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THE OBSERVATION OF LOW CONCENTRATIONS OF Co, Mn AND Cr IN URINE SAMPLES OF PATIENTS WITH BREAST TUMOURS BY GFAAS

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A direct and quick method for the determination of Co, Mn and Cr in urine samples using graphite furnace atomic absorption spectrometry (GFAAS) has been developed. The effect of matrix for all elements studied was investigated on the typical solution according to the Dawson model [1]. Metals in urine reference materials BIO-RAD Lyphochek of Level 1 and Level 2 were used to prove the reliability of the method applied. The detection limits (3σ) estimated were: 0.13 μg l^{-1}, 0.14 μg l^{-1} and 0.18 μg l^{-1} for Co, Mn and Cr, respectively. The method developed was used for the determination of the above-mentioned elements in urine samples of ten patients with breast cancer. Urine samples were analysed before and after surgery and the first chemotherapy. Comparison of Co, Mn and Cr levels prior to and after the treatment were performed using two-way

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analysis of variance (ANOVA) and Student's t-test. The experimental results have indicated that urinary levels of Mn and Cr analysed before surgery were different from those of controls ($p = 0.044$ for Mn, $p = 0.001$ for Cr) while the differences between urinary Co concentrations of the same groups were not found to be statistically significant ($p = 0.060$). Similarly, when levels before and after surgery and chemotherapy are compared, the former results of Mn and Cr were higher than in the later case ($p = 0.034$ for Mn, $p = 0.001$ for Cr) and concentration levels of Co were not found to be significantly different ($p = 0.058$). The urinary Mn was decreased also in control group of patients treated by a common, non-cancer surgery ($p = 0.018$).

Introduction

Cobalt, manganese and chromium play an important role in a number of biological processes as components of enzyme systems or structural parts of biologically active constituents [2–7]. A lot of papers have already been published concerning the role of trace metals in cancer. The attention was focused on clarifying of their role in carcinogenesis and possible use of their assay in biological fluids as diagnostic or prognostic aids in patients with cancer [2]. Many investigations in this area have been carried out for the levels and changes of trace elements in serum, blood, hair and tissues [2,8–18]. Not so much investigation has been done into their occurence in urine [2,19–23]. Urine represents an important clinical material and the biological monitoring of metals in urine has become a matter of wide interest as a means of assessing their importance in biological processes. GFAAS is the method most conventionally used for this purpose. However, the determination of cobalt, manganese and chromium in urine is not easy because of frequent matrix interferences, which occur especially when the instrumentation equipped with deuterium background correction system is applied. Therefore, various chemical modifiers [5,24,25] have been applied to overcome these interferences. However, it is frequently noted that the compounds used as chemical modifiers can represent a possible source of contamination, especially so if the very low concentration (close to the limit of detection) are to be determined. This paper describes the optimisation of the AAS method using an artificial urine matrix in order to enable application of the method of calibration instead of the lengthy method of standard addition. The method applied has put main accent on the time of analysis as well as its accuracy and sensitivity; this is also adequately discussed in this paper.

The method was applied to the pilot study with the aim to obtain information about cobalt, manganese and chromium levels in urine samples of patients suffering from breast tumours and their possible changes after the surgery and chemotherapy.

Experimental

Equipment

Atomic absorption spectrometer Avanta P (GBC, Australia) equipped with GF 3000 graphite furnace, auto sampler PAL 3000 and deuterium arc correction in double-beam arrangement were used for all the measurements. Photron hollow-cathode lamps were used as a source. The wavelengths used were 240.7 nm, 279.5 nm and 357.9 nm for cobalt, manganese and chromium, respectively. Pyrolytically coated graphite tubes (GBC P/N: 56GB725) were used for all the experiments; 20 μl aliquot of the sample was injected.

The water used in experiments was purified using UltraClear (SG, Germany) pure water system to reach the conductivity of 0.05 $\mu S\ cm^{-1}$.

