Microanalytical method development and speciation analysis for investigation of biological systems

PhD thesis

Zsófia Márta Polgári

Semmelweis University
Doctoral School of Pharmaceutical Sciences

Supervisor: Dr. Gyula Záray, DSc
Consultant: Dr. Norbert Szoboszlai, PhD

Reviewers: Dr. Zsuzsanna Hartyáni, CSc
Dr. Péter Horváth, PhD

Head of Examination Committee: Dr. Éva Szökő, DSc
Members of Examination Committee:
Dr. László Lázár, CSc
Dr. András Gergely, CSc

Department of Analytical Chemistry, Institute of Chemistry
Eötvös Loránd University

Budapest, 2011

I. Introduction

Over the past two decades, instrumental analytical methods play more and more significant role in answering biological questions. During the development of this type of methods the character, amount, possible sample preparation of the biological matrix should be considered; therefore, precise, reproducible analytical methods with adequate limit of detection should be developed. For fulfillment of this task, for a chemist it is necessary to learn more about the biological background and methods; in return, for a biologist, to learn more about the analytical methods if reliable answers should be given for the arisen questions. During our investigations, such methods were applied and developed that are suitable for contamination-free investigation of minute (typically 100-300 μg) biological samples (this thesis mainly dealing with cancer cells).

Our work dealt with instrumental analytical method development in field of elemental analysis and speciation.

The first part of the thesis deals with the determination of the "low Z" elements ($Z \leq 23$) in different biological matrices by total reflection X-ray fluorescence (TXRF) method. The Na, Mg, P, S, K, Ca content of human serum, bovine liver, Antarctic krill and spinach certified reference materials were

determined after having applied different sample preparation methods.

In the thesis, further method developments focused on the quantitative determination of iron as well as on the identification of the oxidation state of iron in cancer cells. Nowadays, iron homeostasis of cancer cells is subject of intensive research. Iron is an indispensable element for human body but many studies deals with positive correlation between Fe stores in the body and risk of cancer development. Rapidly dividing cancer cells require higher amounts of Fe than the normal ones; therefore iron chelation therapy can be a new way of chemotherapy. Generally, in experiments performed with iron chelators, changes in the iron content of cells are followed with ^{59}Fe radiolabelled isotopes.

By studying these literature data, the following question arose: Do iron chelators influence the Cu and Zn content of cells? Therefore, the second part of the thesis is related to the above-mentioned topic, as the Fe, Cu and Zn content of cancer cells were determined by TXRF method. Moreover Fe and Cu content of these cells were also determined by graphite furnace atomic absorption spectrometry (GF-AAS) and the necessary sample preparation procedure was also developed.

The relatively easy Fe(III)/Fe(II) conversion makes iron a pivotal and, at the same time, a dangerous element, respectively.

The SR-TXRF-XANES (synchrotron radiation induced total reflection X-ray fluorescence/ X-ray absorption near edge structure) method offers the possibility to investigate the oxidation state and coordination environment of iron in cancer cells.

The third part of the thesis shows the developed sample preparation method for SR-TXRF-XANES analysis and the obtained results.

II. Objectives

1. Determination of „low Z" (Z≤23) elements by TXRF method

The aim of the work was to answer the following questions:

- Is the „low Z" TXRF technique suitable for the quantitative determination of „low Z" elements in biological samples?
- Which digestion method is the most efficient for the sample preparation of biological samples before „low Z" TXRF analysis?

- Can the sulphur content of cancer cells be determined by using „low Z TXRF" method? Can the sulphur content of cells be applied as a reference value?

In order to achieve these objectives, the recoveries of Na, Mg, P, S, K and Ca were determined both in one liquid (human serum) and three solid biological certified reference materials (bovine liver, Antarctic krill and spinach) after having applied different sample digestion methods. Certified reference materials with different organic and inorganic matter content were analyzed by „low Z" TXRF spectrometer without digestion (i.e., the liquid certified reference material), after microwave-assisted acid digestion in conventional and micro-vessels, as well as after vapor-phase acid digestion performed directly on the TXRF carrier plates. Relationship between the number of cells and their sulphur content was also investigated.

