 improved the drying characteristics of the sample[26] and reduced the accumulation of carbonaceous residues.[27]

Analytical Performance

The calibration graph for integrated absorbance was obtained from the results of triplicate 20 μl volumes of acid–EDTA–cobalt standard mixture and 10 μl of $Mg(NO_3)_2$ using the furnace temperature programme and MWO–FI optimized conditions summarized in Tables 1 and 3, respectively. The absorbance increased linearly with element concentration as expressed by the regression equation $A_{Co}=0.002+0.007$ [Co], $r=0.9999$, for the range 0–50 μg l^{-1} of cobalt. A detection limit of 0.3 μg l^{-1} Co (6 pg) was obtained (twice the standard deviation of the integrated absorbance measurements). The characteristic mass, m_o, was 6.1 pg of Co per 0.0044 s. The precision of the data was tested in a series of ten replicate analyses of a 5 μg l^{-1} Co standard solution and a Seronorm certified reference material (CRM) Batch 905 Whole Blood, giving RSDs of 2.6 and 3.1%, respectively. The accuracy of the procedure was investigated by determining the cobalt content in Seronorm CRM Batches 905 (with 5 μg l^{-1} Co) and 906 (with 10 μg l^{-1} Co) Whole Blood samples. The results obtained (4.8 and 9.7 μg l^{-1}) were in good agreement with the certified values.

Table 3 Optimised MWO, mineralization and FI conditions

Parameter	Value
MWO —	
Power control/W	300
Tubing length inside the oven/m	5
Mineralization—	
Nitric acid concentration/mol l^{-1}	0.5
Acid volume/ml	2
EDTA concentration/g l^{-1}	80
EDTA volume/ml	0.4
Sample volume/ml	2
FI—	
Carrier flow rate/ml min^{-1}	30
Sampling pump rate/ml min^{-1}	0.5
Vacuum pressure to the gas diffusion cell/cm Hg	60

Analysis of Whole Blood Samples

The proposed FI–MWO–ETAAS method was applied to the determination of cobalt in the whole blood of twenty healthy subjects not occupationally exposed to the metal and non-smokers. The results, summarized in Table 4, were compared with those obtained by in batch measurements using EDTA (4 mg ml^{-1} of blood), heparin and sodium citrate (5 mg ml^{-1} of blood) as blood anticoagulants. Each value is the mean of four independent sample dissolution analyses. When sodium citrate was used, a continuously high blank level was found, and therefore its use was avoided for further studies. When heparin was added, a high cobalt content was found in all samples (results similar to those previously obtained when zinc and copper were determined in whole blood by FI-MWO-AAS[19]) and its use was also avoided.

Although ultra-pure chemicals and colourless pipette tips were used in this work and checked for contamination, when 'in batch' measurements were performed slightly higher results were always obtained, indicating that some minor sources of external contamination could exist. These could come from the chemicals (specially from the anticoagulant), sample and solutions containers, sample handling, and air-borne contamination.

These experimental evidences support the applicability of the proposed procedure for the determination of cobalt in whole blood with good precision and accuracy. The *in vivo* sampling and on-line mineralization step reduces sampling handling, minimizes the possibility of sample contamination and makes complete automation of the analysis possible.

Generally, the most frequently published reports on cobalt content in body fluids are for blood plasma or serum,[28–31] little information is available on cobalt content in whole blood. Extremely variable cobalt concentrations in human whole

Table 4 Cobalt determination in whole blood of healthy subjects and patients receiving oral cobalt sulfate as treatment for anemia

No. of subjects	Type of subject	No. of determinations	Cobalt content/$\mu g\,l^{-1} \pm$RSD	
			In batch	On-line
20	Healthy	10	1.80 ± 0.05	1.50 ± 0.04
10	Patient	8	4.50 ± 0.13	4.20 ± 0.09

blood of unexposed subjects have been reported as follows: 0.07–0.36 (mean 0.18) (ref. 32); 2.0–2.8 (mean 2.4) (ref. 1); 1.5–2.0 (ref. 6); 1.7–15 (ref. 33); 5–10 (ref. 34); 3.5–63 (mean 43) (ref. 35); and 86 (ref. 36) $\mu g\,l^{-1}$ Co. Although differences in food intake may influence the cobalt content in whole blood, the risk of obtaining high values because of extraneous additions is important in the case of cobalt, and there are good reasons to suppose that these data result either from methodological errors and/or from analyses of improperly handled samples.[28]

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