Doktori (Ph.D.) értekezés tézisei

Development of Trace Analytical Methods of Biological Samples

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I. SCOPE AND OBJECTIVES

In the past few decades the study of macro and micro elemental concentration of biological – mainly human biological – samples become very important. This is due to the discovery that in addition to the macro elements the micro elements are also playing important role in the biochemical processes of the living organisms. These functions are being investigated nowadays. In addition to the essential elements, such elements may appear in the living organisms which are poisonous even in low concentrations.

By determining the concentration of essential and toxic elements in the human body, occupational and environmental intoxications, lack of trace elements, nutritional problems and certain illnesses can be recognised which may change the trace element status. These illnesses can be identified at their early stage, often before the appearance of the clinical symptoms. Hence the determination of the trace element status is of increasing importance as a preventive diagnostic method in the western-european clinical practice. Similar analytical studies were performed in Hungary in some fields, but more extensive studies for clinical applications in the present field are not known.

The topic has extensive international literature, but further studies, performed nowadays, are verified by the discrepancies and problems related to the preparation and analysis of human biological samples, and the evaluation and practical use of the results.

The analysis of human biological samples is a challenging task for the analyst. The concentration of the component to be analysed is usually low in the samples, hence sensitive analytical methods are necessary for its identification and quantitative determination. The high amount of organic compounds may cause further problems during analysis, hence it is necessary to partially or totally remove them from the sample. Changes in the trace element content of the samples should be prevented during sample preparation, i.e. contaminations and sample losses should be avoided.

In addition to the analysis the evaluation of the results may also cause problems. This is due to the high individual deviation of the

biological samples, the lack of unified sample preparation and analytical methods and the considerable differences in the determined reference ranges for healthy controls and patients suffering from certain illnesses.
The human biological analyses are performed from different samples, among them the most widely used are blood (in the form of whole blood, serum or plasma), hair, and other tissues.
The study of the trace element status is especially important for patients whose illness or the regular treatment may be potential source of the dramatic change in the trace element status. During my work patients undergoing regular haemodialysis treatment were selected, because due to the haemodialysis and the probles with the excretion of toxic metals, their trace element status may change easily and the accumulation of toxic elements in their body is possible. Among these toxic elements aluminium is especially important, because it is known to be toxic and its accumulation is in connection with certain diseases (like the Alzheimer-disease). The monitoring of the trace element levels in the body of patients undergoing regular haemodialysis is still not solved.

On the basis of the above, the main goals of my Ph.D. work were the followings:

- to develop suitable sampling and sample preparation methods for the preparation of samples of small amounts in big series.

- to examine several available human biological samples and to decide which are the most satisfactory for the quantitative study of the trace element status.

- to develop highly sensitive, multielemental simultaneous analytical method(s) for the analytical study of small sample amounts.

- to determine reference ranges to the healthy controls and haemodialysed patients on the basis of available literature and own experiments for diagnostic purposes.

- to verify the applicability of the method by studying regularly haemodialysed patients; to follow the changes in the trace element status of the patients, discover the lack or accumulation of trace elements and the possible surces of them; to study their correlation with certain physiological parameters and to establish the analytical background for the regular study of the trace element status of haemodialysed patients.

II. EXPERIMENTAL

To avoid contamination, hair samples were collected by cutting with tantalum carbide scissors and tungsten carbide scalpel. The collected hair samples were stored in polyethylene bags at room temperature till sample preparation. Blood sampling was done with the Vacutainer system (Beckton-Dickinson, USA) which is widely known and applied in the clinical practice. The adsorption of trace elements in the inner wall of the sampling tubes was tested prior to use as well as their dissoluble trace element content. Compared to the trace element content and the concentration changes observed in the case of real samples the above effects are found to be negligible. The blood, serum and plasma samples were stored in the above tubes and in Eppendorf tubes (1,5 cm^3 volume) at -70ºC.

The decomposition of both sample types was performed in closed system with microwave digestion units at high temperature (130-140°C) and pressure (max. 150 bar) (Milestone mls 1200 Mega, Microwave Laboratory Systems, Italy; CEM MARS-5, CEM Microwave Technology Ltd., USA).