2. Determination of Fe, Cu and Zn in HT-29 cells by TXRF and GF-AAS methods

The aim of this part of our work was to determine the Fe, Cu and Zn content in HT-29 colorectal cancer cells by TXRF technique and to develop the sample preparation method in order to accomplish this analytical task. In the lack of certified reference materials for cells, a further objective was the confirmation of the results by performing measurements with

graphite furnace atomic absorption spectrometry (GF-AAS). The study aimed to determine the Fe, Cu and Zn content in HT-29 cells treated with different iron compounds (Fe(II) sulfate, Fe(III) chloride, Fe(III) citrate and Fe(III) transferrin) and iron chelators (Dp44mT, EDTA) by using the developed method.

3. Iron speciation in cancer cells by SR-TXRF-XANES

The aim of this study performed at the synchrotron of Hamburg (HASYLAB, DORIS III, beamline L) was to determine the oxidation state of Fe in human cancer cells by SR-TXRF-XANES technique and to develop the sample preparation necessary to accomplish this analytical task. Furthermore, another goal was to investigate the K absorption edge of Fe in different cancer cell lines (ZR-75-1, HT-29, MDA-MB-231 and HCA-7) being found in different growth phases (lag, log, plateau), after treatments with different chemicals ($CoCl_2$, $NiCl_2$, $MgSO_4$, antimycin A, 5-fluorouracil) and iron compounds ($FeSO_4$, $FeCl_3$, Fe(III) citrate, Fe(III) transferrin) in order to reveal changes in the iron oxidation state and coordination environment in cells.

III. Materials and methods

1. Materials and methods used for the determination of „low Z" (Z≤23) elements by TXRF method

Four reference materials were used: human serum SERONORM^{TM} Trace Elements Serum Serum Level 1, bovine liver NIST 1577a, Antarctic krill MURST-ISS-A2 and spinach IAEA-331.

Microwave-assisted digestion was carried out by using an Ethos 1 equipment supplied by Milestone (Sorisole, Bergamo, Italy). The analysis of the samples was carried out by using a Wobistrax „low Z" TXRF spectrometer.

Three different digestion methods were compared:

- **Conventional method:** about 0.05–0.1 g of the sample was digested with 2 mL concentrated nitric acid in polytetrafluoroethylene (PTFE) vessels (20 min, 220 °C). After cooling down, 10 μL of 1000 mg/L Ti internal standard was added to the samples; the content of the vessels was transferred into 25 mL volumetric flasks and filled up to the mark. From the resulting stock solution, 5 μL was pipetted onto the quartz carrier plate of the spectrometer. This procedure was used for all 4 certified standard reference samples.
- In the case of the SERONORM sample, a **low-volume digestion method** was also used. Two hundred μL of concentrated nitric acid was added to 50 μL of serum sample,

contained in 1.5-mL PTFE micro vessel closed with a tight-fitting screw-cap. The digestion was performed on a hot plate at 120 °C for 20 min. After the digestion, 5 μL of a 50 mg/L Ti internal standard was added to the digestates and 5 μL of the resulting solution was pipetted onto the quartz carrier plate.

- In the case of the SERONORM sample, **direct digestion performed on the quartz carrier plate was also carried out**. A quartz tripod was placed in the PTFE vessels. Then, the quartz plate carrier, onto which the sample had been dropped, was placed on the top of it. In order to protect the sample from cross-contamination, the sample carrier was covered with a quartz lid. Concentrated nitric acid was poured into the PTFE vessels and the digestion was carried out in vapor-phase between 160^{o}C and 200^{o}C for 20 min.

2. Materials and methods used for the determination of Fe, Cu and Zn

The simultaneous determination of Fe and Cu by GF-AAS was performed on a Perkin-Elmer Model SIMAA 6000 atomic absorption spectrometer. The GF-AAS measurements were carried out with the application of $Pd(NO_3)_2$ as a chemical modifier. For the quantitative determination, the spectral lines of Cu 324.8 nm and Fe 305.9 nm were selected. For quantitative determination, external calibration was used.

The TXRF analyses were performed by using an ATOMIKA 8030C TXRF spectrometer. Gallium was used as internal standard. The Kα lines used for determination of Cu, Fe, Zn and Ga internal standard were 8.047 keV, 6.403 keV, 8.638 keV and 9.251 keV, respectively.

