SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Diagnostic analysis of human blood serum sample by atomic spectrometry

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I. Introduction and objectives

The determination of the elemental content of human blood serum is important in medical diagnostics because the human body uses various inorganic compounds, including minerals, in its biological processes. Whole blood elemental composition can provide information about the nutrition of the body, including the functioning of homeostatic mechanisms that regulate transport processes, phase reactions, protection against oxidative damage, and can therefore be indicative of acute or chronic lesions. The proteins in the serum are capable of binding trace elements and thus play a crucial role in their transport processes. Examining the trace elements that bind to a protein can provide a broader picture on the body's normal and diseased trace element status, which can help to diagnose certain difficult-to-recognize degenerative lesions.

During routine laboratory blood tests, a few elements (Na, K, Mg, Fe, Cu) are determined mainly by electrochemical methods, but there are many elemental analytical techniques available today for the analysis of essential and toxic elements in blood. The most commonly used ones are atomic spectrometry devices: flame atomic absorption spectrometry (FAAS), graphite furnace atomic absorption spectrometry (GFAAS), inductively coupled plasma optical emission spectrometry (ICP-OES), and inductively coupled plasma mass spectrometry (ICP).

The physiological functions of trace elements in the human body depend on their attachment to various proteins. Therefore, it is important to determine not only the total element concentration, but also the distribution of the various protein elements, in order to map the physiological processes of the body and the changes occurring during the pathological processes. In addition, the development of reliable analytical methods is essential to develop the biological models needed to understand the relationship between minerals and disease. In the so-called protein-specific analysis, it is first necessary to distinguish between proteins in the blood serum, for which chromatographic techniques are commonly used. For the elemental analysis of fractionated samples, the aforementioned tools can be operated both offline and online, however, these large-scale instrumentation procedures are only available to a limited extent and require adequate skills. A further problem may be that blood is a complex, high organic-content material that can cause potential interferences with known atomic spectrometry techniques.

The aim of our present work is to develop more cost-effective methods for quantitative analysis of the elemental composition of human blood serum samples and to test them in real analytical tasks. Model solutions were designed to investigate the effect of relatively high concentrations of easily ionizable elements in serum on the analytical signal of the test items, which are to be checked by back-testing of a Seronorm® lyophilized blood serum sample. The ultimate goal was to determine the applicability of optimized methods for determining the composition of blood sera from autoimmune patients.

Another aim was to fine-tune an easy-to-use and cost-effective column chromatography technique (anion exchange resin) to separate the most important extracellular transport proteins in serum samples and to determine the essential elemental content of each protein by atomic spectrometry, such as MIP-AES.
Interactions between Gd^{3+}-ions and protein fractions released by the dissociation of [Gd (DTPA-BMA)] complex in human serum (Seronorm®) were also designed by ICP OES.

II. Experimental methods

Optimization experiments were performed on a relatively new instrument, the microwave plasma atomic emission spectrometer (4200 MP-AES, Agilent Technologies). Research was carried out to determine the matrix effects in the nitrogen plasma on the use of blood serum samples. Two model experiments, a full factorial design, and an effect study were performed to investigate the effect of sodium concentration on serum K, Ca, Mg, Cu, Zn, and Fe signal intensity, and a third experiment to analyze the effect of sodium and potassium on each other's analytical signal.

Human blood serum samples were prepared using an ETHOS UP (Milestone) microwave digester for elemental analysis.

Separation of blood serum samples was performed by a simple column chromatography technique using a DEAE Trisakryl® M anion exchange resin for specific protein separation. Proteins were determined by spectrophotometry at 280 nm. For the preparation of fractionated blood serum samples, three types of digestion techniques were compared: atmospheric wet digestion, atmospheric block digestion, and microwave digestion with ETHOS UP (Milestone). The K, Ca and Mg concentrations of the fractionated serum samples were determined with a 4200 MP-AES (Agilent Technologies). Due to the high dilution, elemental analysis of Cu and Zn could not be performed by this technique, and their concentration was determined by graphite furnace atomic absorption spectrometry (240 Z GFAAS, Agilent Technologies).

