Assessment and modifications of digestion procedures to determine trace elements in urine of hypertensive and diabetes mellitus patients

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ABSTRACT

Context: There is accumulating evidence that the metabolism of several trace elements like Cr, Cu, Pb, Cd, Co, Mn and Zn might have specific roles in the pathogenesis and progress of many diseases like hypertension (HTN) and diabetes mellitus (DM).

Objectives: To provide a fast, efficient, sensitive, and reliable analytical procedure for trace element determination in urine samples of HTN and DM patients using inductively coupled plasma optical emission spectrometry (ICP-OES).

Setting and Design: The ICP-OES operating conditions were optimised and carefully selected in order to maximise the sensitivity, precision and accuracy. Factors affecting analytical and biological variability of the concentrations under study were discussed and carefully optimised.

Materials and Methods: Different digestion procedures with acids and oxidising reagents were tested. The suitable procedure ICP-OES was selected, carefully modified and applied. The validity and accuracy of the different elements were determined by spiking of samples with known amounts of multi-element standard solution.

Statistical Analysis: Student t-test and analysis of variance (ANOVA) test were used for analysis. Microsoft Excel was used to assess the significance of the difference between variables. The concentrations obtained were expressed as mean value ± standard deviation (P = 0.05).

Results: The results of this study showed that the mean concentrations of Cd, Zn, Pb, Cu, Cr and Mn in urine from both HTN (study group A) and DM (study group B) patients were higher than the corresponding values observed in the control group. However, while the mean value of Co was low as compared to the control group, the differences found were not significant (P = 0.05).

Conclusion: The method used had excellent sensitivity, multi-element data could be obtained with very short acquisition time. The elements Cr, Cd, Pb and Zn might have specific roles in the pathogenesis and progress of HTN and DM. Further studies are required to investigate the possible roles of these elements in HTN and DM individuals.

Keywords: Diabetes mellitus, digestion procedures, hypertension, urine, trace elements

INTRODUCTION

Hypertension (HTN) is the force exerted by blood against the walls of the blood vessels. It is characterised by the increase of pressure in blood vessels. The prevalence of HTN increases with advancing age. However, nowadays the age criteria have been changed and even the young have HTN problems due to lack of exercise, fast foods, coffee consumption, smoking, alcohol use, etc. Genetic effects may also be a factor.1,2 On the other hand, diabetes mellitus (DM) is a disease that occurs all over the world, however, its prevalence rates differ from one country to another. It is characterised by a disorder of glucose metabolism associated with a reduced ability
of tissues to respond to insulin. The hormone insulin, produced by the pancreas, helps the glucose to enter the cells where it is used as fuel by the body. In DM patients, the body’s metabolic process is completely disturbed either due to lack of insulin or due to ineffectiveness of the insulin produced by the body.\cite{3,4} Moreover, clinical research suggests that the homeostasis of trace elements can be disrupted by different diseases such as HTN and DM.\cite{2,3,5}

Current development of human health related studies requires a growing number of elements to be monitored in biological samples. Few of the elements present in nature play a metabolic role in living organisms.\cite{6} According to their abundance, these elements are classified as macro-, micro- or trace elements. The remaining could be attributed to those elements with unknown biological functions, to others which are present only because of the exposure to polluted environment or to those intentionally introduced into the body for special purposes.\cite{7,8}

Trace elements have recently been attracting the attention of scientists in various systems related to human health such as clinical and environmental analysis. Additionally, the measurement of trace elements is increasingly attracting the interest of physicians because deviations in trace element uptake and/or metabolism are known to be related to certain diseases.\cite{8-10} Analytical studies of trace elements dealing with problems of microanalysis of biological samples also have been increasing due to the expanding health areas. However, great efforts have been made developing analytical procedures for trace element measurement and improving their sensitivity and specificity.\cite{10}

The abnormal metabolism of trace elements plays an important role in health and disease conditions, and studies about them have been attracting significant interest. It has been speculated that trace elements may play a role in the pathogenesis of many diseases.\cite{11} Some of them form part of enzymes and others are involved in the synthesis of hormones. Others are known to be associated with certain diseases if they are present in the body in abnormally low concentrations. Several have been documented as being involved in blood pressure control while some may lead to intoxications in humans if ingested in high concentrations. Many of them are excreted primarily in urine and some are transmitted to blood.\cite{1-4,12-14}