Reagents and Solutions

All reagents were of analytical grade and all solutions were prepared using purified water. Manganese, cobalt and chromium solutions (1 $g\ l^{-1}$) were obtained from Analytica Co. Ltd (Prague, Czech Republic). Standard solutions containing 10 $mg\ l^{-1}$ of Co, Mn and Cr, respectively, were prepared by appropriate dilution of the stocks (1 $g\ l^{-1}$) with deionised water. From these solutions, all working standards were prepared every day by diluting with deionised water. The matrix components used for the preparation of artificial urine solution i.e. sodium chloride, potassium chloride, magnesium chloride, calcium carbonate, hydrochloric acid (36 %), sulphuric acid (98 %) and urea were Suprapur® (Merck, Germany).

All the solutions were stored at 4 °C. Prior to analysis, all the glass and plastic ware was immersed into 2 M nitric acid for 24 hours followed by rinsing with deionised water.

Subjects

Urine samples from a total of ten patients with breast cancer aged 46–70 years (median value = 55.5 years) were collected one week before surgery and seven days after the first chemotherapy, which was applied on the day after surgery. Ten subjects aged 38–78 years (median value = 65 years) of non-cancer and surgically treated females were enrolled as controls. Urine samples of the controls were taken one day before and seven days after surgical intervention. All the samples were analysed by established laboratory methods.

Table I Thermal program for a graphite furnace

Stage	Final temperature °C	Time s		Gas flow l min^{-1}
		Ramp	Hold	
Dry[a)]	60	15	10	3.0
	85	45	5	3.0
	120	10	10	3.0
	250	10	10	3.0
Ash[b)]	1200	17	15	3.0
	1200	0	1	0.0
Atomize	2500	0	0.6	0.0
Clean	2600	1	0	3.0
Cool down	40	25	5	3.0

a) sample was injected at 20 °C
b) ashing temperature 1150 °C was used in the case of cobalt

Samples

Urine samples were collected directly into polyethylene containers and frozen at –20 °C. All plastic ware was cleaned with 2 M nitric acid prior to use. If necessary, urinary sediment was dissolved by ultrasound; no further pretreatment steps were needed for urine samples. The available reference material Lyphochek Level 1-69011 and Level 2-69012, freeze-dried urine (BIO-RAD, Anaheim, USA) was reconstituted according to the manufacturer's instructions.

Procedure

An artificial urine sample suggested by Dawson [1] was used for the purpose of optimizing the conditions of the determination and calibration procedure. The stock sample of artificial urine was prepared by dissolving single components in deionised water to reach the following final concentrations: 50.8 g l^{-1} NaCl, 30.9 g l^{-1} $NH_4H_2PO_4$, 28.6 g l^{-1} KCl, 3.12 g l^{-1} $CaCO_3$, 4.18 g l^{-1} $MgCl_2 \cdot 6H_2O$, 6.7 ml l^{-1} H_2SO_4 (98 %), and 87 ml l^{-1} HCl (36 %). An organic matrix was simulated by the addition of urea (186 g l^{-1} $CO(NH_2)_2$, in accordance to SRM NYC 403 125 by Nycomed). An artificial urine stock solution was diluted 10-fold to obtain "urine equivalent" concentration. Matrix matching calibration was used for all the metals.

The concentrations of the solutions used in the calibration, containing "urine equivalent", ranged from 0.5 to 4 $\mu g\ l^{-1}$ of Co, Mn and Cr. A single urine calibration graph was constructed for each element, from which all calculations were made in a single analytical run. All the calibration plots were linear in the investigated concentration ranges, and the correlation coefficients were 0.9992 for Co, 1.0000 for Mn and 0.9995 for Cr.

The temperature programme for graphite furnace is shown in Table I.

Statistics

A statistical test of combined sample skewness and kurtosis was used for testing of normality of sample distributions for both groups studied. When a normality of sample distribution was not found the statistical transformation was carried out to obtain the corrected estimation of location. Statistical analysis was performed by the analysis of variance (ANOVA) to check whether there were any differences between the cancer and reference groups over the observation period. "Treatment" with two levels (before and after surgery was selected as the first factor, "state" with two levels (cancer and non-cancer group of patients) was selected as the second factor. Additional comparison between groups was performed using Student's *t*-test if variables were normally distributed and the robust tests if not [26].

The ion concentrations were expressed as arithmetic mean ± 2SD or median value. All the calculations and statistical evaluation were carried out by software ADSTAT 1.25 (Trilobyte Statistical Software Ltd., Pardubice, Czech Republic) and NCSS2000 (NCSS, Kaysville, UT). The *p* value (significance level of the test) of < 0.05 was regarded as statistically significant.