The dissociation of the Omniscan Gd (DTPA-BMA) MRI contrast agent in serum and its interaction with the released Gd^{3+}-ion serum proteins were studied in lyophilized human blood serum, where the separation was also performed with the DEAE Trisakryl® M anion exchange resin and proteins were identified by spectrophotometry at 280 nm. For elemental analysis, sample fractions were prepared by atmospheric wet digestion and Gd concentration was determined using 5200 ICP-OES (Agilent Technologies). Samples containing Gd^{3+} ion and serum proteins were filtered with Amicon® Ultra-2 ultrafilter and after centrifugation (3000 rpm, 4 °C), Gd^{3+} ion concentration was also measured by using 5200 ICP-OES (Agilent Technologies).

The results of the various experiments were evaluated by statistical methods. The first model experiment of matrix effect studies was based on a complete 2^n factorial design, which was graphically evaluated by Statgraphics Centurion XVII. software. The effects were examined by the analysis of variance (ANOVA). The effects of sodium and potassium on each other's intensity were evaluated by two-way analysis of variance (ANOVA, General Linear Model - GLM), and graphical representation was also reported in Statgraphics Centurion XVII. software.

Elemental concentrations of real samples (healthy and autoimmune human blood sera) were compared using one-way ANOVA in SpSS IBM 22 for Windows. After data transformation, homogeneity was verified by Levene's test. Significant differences were evaluated using Tukey's multiple response test.

III. New scientific results

1. We have established that the microwave plasma atomic emission spectrometer can be used for cost-effective elemental analysis of human blood serum samples.

1.1. The robustness of the microwave plasma was determined by the intensity ratio of the magnesium atomic and ionic lines (MgII 280.271 nm / MgI 285.213 nm).

By configuring two user-adjustable measurement parameters of the MP-AES device, it was determined that the read time did not significantly affect the measurement, while the nebulizer pressure was the best at the highest value (240 kPa). Based on the robustness test, the intensity ratio of MgII 280.271 nm / MgI 285.213 nm ranges from 8 to 14 for ICP-OES, and from 0.5 to 1.0 for MIP-AES. Explanation for this lies in the configuration differences: plasma temperature and electron density of MIP-AES are significantly lower than ICP-OES techniques. It was found that the intensity ratio obtained by the MIP-AES method is not directly comparable with the results obtained with the ICP-OES.

1.2. Sodium concentration tolerance of the method was determined for the analytical signal of six elements (K, Ca, Mg, Cu, Zn and Fe) that are essential in the serum.

In the case of increasing sodium concentrations, we found that in the case of Mg and Zn the effect of the Na matrix was not significant, but for the other elements the intensity of the signal depended on the matrix composition.

1.3. The effect of the concentration of Na in the serum on the analytical signal of six essential elements (K, Ca, Mg, Cu, Zn and Fe) in the microwave plasma was determined by means of model experiments.

Based on the results of the factorial arrangement, serum sodium had an effect on calcium, copper and iron, whereas had an interaction on zinc, magnesium and potassium. Adding sodium as a matrix to the model solutions at increasing concentrations resulted in a significant increase in the intensities obtained for the elements to be measured. On the basis of the measurement data, the percent differences in the intensity shift were calculated and overall it was found that sodium influences the analytical signal obtained, but that its effect is negligible over the concentration range tested. The effect of increasing sodium concentration on the intensity of the six elements (K, Ca, Mg, Cu, Zn, and Fe) was also studied in the ICP-OES technique. Significant signal increases were also observed with ICP-OES, but to a lesser extent than with microwave plasma.

1.4 The effect of sodium and potassium on each other's analytical signal was studied.

It has been found that the two elements have a small effect on each other's intensity, which can be reduced by the addition of ionization buffer (cesium chloride). During the application of CsCl, it was found that concentrations of 1.0 and 2.0 g L^{-1} in microwave plasma overloaded the excitation source, thus 0.1 g L^{-1} of CsCl is the appropriate.

1.5. We tested the applicability of the MP-AES device for elemental analysis of human blood serum samples with Seronorm® certified lyophilized blood serum.

The recovery of the concentrations of the six elements Ca, K, Mg, Cu, Zn and Fe was performed with and without matrix fitted calibration, where good agreement was found with the literature data.

2. We tested the optimized microwave plasma measurement method with blood samples from autoimmune patients (SLE, SS) and found significant differences between the patient and healthy samples.