Multielemental analysis of the samples was performed with ICP-OES (Spectroflame, Spectro GmbH, Germany) and ICP-MS (Elan-6000, Perkin-Elmer Sciex, Ontario, Canada) systems. The monoelemental aluminium analysis was made with GFAAS method (Varian SpectrAA-10, supplied with GTA-96 graphite furnace unit). Spectrographic method was used for the qualitative analysis of solid samples from the Haemodialysis Centre (Zeiss Q-24 spectrograph, Zeiss SP-2 spectrum projector). PIXE and micro-PIXE methods were applied for the study of longitudinal and transversal distribution of selected elements (Scanning Nuclear Microprobe, Oxford Microbeams, Oxford, United Kingdom).

III. NEW SCIENTIFIC RESULTS

1. Scissors made of tantalum carbide and scalpels made of tungsten carbide were developed and applied for the hair sampling. By using these tools the contamination of the hair samples is restricted to these elements during the sampling procedure, which were not studied. Washing procedure was developed for the removal of the exogeneous contamination from the hair samples including rinsing with organic solvent mixture (diethyl-ether:acetone=3:1), nonionic detergent solution (1% aqueous solution of Decon-90) and ion-exchanged water. The developed method and some selected washing methods from the literature were compared by using homogeneous hair sample as a test sample. It was shown that a more uniform and reproducible exogeneous cleaning can be achieved with the developed method than with the other methods described in the literature.
2. Vacutainer tubes with special inner coating were studied by using dissolution tests. It was shown that the dissoluble contamination from the inner wall of the tubes is negligible compared to the trace element content of the blood samples, hence they are suitable for the sampling and sample storage.
3. Microwave digestion in high pressure closed system was developed for the preparation of hair and blood samples using Teflon vessels. The original Teflon vessels were modified to decrease the required sample amount, to minimize the dilution of the samples and to increase the number of samples digested in one cycle. Surface-treated quartz tubes were inserted to the Teflon vessels (three tubes to every vessel), and the samples were decomposed in these quartz tubes. The sample amount decreased from 500 mg to 100 mg and the number of samples digested in one cycle increased from 6 to 18. It was shown that by using the described sample amount and microwave program the samples do not run out of the tubes during digestion.
4. By using different certified reference materials it was shown that the developed microwave digestion method is suitable for the digestion of very low sample amounts without considerable

contaminations or analyte losses. The lowest sample amount was 1 mg, for which the measured concentrations after digestion and the certified values were in good agreement (95-105%), similarly to the bigger sample amounts.

5. During the analysis of blood serum samples extremely high aluminium concentratios were measured. The source of this aluminium was the aluminium contamination of the high purity HNO_3 and HF, which cannot be removed by subboiling-point distillation. This problem was succesfully avoided with a simple and fast sample preparation method by using trimethyl-amine solution (25% (m/m), aqueous) as the digesting agent.
6. Hair samples from healthy controls and haemodialysed patients were studied with proton microprobe (micro-PIXE). The concentration and distribution of Ca, Cl, Fe, K and Zn was determined on the surface along the single hairs and in the cross-sectional samples both for washed and unwashed samples. It was shown with measurement data and distribution maps that the distribution of the selected analytes is not homogeneous in the cross-section of the hair and the elements can be accumulated in different layers in the hair. Hence a washing procedure may influence not only the concentration, but also the ratio of the elements in the hair samples by washing out the more available and less fixed analytes from the hair.
7. Serum samples of healthy subjects were analysed and reference ranges were determined for Al, Cr, Mn, Fe, Co, Ni, Cu, Zn, Sr, Mo, Cd and Pb. In the case of some analytes serious differences were found between the determined reference ranges and the ranges found in the litarature, used in the clinical practice and recommended by WHO. Serum samples from haemodialysed patients were analysed and the concentration of Cr, Fe, Co, Mn, Ni, Cu, Sr and Mo was significantly different from the ranges measured in healthy controls.
8. The water purification system and the purified water used for haemodialysis was analysed to determine if they can be a potential source of aluminium for the patients. I found that certain parts of the purification system are made of aluminium

alloy, and the activated charcoal used for dechlorination also contains aluminium in high concentration. Samples were taken from the inlet tap water, after the water cleaning steps throughout the system and the resulted purified water. The analytical results of the water samples show that the aluminium concentration in the inlet tap water may have an effect on the aluminium content of the purified water. However, the aluminium concentration of the purified water remained well under the limits in all cases.