Results and Discussion

Operating Conditions

Pyrolysis temperature varied between 400 – 1400 °C. The pyrolysis curves were recorded for three following different types of fluids: aqueous solutions (i), solutions containing "urine equivalent" (ii) and diluted (1:1) artificial urine solution (iii). The concentrations of the metals were 10 $\mu g\ l^{-1}$ cobalt, 2 $\mu g\ l^{-1}$ manganese and 2 $\mu g\ l^{-1}$ chromium. When the solutions containing a urine matrix were analysed, for the temperatures bellow the 1000 °C, the scatter signal was observed for all the metals studied due to insufficient compensating ability of the corrector. It was found that interferences originated mainly from matrix of alkali metal chlorides and phosphorus compounds. In order to remove this problem, an

ash temperature of 1000 °C had to be applied. Our experiments demonstrated that a diluted artificial urine solution adequately simulated the real samples and that it is well useful for the optimisation purpose. The optimal ash temperature found for cobalt aqueous solution was 700 °C, for manganese 800 °C and for chromium 1000 °C. For the analysis of solutions containing the artificial urine matrix, an ash temperature of 1150 °C for cobalt and 1200 °C for manganese and chromium could be applied. Probably this was possible because MgO produced by hydrolysis of $MgCl_2$ (matrix component) stabilized the analyte. A significant reduction of the background signal was observed for all the elements when these temperatures were applied.

The atomisation temperature chosen was 2500 °C. Three calibration curves in all cases were recorded for: (i) aqueous solutions, (ii) solutions containing "urine equivalent" and (iii) solutions containing "urine equivalent solution" diluted twice. The slopes obtained for all the elements were not found to be significantly different when (ii) and (iii) are compared. These slopes were also not different from slopes obtained in the case of the standard addition method (less than 10 %), which was applied in several cases of real urine samples; therefore, the matrix matched calibration containing an artificial urine matrix diluted 1:1 was used in all the cases.

The detection limits (3σ) for the elements determined were 0.13 $\mu g\ l^{-1}$, 0.14 $\mu g\ l^{-1}$, and 0.18 $\mu g\ l^{-1}$ for Co, Mn, and Cr, respectively, which is adequate for the determination of all metals at the normal levels in the general population. The reproducibility assessed by the relative standard deviations and evaluated from ten repeated measurements of 0.5 $\mu g\ l^{-1}$ of Co, Mn and Cr in urine solution was better than 5 % for all the elements.

Precision and Accuracy

Two lyophilised reference control urine samples with certified levels were used to assess precision and accuracy. The results were in a good agreement with declared values (Table II).

Metal Concentrations in Urine Samples

Using the described procedure, all of the above-mentioned metals were determined in urine samples obtained from breast cancer patients and non-cancer surgically treated controls. Table III shows and compares the results of cobalt, manganese and chromium concentrations in urine samples before and after surgery and chemotherapy.

In the analysis of variance, there was a statistically significant effect of state

Table II Determination of cobalt, manganese and chromium in certified BIO-RAD Lyphochek standards[a)] by GFAAS method given in μg l^{-1}

	SRM	Declared	Found
Cobalt	Level 1–69011	3.3 ± 0.7	3.9 ± 0.4
	Level 2–69012	10.6 ± 2.1	10.6 ± 1.1
Manganese	Level 1–69011	4.7 ± 1.0	4.5 ± 0.2
Chromium	Level 1–69011	3.9 ± 0.8	3.4 ± 0.3

[a)] Each value is the mean ±2SD

Table III Cobalt, manganese and chromium content[a, b)] in urine samples of patients with breast tumour determined before and after surgery and chemotherapy and control group given in μg l^{-1}

	Cancers		Controls	
	Before	After	Before	After
Cobalt	0.64 ± 0.45	0.43 ± 0.14	0.36 ± 0.08	0.29 ± 0.12
	(0.53)	(0.46)	(0.38)	(0.30)
	(0.13 – 2.15)	(0.13 – 0.61)	(0.15 – 0.98)	(0.11 – 0.64)
Manganese	1.11 ± 0.62	0.58 ± 0.32	0.52 ± 0.08	0.39 ± 0.04
	(1.10)	(0.53)	(0.46)	(0.38)
	(0.37 – 1.90)	(0.21 – 1.28)	(0.31 – 1.12)	(0.30 – 0.77)
Chromium	0.37 ± 0.13	0.18 ± 0.09	0.17 ± 0.05	0.22 ± 0.04
	(0.37)	(0.18)	(0.16)	(0.21)
	(0.19 – 0.60)	(0.08 – 0.38)	(0.10 – 0.45)	(0.10 – 0.47)

