Electrothermal atomic absorption spectrometric determination of cobalt in human serum and urine

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Problems and possibilities of the determination of Co in serum and urine samples by electrothermal atomic absorption spectrometry (ETAAS) are described. Optimal instrumental parameters as well as a suitable atomizer, calibration procedure and hydrogen peroxide as modifier are proposed for direct ETAAS measurement of Co in serum and urine. The detection limit achieved was 0.1 μg L^{-1} for both matrices and relative standard deviations varied in the range 5–20% depending on the Co concentration in the sample. Validity of the method was verified by the analyses of standard reference materials. For serum samples with a Co content lower than the detection limit, a separation and preconcentration procedure based on liquid/liquid extraction is suggested prior to determination of Co in the organic phase by ETAAS. This procedure permits determination of 0.02 μg L^{-1} Co in serum samples with a relative standard deviation of 10–18%.

Current development of human health related studies requires a growing number of elements to be monitored in biological matrices, including all essential and well-recognized toxic metals (1, 2).

Cobalt is an essential element in the human body as a component of vitamin B$_{12}$ and it is clear that monitoring of body fluids for cobalt is essential for the control of nutritional deficiencies, and perhaps prevention of its toxic effect in cases of occupational exposure. The most serious problems of cobalt determination in serum and urine samples are: (*i*) the extremely low concentration of this element in body fluids (normal serum and urine levels are in the range 0.1–0.5 μg L^{-1}); (*ii*) possible sample contamination during blood collection; (*iii*) high content of organic matrix causing numerous physical and chemical interferences. Double focusing sector field inductively coupled plasma mass spectrometry is a very useful and superior technique for this analysis (3–10). However, these instruments are not available in all laboratories. Another option is to use electro-

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thermal atomic absorption spectrometry. Therefore an accurate measurement of cobalt at concentrations 0.5–1 μg L^{-1} is still a challenge (11–13).

In the present study, the possibilities of electrothermal atomic absorption spectrometry (ETAAS) are investigated for Co determination in serum and urine samples from healthy persons and patients on haemodialysis. The originality of the paper is that procedures based only on direct determination of Co without any preliminary sample pretreatment are proposed. This makes it possible to achieve very low detection limits due to the very low blank sample level. The methods of direct determination and the procedures of extractive separation of cobalt from serum have been optimized (type of graphite tube, modifier, temperature programs, calibration procedure). Analytical quality assurance of the method was carried out by analyzing standard reference materials.

<div align="center">EXPERIMENTAL</div>

Instrumentation

The atomic absorption spectrometer Varian Spectra AA 640Z Zeeman AAS equipped with GTA100 graphite furnace (Varian, USA) and PSD-100 autosampler (Varian) was used. Pyrocoated tubes and tubes with centre fixed platforms were used as atomizers. A Varian cobalt hollow cathode lamp was used and the measurements were performed at 242.5 nm. Argon was applied as protective gas and 10 μL serum or urine samples were injected into the graphite furnace (GF). The graphite furnace operating parameters are presented in Table I. Only integrated absorbance values (peak area) were used for quantification.

Reagents and samples

The working standard solutions were prepared by dissolving the Merck (Germany) stock solution containing 1 g L^{-1} cobalt in nitrate form. Hydrogen peroxide produced in the Laboratory for High Purity Substances (University of Sofia, Bulgaria) was additionally purified by ion exchange. Mg(NO$_3$)$_2$ *p.a.* (Merck), NH$_4$F and NH$_4$SCN *p.a.* (Fluka, Switzerland) were additionally purified by recrystallization. Ammonium pyrrolidinedithiocarbamate, APDC *p.a.* (Merck) and isobutylmethyl ketone, IBMK *p.a.* (Merck) were used for the extraction procedures.

Lypochek Urine Metals Control Level 1 (Lot 69031, Bio Rad, Germany) and Clin Rep Level 1 serum (Recipe, Germany) samples were used as reference materials for method validation.

All disposable devices were rigorously cleaned shortly before use by immersion in hot concentrated nitric acid and rinsing with doubly distilled water.

Serum and urine samples were obtained from 20 presumably healthy volunteers and additional serum samples were obtained from 15 dialyzing patients and were transferred into plastic tubes. Serum samples were collected with a plastic iv canula No. 24 (TIK, Slovenia) with injection valve. Urine samples were taken as spot samples. The samples were acidified up to pH 2 and were kept frozen (–18 °C) until analysis.