Cell culture and sample preparation

All cell lines were cultured at the Department of Clinical Research of the National Institute of Oncology, Budapest. The HT-29 human colorectal adenocarcinoma cells were cultured to 80% confluency in 6-well plates (10^6 cells/well). Cells were incubated for 4 h with increasing concentrations (10, 20, 50 and 100 μmol/L expressed as Fe in medium) of different iron compounds: Fe(II) sulfate, Fe(III) chloride, Fe(III) citrate and Fe(III) transferrin. Similar treatments were also carried out in fetal calf serum (FCS) containing and FCS-free medium. In the case of each experiment, untreated cells (grown only in culture medium) were used as controls. Concentration of the treatments in the chelator experiments were the following: 20 μM of Fe(II) sulfate, 50 μM of Dp44mT or EDTA.

After incubation, cells were harvested with a trypsin-EDTA solution. Trypsinization was stopped by dilution with phosphate buffered saline (PBS) and then, the cells were pipetted into Eppendorf tubes. The cells were washed two

times with 1 mL PBS. The cell number was counted before the second centrifugation by using a Bürker chamber. The cells were centrifuged in Eppendorf tubes. After the second centrifugation, 20 μL of 30% H_2O_2, 80 μL of 65% HNO_3 and 15 μL of 10 μg/mL Ga were added to the cells and digested for 24 h at room temperature (partial digestion). From the resulting solutions, 10 μL were pipetted on the quartz reflectors used for TXRF analysis. The reflectors were dried on a hot plate at 80 ºC for 10 min. For GF-AAS analysis, 10 μL was directly dispensed into the graphite tube by the autosampler.

3. Materials and methods used for iron speciation studies

For the investigation of the oxidation state of Fe in cells, the XANES measurements in fluorescence mode was applied. The absorption measurements were carried out in vacuum by using the setup at the beamline DORIS III L at HASYLAB (DESY, Hamburg) with grazing incidence geometry at the Fe K edge.

Sample preparation, treatment of cells

10^5–2×10^5 cells (HT-29 cells, ZR-75-1 human breast cancer, HT-1080 human fibrosarcoma and MDA-MB-231 human breast cancer cells) were treated at 80% confluency in 6-well plates in sterile PBS (phosphate buffered saline) for 20 min or 4 h. The concentration of the chemicals used for the treatments was the following: 1 mM of $NiCl_2$, 2.4 and 57.6 mM of $CoCl_2$,

10 mM of $MgSO_4$, 25 and 300 μM of antimycin A and 130 μM of 5-fluorouracil. The treatments with different iron compounds (50 μM of Fe(II) sulfate, Fe(III) chloride, Fe(III) citrate and Fe(III) transferrin) were carried out for 4 hours in a FCS-free medium.

After treatments, cells were harvested with trypsin, washed twice with isotonic NaCl solution and centrifuged at 20,000 g at 4 °C for 15 min. The cell number was counted by using a Bürker chamber. After the second centrifugation, cells were resuspended in 10 μL isotonic NaCl solution and 5 μL of cell suspension were pipetted onto quartz carrier plates. The estimated cell concentration was $10^4 - 2\times10^4$ cells/μL. The excess of isotonic NaCl solution was removed by pipetting. After this procedure the cell monolayer was checked microscopically and dried at room temperature. All quartz carrier plates were placed into Ar-filled vessels and transported to the HASYLAB at DESY.

IV. Results

1. Determination of „low Z" (Z≤23) elements by TXRF method

In the case of the serum sample, the direct determination (without digestion) of K and Ca can be performed. However, the self-absorption effect observed at „low Z" elements

hampers their accurate determination. Thus, for P and S determination, the use of matrix-matched response factors can be recommended.

The conventional digestion method is a very efficient method for the analysis of biological samples with high dry weight content if the concentration of the analytes in the final solution after dilution exceeds the quantification limit. Recovery values for spinach and Antarctic krill samples were significantly worse than those for the bovine liver and human serum certified reference materials. The reason of this phenomenon may be the high inorganic matter content of the first two above-mentioned reference materials.

In the case of low-volume digestion, the organic matter cannot be properly eliminated and the dilution of the inorganic matrix is too low; thus, higher background and self-absorption of the fluorescent radiation occurred, deteriorating the analytical performance.

In the case of the vapor-phase digestion, the presence of the undiluted inorganic compounds and the partial flush-off of the sample do not facilitate any quantitative analysis.

On the basis of the results, the conventional acid digestion can be mainly recommended for the determination of „low Z" elements in biological matrices.