9. Blood samples were taken from many patients before, during and after haemodialysis treatment. The analytical results show that the concentration of Al, Mn, Sr, Cd and Pb is increased, the concentration of Zn and Mo is decreased, while the concentration of Fe, Co, Ni and Cu remained nearly constant during the haemodialysis. On the basis of the analytical results it is possible that the samples were contaminated with chromium during sampling due to the stainless steel needle used for sampling. Hence the chromium was excluded from the further studies.
10. By comparing the results of the clinical tests and the trace element analysis it was shown that there is a close correlation between the concentration of cobalt and nickel in the serum and the high homocystein level. The high homocystein level is an individual risk factor of the cardiovascular diseases which are very common for the HD patients. In the case of those patients where the concentration of Co was higher than the reference range the homocystein level was significantly higher and the vitamine B_{12} level was significantly lower than the normal values. Opposite effect was observed as a function of the Ni concentration. If the nickel concentration is increased, the homocystein level in the serum was significantly lower, while the B_{12} concentration was significantly higher than in the previous case. These results show that the increase in Ni concentration of the serum enhances the metabolism of homocystein (which is indicated by the lower homocystein and higher B_{12} concentration). At the same time, the small increase in

the concentration of Co (which was observed in the case of some patients) does not have such a positive effect.

11. Correlation was found between the concentration of certain metal ions and the activity of superoxide dismutase (SOD) and xantine oxidase (XO). These enzymes have a great importance in the formation of oxidative stress. The activity of SOD was lower, while the activity of XO was higher in the serum samples of HD patients than in the serum of healthy controls. The change in the activity of SOD was in correlation with the concentration of Zn, Cu and Mo in the serum. The activity of XO was in correlation with the concentration of Mo and Mn.
12. The concentration of certain trace elements in the serum of HD patinets is in correlation with the disease initiating the kidney failure, e.g. for vascular problems the concetration of Mn and Zn was low, while in the case of diabetes mellitus the concentration of Mo was low.

IV. PRACTICAL USE OF THE RESULTS

During my work I studied the usability of human hair, blood, plasma and serum samples for the determination of the trace element status of the body. I called the attention to certain problems related to sample preparation, analysis and evaluation; for some of them I found a solution.

I developed analytical methods for the analysis of the above samples. I found that considering the trace element concentrations, the technical, security and health issues related to sample storage and handling, the hair samples are the most suitable for the study of trace element status. However, actually there are no sample preparation methods available which can guarantee the removal of the exogeneous contaminations from the surface of the hair sample and the endogeneous composition remains intact. Despite of the high number of publications on that field, the clinical application of hair analysis is not frequent due to the lack of approved clinical reference ranges.

On the contrary the analysis of blood, serum and plasma samples is widely applied in the clinical practice, hence the necessary reference ranges are available. Due to the above, after comparing the different samples I focused my work to the analysis of human serum samples. The method, optimized during my work, is suitable to perform the analysis of such samples in huge series. This was shown with the samples of a selected group of patients (patients undergoing regular haemodialysis).

By using the developed method and the existing clinical reference ranges the regular monitoring of the trace element status of healthy subjects and the diagnostic analysis of patients suffering from different diseases can be performed.