All patients signed agreement for cobalt testing of their serum during a systematic medical checkup. Clinical experiments were performed according to the Ethics Committee provisions of the Institute of Preventive Medical Care and Toxicology at the Military Health Institution Center (Skopje, Macedonia).

Procedures

Direct ETAAS determination of Co in serum and urine

The serum or urine samples of $10 \, \mu L$ were directly introduced into the graphite furnace together with equal volume of modifiers. Calibration curves (1–5 $\mu g \, L^{-1}$ Co) were prepared by using aqueous standard solutions of Co.

Extraction-ETAAS determination of Co in serum

Extraction of Co as chelate complex with APDC. – Serum sample (3.0 mL) was placed in the extraction tube, 1 mL 1% solution of APDC was added and extraction was performed for 3 min in $500 \, \mu L$ IBMK. Cobalt was measured in the organic phase by ETAAS.

Extraction of Co as ion associate complex with NH_4SCN. – Serum sample (5.0 mL) was placed in the extraction tube, 0.2 g NH_4SCN and 0.05 g NH_4F were added and extraction was performed for 3 min in $500 \, \mu L$ IBMK. Cobalt was measured in the organic phase by ETAAS.

Recoveries for both procedures were calculated against organic standard solutions of Co.

Data analysis. – Detection limit and determination limit were calculated based on the variability of the blank (3 SD_b and 6 SD_b, respectively). Common statistical tests (ANOVA, Student's *t*-test, 95% confidence level) were used.

RESULTS AND DISCUSSION

ETAAS determination of Co in serum and urine

GFAAS program optimization. – Serum and urine samples were spiked with 4 $\mu g \, L^{-1}$ Co, ashing and atomization curves were constructed for both atomizers investigated in the presence of the modifiers studied. The results obtained are presented in Fig. 1. It was found that ash temperature of 1200 °C for pyrocoated tubes and 1300 °C for tubes with centre fixed platforms can be used without any loss of Co even in the absence of a modifier. These temperatures are high enough to ensure complete matrix decomposition and removal during the pyrolysis step. The modifiers used do not influence the thermal behaviour of Co. The only advantage achieved with H_2O_2 as modifier is the elimination of carbon residue formed at the end of the atomization cycle. It is very important to keep the atomization temperature as low as possible, because a rise in the atomization temperature leads to higher values of nonspecific absorption: for pyrocoated tubes the optimal atomization temperature is around 2100 °C and for tubes with centre fixed platform

Table I. Instrumental parameters for determination of Co in serum and urine samples by ETAAS

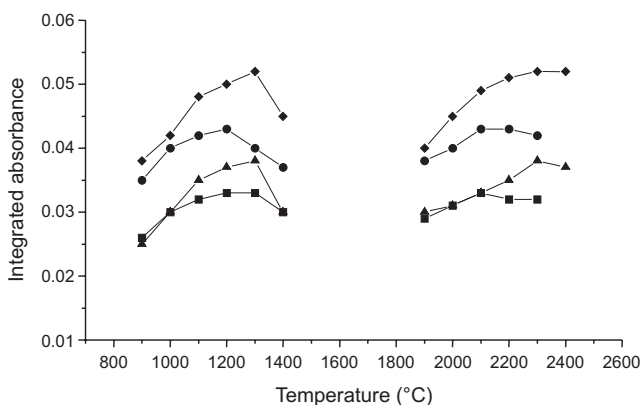| Step	Temperature (°C)		Time (s)	Argon flow (L min^{-1})
	Pyrocoated tubes	Tubes with centre fixed platforms		
Drying				
1	85	85	5	3
2	95	95	40	3
3	120	120	10	3
Pyrolysis				
4	1200	1300	5	3
5	1200	1300	5	3
6	1200	1300	2	0
Atomization				
7	2100	2300	2	0
8	2100	2300	2	0
Cleaning				
9	2300	2400	2	3

Fig. 1. Ashing and atomization curves for Co: in serum: (●) – pyrocoated tube, (▲) – tube with centre fixed platform, and in urine (■) – pyrocoated tube, (◆) – tube with centre fixed platform.

around 2300 °C. The optimal instrumental parameters for Co determination in serum and urine are summarized in Table I.