The S content in HT-29 cells determined by Wobistrax „low Z" TXRF spectrometer was in good agreement with the results obtained by ATOMIKA 8030C instrument. Linear correlation was observed between the cell number and their sulphur content. Later on, this correlation was applied to check the cell number in further experiments.

2. Determination of Fe, Cu and Zn in HT-29 cells by TXRF and GF-AAS methods

The developed multielemental TXRF and simultaneous GF-AAS methods are suitable for determination of Fe, Cu in HT-29 human colorectal cancer cells treated with different iron compounds (Fe(II) sulfate, Fe(III) chloride, Fe(III) citrate and Fe(III) transferrin) after a relatively simple sample preparation protocol consisting of treatment of cell sample with a mixture of 65% nitric acid and 30% hydrogen peroxide for 24 h in the same vessels used for centrifugation. Although complete digestion of the samples could not be achieved, the selected analytical methods (TXRF and GF-AAS) allowed an accurate determination of these types of biological matrices minimizing their contamination. Thus, results obtained for Cu and Fe by TXRF and GF-AAS method were in good agreement. Determination of Fe in high concentration and Zn were possible only by TXRF.

The developed sample preparation method is suitable for the TXRF or GF-AAS determination of Fe, Cu, Zn in 0.2 -10 × 10^6 cells.

According to the Fe uptake studies, HT-29 cells incubated in FCS-free medium contained cca. 5–50 times higher Fe amount comparing to cells cultured in FCS supplemented medium and notable differences could be observed in the iron uptake according to the different Fe compounds used.

By using the developed methods, it is possible to follow the Fe, Cu and Zn content in cancer cells, which was applied in our experiments. On the basis of the experiments using the novel iron chelator Dp44mT, it can be concluded that this chelator influences not only Fe but also Cu and Zn homeostasis of cancer cells. Due to this fact, the determination of Cu and Zn content of neoplastic cells in iron chelator studies is recommended, because these elements may also play role in the antiproliferative activity of iron chelators.

3. SR-TXRF-XANES analysis of cancer cells

The developed sample preparation method consisiting of pipetting the cell suspension onto the quartz reflectors and the SR-TXRF-XANES geometric arrangement are well applicable for XANES studies aiming at determination of iron oxidation state in cancer cells and semiquantitative determination of

elemental composition, too. The method is simple, relatively fast and the risk of contamination is minimal due to the small number of sample preparation steps . A drawback of the procedure is that the cell layer should be checked for each sample by a microscope, and its thickness should not exceed that of a monolayer. The method may be suitable for studying species of other elements like Cr, Co, Ni, Cu or Zn.

From the XANES analysis it can be concluded that the molecular environment of iron remained the same during all growth phases of the cells and there were no significant differences among the investigated cell lines.

The oxidation state of iron in cells treated with $CoCl_2$, $NiCl_2$, $MgSO_4$ was practically unchanged and their XANES spectra were very similar to that of ferritin. Differences in the position of the absorption edge were observed in XANES spectra of cells treated with 5-fluorouracil and antimycin A (the edge position of 5-fluorouracil treated cells is shifted towards higher energies, meanwhile the antimycin A treatment influenced the shape of the spectrum).

Differences could be observed in the shape of XANES spectra of cells treated with different iron compounds but the interpretation of these results needs further experiments.

V. Conclusions

1. For the determination of „low Z" elements (Na, Mg, P, S, K and Ca) in biological samples by TXRF, the conventional acid digestion can be mainly recommended.

2. On the basis of our results, the determination and application of matrix matched response factors can be recommended to achieve the best analytical reproducibility and recovery in the case of „low Z" element determination in biological samples.

3. The developed sample preparation method is suitable for the TXRF or GF-AAS determination of Fe, Cu, Zn in 0.2 -10 × 10^6 cells.

4. The most important parameters of the analytical capability of the developed TXRF and GF-AAS methods for the determination of Fe, Cu and Zn in HT-29 cells are the following:

	TXRF	**GF-AAS**
Reproducibility	<10 %	<5 %
Recovery	87-105 %	98-101 %
Limit of detection		
Cu	10.3 ng/mL	0.7 ng/mL
Fe	14.5 ng/mL	9.7 ng/mL
Zn	9.8 ng/mL	-

5. HT-29 cells incubated in FCS-free medium contained cca. 5–50 times higher Fe comparing to cells cultured in FCS supplemented medium and notable differences could be observed in the iron uptake according to the different Fe compounds used in the uptake experiments.