We found no significant differences in Mg and Fe concentrations compared to the control group, but Ca and K levels were significantly lower in the SS and SLE samples. The Zn content was significantly decreased, while the Cu content was significantly increased in SS and SLE samples, according to competitive antagonism known from pharmacology. The two disease groups showed a similar pattern with respect to the observed changes in the concentration of the tested elements.

3. Cost-effective, off-line protein speciation analysis was optimized for MIP-AES and the binding of elements from serum samples from SS and SLE patients to the most important transport proteins.

In the samples from the control group, zinc was detected only in the Alb fraction above the limit of detection, while in the SS and SLE groups it was present in IgG and Cp containing proteins, and the concentration of Zn in the Alb fraction decreased simultaneously. Cu was found to be the most abundant in the Cp-containing protein in the control group, whereas the Cu content of the Cp fraction of SS and SLE samples was significantly reduced and increased in the Alb-containing fraction. We concluded that the concentration of Cu increases with the decrease of the Zn content of the Alb fraction in the autoimmune samples.

There was no significant difference in Mg concentration between the control and the two autoimmune groups, while K was present in the Trf protein fraction at higher concentrations than in the control and SLE groups.

4. In the case of Omniscan [Gd (DTPA-BMA)], we propose a model for the interpretation of the interactions between the serum proteins and Gd^{3+} ion released by the dissociation of the complex.

4.1. We found that the Gd^{3+}-ion released during the dissociation of the [Gd (DTPA-BMA)] complex interacts with the serum proteins (IgG, Trf, Alb, Cp).

Two experiments were performed: where 5 ml of Seronorm ™ (5 ml of 0.025 M $NaHCO_3$, pH 7.4) were prepared containing 1 mM Omniscan (1 mM [Gd (DTPA-BMA)) and 50 μM Ca (DTPA BMA) Gd^{3+} was almost exclusively found in the IgG protein fraction, whereas it was negligible in the Trf, Alb, and Cp protein fractions. After elemental analysis of the fractionated serum proteins incubated for 19 h at 37 °C, the concentration of Gd^{3+} was found to be the highest in the IgG protein fraction, whereas the Gd^{3+} content of the Alb and Cp fractions significantly exceeded the Gd^{3+} ratios found in the same protein fractions of the unincubated sample. Because the [Gd(DTPA-BMA)] complex is neutral under physiological conditions, the large amount of Gd^{3+} ion present in the IgG fraction corresponds to the intact [Gd(DTPA BMA)] complex. This is due to the weak (virtually negligible) interactions between the DEAE Trisakryl® anion exchange resin, the [Gd(DTPA BMA)] complex and IgG. The amount of Gd^{3+} found in the Alb and Cp protein fractions in the incubated sample is significantly higher than that in the unincubated sample in the same proteins, which interact with the Gd^{3+}-ions produced by the dissociation of [Gd (DTPA-BMA)]. The investigation of the interactions between Gd^{3+} ions released by [Gd(DTPA-BMA)] and serum proteins revealed that Trf, Alb and Cp are involved in the coordination of free Gd^{3+}.

4.2. The association between Gd^{3+} ion and human serum albumin (HSA) serum protein was confirmed.

To support our results on the interaction between the above-described proteins and the Gd^{3+} ion, we investigated the interaction between Gd^{3+} and human serum albumin (HSA). The concentration of free metal ion in the Gd^{3+} -HSA system was determined by ICP-OES. Based on the data obtained, it is possible to postulate the subsequent formation of Gd(HSA), Gd_2(HSA) and Gd_3(HSA) complexes. The apparent stability constants of the complexes (pH 7.4, 0.025 M $NaHCO_3$, 0.15 M NaCl, 25°C) were calculated by fitting the experimental data. We proved that assuming the formation of the 1: 1 and 3: 1 complex provides the best statistical description of the experimental data, while the apparent stability product of the 2:1 complex could not be estimated. The estimated apparent stability product of the Gd(HSA)- and Gd_3(HSA)-complexes was β_{11}=1040±70 M^{-1}, β_{31}=(1,06±0,03)×10^9 M^{-3}.

IV. Possible applications of the results

The results obtained in this doctoral thesis may help in the application of the microwave plasma device, which is considered new in its present form, in epidemiological studies. In addition to sample-specific determination of analytical performance characteristics and measurements using reference materials, the technique may be an appropriate alternative to routine analysis. The matrix effect studies performed can provide a useful basis for optimizing the elemental analysis method of other similar matrix types.