Calibration. – Matrix interferences were evaluated according to the slopes of calibration curves obtained in the presence of the matrix (serum or urine), and for aqueous standard solutions. The results obtained are recorded in Table II. As can be seen, tubes with centre fixed platforms are perfect atomizers for Co determination in urine – the slope of aqueous standard calibration curve (3.9 ± 1.2%) and the slope of the calibration curve in presence of urine (3.8 ± 1.4%) are practically equal. This means that calibration against aqueous standard curves could be used, simplifying in this way the analytical procedure. Pyrocoated tubes are preferable atomizers for serum samples, but in this case

significant differences were observed between the slopes of the calibration curves for aqueous standards (3.8 ± 1.2%) and in the presence of serum (2.5 ± 2.0%). The standard addition method was applied for calibration. However, further experiments performed showed that no significant differences were found between the slopes of calibration curves for different serum samples; consequently, the analytical procedure requires at least a single standard addition to one of the serum samples analyzed. It should be mentioned that statistically different slopes of the standard addition curves were obtained when standard solution was added beforehand to the sample and then injected into the furnace and for the standard additions made directly into the graphite furnace by using the facilities of the auto sampler. In the former case, the slope was remarkably higher. Depression of Co atomization was probably due to the formation of some complexes with serum components; this requires standard addition before sample injection.

Table II. Slopes (A μg^{-1}) of the best fit linear regression models for calibration curves

| Sample | Slopes of calibration curves | | | |
| | Pyrocoated tube | | Tube with center fixed platform | |
	Mean value	RSD (%)[a]	Mean value	RSD (%)[a]
Aqueous standard solutions	3.8	1.2	3.9	1.2
Serum	2.5	2.1	2.1	2.3
Serum in the presence of H_2O_2	2.7	2.0	2.2	2.1
Urine	1.9	2.4	3.8	1.4
Urine in the presence of H_2O_2	2.0	2.3	3.9	1.3

[a] $n = 5$

Analytical figures of merit. – The linearity range for Co determination is 0.2–30 μg L^{-1} for both matrices. The detection limit for cobalt in serum and urine was 0.1 μg L^{-1} and the determination limit was 0.2 μg L^{-1}. Within-batch precision strongly depends on analyte concentration in the measuring solution. For direct ETAAS, for Co in the range 0.1–0.15 μg L^{-1}, within-batch precision is 16–20% while for Co in the range 0.15–0.20 μg L^{-1} it is 5–8%. Between-batch precision (calculated as the standard deviation for results obtained for parallel samples analyzed on different days) is 12–17%.

The accuracy of the proposed procedure for ETAAS determination of Co in serum and urine samples was checked by spike recovery experiments. Serum and urine samples were spiked with Co in the concentration range 0.5–10 μg L^{-1} Co. The recovery values obtained ranged between 96.0 and 98.0% for all spikes.

The accuracy of the analytical methods developed was also checked by analyzing certified reference materials for urine and serum. A comparison of the results given in Table III shows good agreement with the certified values, thus confirming the validity and versatility of the analytical procedure.

Table III. Analysis of Co in reference materials by direct and extraction ETAAS methods

Standard reference material	Certified value ($\mu g\ L^{-1}$)	Determined by ETAAS[a]	
		Direct ETAAS ($\mu g\ L^{-1}$)	Extraction ETAAS ($\mu g\ L^{-1}$)
Lypocheck Urine Metals Control Level 1	3.7 (2.9–4.4)	3.9 ± 0.3	3.8 ± 0.4
Clin Rep Level 1 serum	5.0 (3.7–6.7)	5.1 ± 0.4	5.2 ± 0.5

[a] Mean ± SD, *n* = 5

Extraction-ETAAS determination of Co in serum

Since cobalt content in some serum samples is lower than the determination limit achieved by ETAAS, separation and preconcentration procedures should be proposed for these samples. Liquid/liquid extraction was tested as an effective analytical procedure for Co preconcentration directly from undigested serum samples. Two extraction reagents were studied for this purpose.