6. On the basis of the experiments using the novel iron chelator Dp44mT, it can be concluded that this chelator influences not only Fe but also Cu and Zn homeostasis of cancer cells and the changes in Cu and Zn content of cells can also play role in the antiproliferative effect of iron chelators.

7. The developed sample preparation method consisting of pipetting the cell suspension onto the quartz reflectors and the SR-TXRF-XANES geometric arrangement are suitable for the determination of oxidation state of iron in cell lines as well as for semiquantitative determination of elemental composition.

8. From the SR-TXRF-XANES analyses, it can be concluded that the molecular environment of iron remained the same during all growth phases of the cells and there were no significant differences among the investigated cell lines.

9. The oxidation state of iron in cells treated with $CoCl_2$, $NiCl_2$, $MgSO_4$ was practically unchanged and their XANES spectra were very similar to that of ferritin. Differences in the

position of the absorption edge were observed in XANES spectra of cells treated with 5-fluorouracil and antimycin A.

10. Differences can be observed in the shape of XANES spectra of cells treated with different iron compounds but the interpretation of these results needs further experiments.

VI. Publications

1. Szoboszlai N, Polgári Z, Mihucz VG, Záray G. (2009) Recent trends in total reflection X-ray fluorescence spectrometry for biological applications. Anal Chim Acta 633: 1-18. *IF: 3.757*

2. Polgári Z, Meirer F, Sasamori S, Ingerle D, Pepponi G, Streli C, Rickers K, Réti A, Budai B, Szoboszlai N, Záray G. (2011) Iron speciation in human cancer cells by K-edge total reflection X-ray fluorescence-X-ray absorption near edge structure analysis. Spectrochim Acta Part B At Spectrosc. 66: 274-279. *IF: 3.549*

3. Polgári Z, Szoboszlai N, Óvári M, Záray G. (2011) Possibilities and limitations of the total reflection X-ray fluorescence spectrometry for the determination of low Z elements in biological samples. Microchem J. DOI: 10.1016/j.microc.2011.06.002 *IF: 2.48*

4. Polgári Z, Ajtony Z, Kregsamer P, Streli C, Mihucz VG, Réti A, Budai B, Kralovánszky J, Záray G, Szoboszlai N. (2011) Microanalytical method development for Fe, Cu and Zn determination in colorectal cancer cells. Talanta DOI:10.1016/j.talanta.2011.07.015 *IF: 3.722*

VII. Acknowledgements

I would like to express my gratitude to my supervisor, professor Gyula Záray for directing and financing my work, and for the countless advice given to me for the writing of the thesis.

I gratefully thank professor Éva Szőke, head of Doctoral School of Pharmaceutical Sciences who made my PhD work possible. I, hereby, acknowledge the predoctoral scholarship given by the Semmelweis University School of PhD Studies .

I would like to thank my consultant, Norbert Szoboszlai, who helped me to plan, accomplish, evaluate and interpret the experiments. I am grateful to Mihály Óvári for the help with the „low Z" experiments and instrument. I also thank Victor Gábor Mihucz for his altruism and encouragement during my PhD work.

I warmly thank Judit Kralovánszky, head of Department of Clinical Research of the National Institute of Oncology, who

allowed me to carry out all the experiments related to cancer cells at her department. I really appreciated her help and renewed interest in my research. I would like to thank Barna Budai for his help given for the statistical data evaluation and his useful remarks. I am grateful to Andrea Réti for introducing me into the mysterious world of cell culturing and for her friendly encouragement.

I would like to thank professor Christina Streli, professor Peter Wobrauschek and Peter Kregsamer, who made always possible to measure my samples in the Atominstitut in Vienna. I really enjoyed the peaceful, friendly atmosphere in their laboratory. I am grateful to Florian Meirer for the evaluation of XANES measurements and for his enthusiastic and detailed answers of my questions.

I would like to thank Zsolt Ajtony who made possible the GF-AAS measurements in his department, and thanks for his help, too. I am also indebted to Zsolt Bendő and Csanád Pénzes for the SEM images and for the AFM measurements, respectively.

Last but not least, I would like to thank my parents for all their love and encouragement and to Bálint for his long-lasting patience and for creating day by day a peaceful background environment.