Urine usually is used for the diagnosis of chronic degenerative disease that is caused by some trace elements. Therefore, urine analysis can provide important information to a clinician that may not be readily available with blood analysis. The levels of trace elements in the blood and urine are tightly controlled via metabolic, reabsorptive and excretory mechanisms.\cite{15-17}

Bone and teeth, hair and nails, organs and blood and its components (urine, cerebrospinal, amniotic, synovial fluids and tears, saliva, perspiration, bile, milk) are good indices of exposure to elements and can easily be assessed thus making them suitable to be used as bioindicators for these purposes.\cite{18} According to the above facts, it is important to determine the trace element concentrations in human urine having physiological disorders, like HTN and DM. Therefore, human urine was chosen for this study as probability (representative) sampling. Sample collections consisted of a number of healthy (control group) and HTN and DM individuals (study groups A and B) of different ages (30 - 75 yrs), which were selected from occupants of urban populations from Taif, Saudi Arabia on personal request. A questionnaire was employed to collect details concerning physical data, ethnic origin, health, dietary habits and consent of donor. Some factors affecting analytical and biological variability of the concentrations to be determined, such as the route of absorption, the presence of sources of environmental pollution in certain areas of residence, physiological variables and lifestyles, were also discussed.

There are several modern techniques for the determination of trace elements in urine. The digestion procedures vary according to the nature of the samples, the available method of analysis, elements to be determined and their concentration levels. Most techniques generally require the element to be in soluble forms. In all cases, samples demand manipulation prior to other processing and detection.\cite{8} In most inorganic determinations in clinical researches, the sample is digested or leached by oxidising acidic mixtures aided by heat or ultrasound or microwave radiation for oxidising the organic matter.\cite{19} The main advantages of microwave-assisted procedures were that it required smaller amounts of sample and oxidising materials, shorter digestion times, and easiness of sample handling. These procedures had to be validated to ensure that no contamination and/or losses occurred. The presence of these problems could affect the accuracy and provision of the final results. Therefore, the validation of the whole procedure was made by using a certified reference materials and/or standard additional method and/or by comparing the results of two different certified analytical procedures.\cite{10,20}

Biomonitoring of such trace elements present in complex samples required sensitive analytical methods with outstanding precision and high sample throughput. This was to cope with the low element...
concentrations and the large number of samples that were to be processed, eventually following an emergency. The most common analytical technique for measuring trace elements concentrations in biological samples like urine are flame or electrothermal atomic absorption spectroscopy, inductively coupled plasma optical emission spectroscopy (ICP-OES), inductively coupled plasma mass spectrometry and high performance liquid chromatography. It follows that analytical methods for determining minor and trace elements in biological matrices such as urine and blood should involve minimal sample handling and achieve relatively low detection limits, to permit easy and reliable determination of elements. Considering these requirements, ICP-OES was a good solution, because it allowed rapid and precise multi-element determination in a single solution, with sufficiently low detection limits and wide dynamic range and high accuracy.

Although potentially harmful effects of trace elements are generally well-known, limited studies are available regarding the investigation of the relationship between these elements and diseases. This will be indicated by determining the concentrations of selected trace elements like Cd and Pb in urine of HTN and DM individuals (study groups A and B). These concentrations will then be evaluated to determine the increase or decrease of these elements as compared to the control group. A total of 150 samples of urine were analysed after ‘wet digestion' for seven trace elements using ICP-OES.

**MATERIALS AND METHODS**

**Instrumentation and conditions**

A Varian 725-ES, ICP-OES with radial viewing configuration was used to analyse the standard and sample solutions of Cd, Co, Cr, Cu, Mn, Pb and Zn. The ICP-OES operating conditions were well optimised and carefully selected in order to maximise the sensitivity for the desired elements and to obtain the best precision and accuracy. Details of the operating conditions are summarised in Table 1. Each element was measured at two specific lines (nm) atomic (I) and/or ionic (II) line characteristics of a particular element that gives maximum sensitivity. Lead was measured only at atomic (I) line. The intensity of this emission is indicative of the concentration of the element within the samples. Selected emission lines (nm) for each element are summarised in Table 2.