[a)] Each value is the mean ±2SD, median and the range are shovn in parentheses
[b)] Results were recalculated to the normal urine density 1.018 g cm^{-3}

(ill or healthy) ($p = 0.0012$) and treatment ($p = 0.0018$) on urinary cobalt levels as well as a significant interaction between both factors ($p = 0.0338$). The difference between values before and after chemotherapy treatment was found to be statistically insignificant ($p = 0.058$). Also the control group showed no changes over the observation period ($p = 0.075$). There were no differences for the urinary cobalt in cancer patients before the operation and chemotherapy compared to the reference ones ($p = 0.060$). After all medical treatments (operation and chemotherapy) both were equal, as the difference was not statistically significant ($p = 0.064$). As it has been mentioned, the effect of state as well as the effect of

treatment in the analysis of variance was found to be statistically significant, but when the Student's *t*-test was used, the differences in the groups and between the values of cancer and reference group were not found to be statistically significant. It is evident that p values obtained using *t*-tests are very close to the selected significant level 0.05. The main reason of these discrepancies is in that the values of cobalt for cancers before the treatment were in a wide range. In our case, when the sample size is relatively small, the statistical tests should be more useful.

In the case of manganese in the analysis of variance, there was a significant effect of the group ($p = 0.0002$), treatment ($p = 0.0003$) alike the significant interaction ($p = 0.0431$) over the observation period. Differences between the values obtained before surgery and chemotherapy and after the treatment ($p = 0.034$) were found. The manganese level in the reference group was also decreased after surgery ($p = 0.018$). After the observation period the manganese levels in all groups were the same ($p = 0.160$). Manganese levels in cancers analysed before surgery were significantly higher compared to controls ($p = 0.044$).

In the case of urinary chromium, two-way ANOVA demonstrates a significant effect of treatment ($p = 0.0288$) as well as significant interaction between both factors ($p = 0.0004$). The concentrations of chromium were significantly higher in the patients before treatment than after that ($p = 0.001$). No statistical difference was found between the urinary chromium levels before and after surgical intervention in the reference group ($p = 0.223$). The chromium level in cancer group analysed before surgery was significantly higher than the values obtained for controls ($p = 0.001$). Thereafter, they were equal ($p = 0.054$).

It is difficult to compare our results with results presented in the literature. A lot of papers have been published dealing with determination of trace metals in urine samples of occupationally non-exposed or exposed population, or people with various diseases [6,27–30]. Most papers dealing with determination of metals in urine of cancer patients are dedicated to zinc [19,20,23]. Among the papers reviewed [22] only one (in the 1970s) concerns determination of several trace metals in urine (blood) of patients with various types of cancer prior to and after treatment. The authors have determined Al, Mn, Co, Pb and Zn. With the exception of zinc, all metals were excreted at a subnormal rate. It is necessary to point out that urinary levels of these metals are very low and close to the limit of detection and also belong among common contaminants. This fact, frequently mentioned in the literature, is the main reason of the discrepancies between reports of various authors. Therefore, it is evident that further studies, with larger sample size, produced in various laboratories should be carried out.

Conclusion

The development of an atomic absorption procedure for the direct determination of cobalt, manganese and chromium in human urine has been described. The method offers a short analysis time, good sensitivity, low sample volume, low cost of determination, and is suitable to screen these metals in occupationally and non-occupationally exposed persons.

The method was applied for the determination of cobalt, manganese and chromium in urine samples of patients with breast cancer. The results showed that the levels of chromium and manganese were significantly higher in the patients before surgery and chemotherapy, while difference between the values of cobalt determined after surgery and chemotherapy was not found to be statistically significant. In the case of manganese, this difference was probably caused owing to operation because the same trend was observed for surgically treated non-cancers controls. Manganese and chromium levels determined before treatment were found to be different from healthy controls.

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