Depending on the results obtained from the elemental analysis of serum samples from autoimmune patients, further biomarkers may be found. Complementary comparative analyzis with additional types of systemic diseases may provide data to map difficult-to-diagnose pathways. Elemental analysis of protein speciation can provide a deeper understanding of the biochemical processes underlying degenerative lesions.

In the Omniscan [Gd (DTPA-BMA)] contrast media experiment, it was found that Gd^{3+} ions released by dissociation of the Gd^{3+}-complexes are involved in complex formation with serum proteins (Trf, Alb, CP), and the corresponding species may accumulate in different tissues (liver or brain) through active and passive transport.

The results presented in this dissertation prove the importance of elemental analysis in medical diagnostic studies and present cost-effective alternatives that are easily adaptable to descriptive and comparative experimental work.

V. ABBREVIATIONS IN THE DISSERTATION

MIP-AES:	microwave plasma atomic emission spectrometry
ICP-OES:	inductively coupled plasma optical emission spectrometry
GFAAS:	graphite furnace atomic absorption spectrometry
FAAS:	flame atomic absorption spectrometry
ICP-MS:	inductively coupled mass spectrometry
SLE:	systemic lupus erythematosus
SS:	Sjögren's syndrome
DTPA-BMA:	diethylenetriamine-N,N,N',N",N"-pentaaceticacid-bismethylamide
MRI:	magnetic resonance imaging
IgG:	immunoglobulin G
Trf:	transferrin
Alb:	albumin
Cp:	ceruloplasmin
Tris:	tris- (hydroxymethyl) aminomethane $(OH\text{-}CH_2)_3\text{-}C\text{-}NH_2$
NSF:	nephrogenic systemic fibrosis
HSA:	human serum albumin

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List of publications related to the dissertation

Foreign language scientific articles in international journals (2)

1. Fehérné Baranyai, E., **Tóth, C. N.**, Fábián, I.: Elemental Analysis of Human Blood Serum by Microwave Plasma-Investigation of the Matrix Effects Caused by Sodium Using Model Solutions.
   Biol. Trace Elem. Res. 191, 1-11, 2019. ISSN: 0163-4984.
   DOI: http://dx.doi.org/10.1007/s12011-019-01743-1
   IF: 2.431 (2018)

2. **Tóth, C. N.**, Fehérné Baranyai, E., Csípő, I., Tarr, T., Zeher, M., Posta, J., Fábián, I.: Elemental Analysis of Whole and Protein Separated Blood Serum of Patients with Systemic Lupus Erythematosus and Sjögren's Syndrome.
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   DOI: http://dx.doi.org/10.1007/s12011-017-0945-y
   IF: 2.361

List of other publications

Foreign language international book chapters (1)

3. Buzetzky, D. B., **Tóth, C. N.**, Nagy, N. M., Kónya, J.: Sorption of phosphate and arsenite ions on modified bentonites.
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Hungarian scientific articles in Hungarian journals (1)

4. Lós, R., Izbékiné Szabolcsik, A., Ungvári, C., **Tóth, C. N.**, Fehérné Baranyai, E., Bodnár, I.: Szintetikus fürdővizek makro- és mikroelem tartalmának vizsgálata. *Int. J. Eng. Manag. Sci. 4* (2), 61-69, 2019. EISSN: 2498-700X. DOI: http://dx.doi.org/10.21791/IJEMS.2019.2.7.

Foreign language scientific articles in Hungarian journals (1)

5. Buzetzky, D. B., **Tóth, C. N.**, Nagy, N. M., Kónya, J.: Application of Modified Bentonites for Arsenite (III) Removal from Drinking Water. *Period. Polytech.-Chem. Eng. 63* (1), 113-121, 2019. ISSN: 0324-5853. DOI: http://dx.doi.org/10.3311/PPch.12197 IF: 1.382 (2018)

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6. **Tóth, C. N.**, Harangi, S., Károlyi, A., Fábián, I., Fehérné Baranyai, E.: Method development for the elemental analysis of organic rich soil samples by microwave plasma atomic emission spectrometry. *Stud. Univ. Babes-Bolyai Chem. 62* (4), 483-494, 2017. ISSN: 1224-7154. DOI: http://dx.doi.org/10.24193%2Fsubbchem.2017.4.41 IF: 0.305

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