**Reagents and glassware**

All reagents and chemicals were of analytical grade from Darmstadt, Germany. Mineral acids, chemical reagents, and oxidising agents [95 - 98% (m/m) H$_2$SO$_4$ (d = 1.84 - 1.85 kg l$^{-1}$), 69 - 72% (m/m) HNO$_3$ (d = 1.41 - 1.51 kg l$^{-1}$), 36.5 - 38% (m/m) HCl (d = 1.18 - 1.19 kg l$^{-1}$), 30% (m/m) H$_2$O$_2$ (d = 1.11 - 1.45 kg l$^{-1}$), etc.] were used. A multi-element stock standard solution (1,000 mg l$^{-1}$) was also used. Calibration standard solutions were obtained from the stock solution by suitable dilutions. Deionised doubly distilled water (DDDW), was also used throughout the analyses for preparing reagent, standard and sample solutions. DDDW was also used for washing and rinsing of all apparatus and glassware. Acid-washed plastic (polypropylene) vessels were used for preparing and storing solutions. All solutions were stored at −5°C until needed for analysis. Plastic, glassware and the auto sampler cups were cleaned by soaking in 5 mole l$^{-1}$ HNO$_3$ for about 24 hrs, rinsing five times with DDDW, dried, and stored in a class (100 laminar) flow hood.

**Collection of samples**

For the present study, samples of human urine were collected from healthy non-smoking control (n = 50), HTN (study group A; n = 50) and DM (study group B; n = 50) individuals in a polyethylene storage bottles (acid cleaned bottles). Samples were taken from males and females of different ages ranged from 30 - 75 yrs from occupants of urban populations from Taif, Saudi Arabia.
Microwave acid digestion

Triplicate of 0.5 ml of human urine samples, of each HTN (study group A), DM (study group B) and control group were directly placed into a porcelain crucible. Three millilitres of a freshly prepared mixture concentrated HNO₃ – H₂O₂ (2:1, v/v) was added to each crucible. The crucibles were covered and kept at room temperature (~35°C) for about 5 mins as a predigestion time, then placed in a microwave oven. Then, crucibles were heated following a one-stage digestion program at 30% of total power (900 W). Complete digestions of all samples required 2 - 3 mins. After the digestion was completed, the crucibles were left to cool at room temperature and the resulting solution (about 0.5 ml of semi-dried mass) was dissolved by 5 ml of 0.1 mole l⁻¹ HNO₃. This was then, transferred quantitatively to 10 ml volumetric flasks, diluted with DDDW up to mark and transferred to a polyethylene storage bottle for further analysis. Blank and spike sample solutions were carried out simultaneously through the complete digestion procedures and similar acid matrices. The presence of Ca. 0.1 mole l⁻¹ HNO₃ in the final solution was necessary to maintain acidic environment and avoid formation of insoluble hydroxides before measurement steps. This procedure is similar to that stated by Kazi, Afridi and Memon with some modifications in digestion time and microwave oven program. The validity of the digestion procedure was checked by spiking of different samples with known amounts of multi-element standard solution before and after digestion procedures. All selected trace elements were determined in the prepared solutions by ICP-OES.

Statistical analysis

All results were statistically evaluated by Student t-test, and ANOVA test (P = 0.05). In addition, Microsoft Excel and Origin software’s were also used to assess the significance of the differences between the variables investigated in patients and control individuals. The concentration values obtained were expressed as average value ± confidence interval (P = 0.05). All statistical analysis was based upon triplicate measurements of all standard and sample solutions.

Analytical figures of merit

The validity and efficiency of the microwave digestion method was checked by analysing spike solutions with a multi-element standard solution. The spike solutions were added to known amounts of the samples before and after digestion, which had also been through the digestion steps. The detection limit (LOD) was defined as 3 s m⁻¹, where SD is the standard deviation corresponding to 10 blank injections and m is the slope of the calibration graph. The LOD was 5 μg l⁻¹ for Pb, 1 μg l⁻¹ for Cu and Co, 0.9 μg l⁻¹ for Cr, 0.6 μg l⁻¹ for Cd, 0.5 μg l⁻¹ for Zn and 0.08 μg l⁻¹ for Mn.

RESULTS AND DISCUSSION

All results were expressed as x ± SD, where x is the mean values and SD is the standard deviation. To ensure that no contamination and/or loss of elements occurred during sample preparations and measurement methodology, a recovery test was demonstrated by standard addition methodology. It was performed using a bulk sample which had also been through all the digestion procedures. Multi-element standard solution spike were added to a known amount of the samples both before and after digestion, to assess the validity of the digestion procedure. The recoveries of the predigested spiked sample ranged between 95.8 - 103.7%; while, the recoveries of the postdigested spiked samples were between 95.7 - 102.8%. This indicated that there was no occurrence of contamination and/or loss of elements during sample preparations and measurement steps. Therefore, no significant differences were observed in measured values (P = 0.05).