Ammonium pyrrolidinedithiocarbamate (APDC). – This reagent forms chelate complexes with Co and ensures quantitative extraction of Co in a wide pH range from 4 to 7 (14). Recoveries achieved for extraction of Co with APDC from 3-mL serum samples into 500 μL IBMK are in the range 95–97%, which means that this extraction system could be used for Co separation and preconcentration from serum samples. However, in some cases relatively high values of non-specific absorption were observed during the ETAAS measurement due to the high iron content co-extracted in the organic phase. To eliminate these interferences another extraction procedure was suggested.

NH_4SCN. – Quantitative extraction of Co could be achieved from 0.5 mol L^{-1} solution of NH_4SCN into IBMK. Iron as Fe^{2+} or Fe^{3+} strongly depressed the degree of extraction. Therefore, for serum samples, NH_4F is proposed as a reagent forming stable complexes with iron ions and thus eliminating this interference. ETAAS measurement of Co in the organic phase is free of matrix interferences because Fe is blocked in the aqueous phase. This extraction system ensures high recoveries (96–98%) and interference-free determination of Co. Hence, extraction of Co from a 5.0-mL serum sample in the presence of 0.2 g NH_4SCN and 0.05 g NH_4F into 500 μL IBMK is proposed for Co separation and preconcentration.

Analytical figures of merit. – The extraction analytical procedure with NH_4SCN allows detection of 0.01 μg L^{-1} Co and determination of 0.02 μg L^{-1} Co in serum samples. Within-batch precision for Co in the range 0.02–0.1 μg L^{-1} is 10–18%.

The accuracy of the results obtained by the proposed extraction procedures was verified by comparative analysis of serum samples with a relatively high content of Co by direct ETAAS and by the suggested extraction ETAAS procedure. Very good agreement between the results obtained (Table III) for reference standard samples confirmed the accuracy of the proposed preconcentration method.

Analytical application

The analytical methods developed were applied to the determination of Co in serum and urine (direct method and extraction method with NH_4SCN for a low concentration of Co) samples obtained from 20 presumably healthy volunteers and in serum samples from 10 patients on dialysis. The results obtained revealed that the Co content in serum of healthy volunteers varied in the range 0.04 to 0.09 µg L^{-1} and in urine in the range 0.11 to 1.22 µg L^{-1}. As could be expected, the Co content in the serum samples of persons on dialysis was significantly higher than in healthy persons, with serum Co ranging from 1.70 to 4.70 µg L^{-1}.

CONCLUSIONS

The results of this study indicate that direct ETAAS determination of cobalt in serum and urine samples is possible in the range 0.2–30 µg L^{-1} by using graphite tubes with a centre fixed platform for urine samples and pyrolytically coated graphite tubes for serum samples. The suitable modifier proposed is H_2O_2, which prevents carbon residue formation in the graphite tube. Pretreatment temperature of 1200 °C (1300 °C) and atomization temperature of 2100 °C (2300 °C), respectively, for pyrolytically coated graphite tubes and graphite tubes with centre fixed platforms are recommended.

For serum samples with a cobalt content below 0.2 µg L^{-1}, the extraction procedure with NH_4CSN, in the presence of NH_4F as a masking reagent for iron, into IMBK was developed. ETAAS measurements were carried out using the same temperature program.

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S A Ž E T A K

Određivanje kobalta u ljudskom serumu i urinu elektrotermičkom atomskom apsorpcijskom spektrometrijom

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U radu je opisano određivanje Co u ljudskom serumu i urinu elektrotermičkom atomskom apsorpcijom (ETAAS). Za izravno mjerenje Co ETAAS metodom predloženi su optimalni instrumentalni parametri kao i pogodni atomizer, postupak kalibracije i vodikov peroksid kao modifikator. Utvrđena je granica detekcije od 0,1 µg L^{-1} za obje matrice, a relativna standardna devijacija varirala je od 5 do 20%, ovisno o koncentraciji Co u uzorku. Ispravnost metode potvrđena je analizama standardih referentnih materijala. Za uzorke seruma s koncentracijom Co nižom od granice detekcije, predložen je postupak odjeljivanja i koncentriranja Co ekstrakcijom tekuće/tekuće prije njegovog mjerenja u organskoj fazi ETAAS-om. Ovim postupkom moguće je odrediti 0,02 µg L^{-1} Co u uzorcima seruma uz relativnu standardnu devijaciju od 10 do 18%.

Ključne riječi: kobalt, ljudski serum, urin, ekstrakcija, ETAAS

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