V. PUBLICATIONS

Publications in connection with the dissertation:

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2. J. Dombovári, Lajos Papp, Imre Uzonyi, Ildikó Borbély-Kiss, Zoltán Elekes, Zsuzsa Varga, János Mátyus, György Kakuk "Study of cross-sectional and longitudinal distribution of some major and minor elements in the hair samples of haemodialysed patients with micro-PIXE" Journal of Analytical Atomic Spectrometry (1999) 14:553-557
3. Dombovári János, Papp Lajos, Varga Zsuzsa, Mátyus János, Kakuk György "Haemodializált betegek és egészséges kontrollszemélyek vér-, plazma- és hajelemzései valamint a dialízishez használt víz vizsgálata ICP-OES, GAAS és spektrográfiás módszerrel" Magyar Kémiai Folyóirat (2000) 106(5-6.):230-237
4. J. Dombovári, J.S. Becker, H.-J. Dietze "Multielemental analysis in small amounts of environmental reference materials with inductively coupled plasma mass spectromety" Fresenius Journal of Analytical Chemistry (2000) 367:407-413
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3. J. Dombovári, J.S. Becker, A.J. Kuhn, W.H. Schröder, "Multielement analysis of small plant tissue samples using inductively coupled plasma mass spectrometry" Entwicklung und Anwendung massenspektrometrischer Metoden zur Spuren-, Ultraspuren und Oberflächenanalytik für Forschungsaufgaben des Forschungszentrum Jülich, 2000, 3821/Teil 2, 79-90. Jülich, Németroszág, ISSN 0944-2952
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5. S. Boulyga, J. Dombovári, J.S. Becker, H.-J. Dietze "Determination of selenium in biological samples using ICP-QMS", Atomic Spectroscopy (2000) 21(5):149-155
6. J. Dombovári, J.S. Becker, "Isotope Ratio and Reverse Isotope Dilution Measurements of Magnesium in Small Amounts of ^{26}Mg-spiked Nutrient Solutions with Inductively Coupled Plasma Mass Spectrometry", Entwicklung und Anwendung massenspektrometrischer Metoden zur Spuren-, Ultraspuren und Oberflächenanalytik für Forschungsaufgaben des Forschungszentrum Jülich, 2000, 3821/Teil 2, 135-150. Jülich, Németroszág, ISSN 0944-2952

VI. CONFERENCE PRESENTATIONS

1. J. Dombovári, L. Papp "Development of a new analytical method for the determination of trace element content of human hair" VIII. Hungarian-Italian Symposium on Spectrochemistry, Debrecen, 1997 június 29 - július 4, Book of Abstracts, MKE-MTA kiadvány, Budapest, HI-25.
2. J. Dombovári, L. Papp, I. Uzonyi, I. Borbély-Kiss, Z. Elekes, Zs. Varga, J. Mátyus, Gy. Kakuk, "Study of cross-sectional and longitudinal distribution of some major and minor elements in the hair samples of haemoidialysed patients with Micro-PIXE", 8th Solid Sampling Spectrometry Colloquium, Budapest, Hungary, September 1-4, 1998, MKE-MTA kiadvány, Budapest
3. Dombovári J., Varga Zs., Mátyus J., Kárpáti I., Kakuk Gy., Papp L. „A vér és plazma nyomelem összetétele hemodializált vesebetegeknél", A Magyar Nephrologiai Társaság 1998. évi Nagygyűlése, Budapest, 1998. október 14-16, Hypertonia és Nephrologia, 1998, S2(3), 90. o.
4. J.S. Becker, J. Dombovári, H.-J. Dietze "Multielement analysis in small amounts of environmental materials" The 26th Annual Conference of the Federation of Analytical Chemistry and Spectroscopy Societies (FACSS), Kanada, Vancouver, 1999 október
5. Mátyus J., Dombovári J., Kárpáti I., Papp L., Kakuk Gy., Varga Zs. „Hemodializált vesebetegek nyomelem és antioxidáns státusa", A Magyar Nephrologiai Társaság 1999. évi Nagygyűlése, Szeged, 1999. október 14-16, MNT kiadvány, Budapest, 83. o.
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10. L. Papp, J. Dombovári, „Development of new microanalytical methods for the analysis of human biological materials” Colloquium Spectroscopicum Internationale (CSI) XXXII. Pretoria, South Africa, 8-13 July 2001., Volume of Abstracts P6-4