Table 3 shows the results that were obtained for the determined trace elements in urine samples of the control group, HTN (study group A) and DM (study group B). According to the results, it was found that the levels of Zn (1.64 ± 0.25 mg l⁻¹) and Cu (0.48 ± 0.08 mg l⁻¹) in urine of HTN (study group A) were high as compared with to the corresponding values of control group (0.95 ± 0.14 mg l⁻¹) and (0.18 ± 0.04 mg l⁻¹), respectively. Likewise, the levels of Zn (1.78 ± 0.28 mg l⁻¹) and Cu (0.67 ± 0.11 mg l⁻¹) in urine of DM (study group B) were high as compared with the corresponding values of control group (0.95 ± 0.14 mg l⁻¹) and (0.18 ± 0.04 mg l⁻¹).
0.04 mg l⁻¹), respectively, but the differences found were not significant \((P = 0.05)\).

Furthermore, it was found that the Cr levels in urine of HTN (study group A; 0.13 ± 0.03 mg l⁻¹) and DM (study group B; 0.11 ± 0.03 mg l⁻¹) were high as compared to the control group (0.05 ± 0.02 mg l⁻¹) with no significant differences \((P = 0.05)\). Moreover, very close Mn values were detected in urine of HTN (study group A; 0.09 ± 0.03 mg l⁻¹) and DM (study group B; 0.07 ± 0.02 mg l⁻¹) as compared to the control group (0.04 ± 0.02 mg l⁻¹) but the differences found were non-significant \((P = 0.05)\).

In contrast, low Co values were detected in urine of HTN (study group A; 0.45 ± 0.06 mg l⁻¹) and DM (study group B; 0.98 ± 0.18 mg l⁻¹) as compared to the control group (1.22 ± 0.24 mg l⁻¹), but the differences found were insignificant \((P = 0.05)\).

On the other hand, high levels of both Cd and Pb were detected in the urine of HTN (study group A; 1.92 ± 0.29 mg l⁻¹) and (study group A; 1.01 ± 0.21 mg l⁻¹) and DM (study group B; 1.02 ± 0.17 mg l⁻¹) and (study group B; 1.45 ± 0.26 mg l⁻¹), as compared to control group (0.12 ± 0.03 mg l⁻¹) and (0.34 ± 0.07 mg l⁻¹) respectively, but the differences found were not significant \((P = 0.05)\).

Figure 1 shows the distribution of concentrations of seven selected trace elements in the urine of control, HTN and DM groups under study. It showed that lower concentrations were observed for Mn, Cr, Cu and Co. The opposite was true for Cd, Zn and Pb. Elevated value of Cd was observed in the urine of HTN individuals.

There are wide variations in the published data for the trace elements concentrations in biological samples such as urine of HTN and DM individuals of different countries.¹²₄₆₂₃₃₃⁴⁻⁰ To compare the reference ranges determined in the present study with those found by other authors is difficult, because there is a lack of coherence in the levels of trace elements found by various laboratories. One possible explanation for the different ranges of trace elements may come from the fact that with higher analytical sensitivity, the presence of contaminants becomes increasingly important. This is especially the case with elements that are physiologically present at very low concentrations, such as Mn, Cd, Pb and Zn.

CONCLUSION

The main goal of the work described here is to provide a fast, sensitive, and reliable method for trace elements analyses in a range of clinical matrices, i.e., urine of HTN and DM individuals) using high resolution ICP-OES. To assess this, a suite of clinically important trace elements such as Cd, Co, Cr, Cu, Mn, Pb and Zn was quantified. However, after all conditions had been established, measurements became very efficient. Since the ICP-OES has excellent sensitivity, multi-element data can be obtained with very short acquisition time. Three replicates for seven trace elements were performed in only about 30 seconds, 150 samples of each of the seven trace elements were measured in a few hours (~2 hrs). We can conclude that there is accumulating evidence that the metabolism of several trace elements like Cr, Cu, Pb, Cd and Zn might have specific roles in the pathogenesis and progress of many diseases like HTN and DM.¹¹

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