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"Petru Poni"
Institute of Macromolecular Chemistry

NMR Techniques in the Study of Biofluids

ABSTRACT OF THE PhD THESIS

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Domnului/Doamnei.....

Vă facem cunoscut că în ziua de **25 aprilie 2016**, ora **12:00**, în biblioteca Institutului de Chimie Macromoleculară „Petru Poni" din Iaşi va avea loc susţinerea publică a tezei de doctorat **"Tehnici RMN în studiul biofluidelor",** autor inginer chimist **Alina Elena Nicolescu**, în vederea conferirii titlului ştiinţific de doctor.

Comisia de doctorat are următoarea componenţă:

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CONDUCĂTOR ŞTIINŢIFIC	**Acad. Bogdan C. Simionescu**, Institutul de Chimie Macromoleculară „Petru Poni" din Iaşi
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În conformitate cu Regulamentul privind organizarea şi desfăşurarea doctoratului pentru acordarea titlurilor ştiinţifice în Academia Română, vă trimitem rezumatul tezei de doctorat cu rugămintea de a ne comunica în scris aprecierile şi observaţiile dumneavoastră.

Cu această ocazie vă invităm să participaţi la susţinerea publică a tezei de doctorat.

Director,
Dr. Anton Airinei

ACADEMIA ROMÂNĂ INSTITUTUL DE CHIMIE MACROMOLECULARĂ "PETRU PONI" IAŞI

CONTENT

KEYWORDS

- NMR spectroscopy
- Metabolites
- Markers
- Rare metabolic diseases
- Diabetes
- Urine
- Cerebrospinal fluid
- Amniotic fluid
- Aminoacids
- Organic acids
- Amines
- Aminoxides
- Pyrrolobenzimidazoles
- Pyrroloquinoxalines

“Why just NMR? – Because there is hardly another technique that is so informative for so many different types of applications, and because there is no other technique that provides so much fun”.

Prof. Richard R. Ernst,
ETH Zurich, Nobel Laureate 1991.

Introduction

The main purpose of the thesis is the development of medical diagnostics methods based on NMR spectroscopy, for the diagnosis of pathologies for which the classical diagnostic is very difficult to obtain or where conventional methods provide limited information.

The specific objectives are:

- Provide immediate medical diagnostics in various cases of rare metabolic diseases, which is very useful for medical doctors.

- Obtain relevant information to understand the mechanism of diabetes and identifying useful markers for early diagnosis of this pathology.

- Explore the potential of NMR spectroscopy in the study of less accessible biofluids (cerebrospinal fluid and amniotic fluid).

- The advancement of knowledge in the field of biomedical applications of NMR spectroscopy and publishing the results in scientific journals.

- Development of a NMR spectral database with the main metabolites and study the influence of experimental conditions on the spectral profile of biological fluids.

- Explore the potential of ^{15}N NMR spectroscopy in the study of metabolites and active biological compounds.

Part 1. Review.

CHAPTER 1. NMR TECHNIQUES AND BIOMEDICAL APPLICATIONS

The thesis includes an overview of the main types of pulse sequences used in NMR spectroscopy both from a historical perspective as well as the utility and the information generated by one- and two-dimensional experiments. It also discusses the most important parameters of the pulse sequences: pulses, decoupling pulses, relaxation delays, acquisition time, etc.

The second chapter of the literature study presents a description of the main body biofluid. Their composition, functions, methods of storage and stability are presented. Several literature studies on the biomedical applications of NMR spectroscopy are reviewed.

Part 2. Original results.

CHAPTER 2. NMR URINARY MARKERS IN THE STUDY OF METABOLIC RARE DISEASES

In this chapter the results of the NMR studies on diagnosis of some rare metabolic diseases are presented. The rare metabolic diseases are known as inborn errors of metabolism – IEM. These results were obtained from the analysis of urine samples from children diagnosed with various inborn errors of metabolism.

The aim was to establish the diagnosis and assess the potential of NMR techniques to study the metabolic profile induced by these diseases. The research directions were:

1. Identification of some urinary markers for several rare metabolic diseases.

2. Establishing the diagnostic for the analyzed cases.

3. Following-up of the treatment response for some cases of organic acidurias, thorough quantification of the selected markers.

The major advantage of NMR spectroscopy is that it offers the "global metabolic fingerprint" of the analyzed sample, unlike classical methods of analysis which require special kits or special techniques, available only for a limited number of metabolites (markers selection). This feature becomes important in the study of inborn errors of metabolism as it provides access to more metabolites, which can then be associated with various metabolic defects. By conventional methods, if the preselected marker for a specific inborn error of metabolism is not detected, the result is negative and there are no doubts in this respect. Using NMR techniques, because it is not necessary to select a specific marker, in the case of metabolic illnesses, the spectral profile will change. In this way, although the result is negative for the suspected marker, the overall result is false negative in relation with general health.

During the PhD period, 18 cases of rare metabolic diseases were identified through NMR Spectroscopy. For 3 cases the diagnostic was firstly established using NMR spectroscopy. For all the cases, the diagnostic was later confirmed abroad, using classical methods.

In the theses, I showed that if the intuitive treatment is applied for metabolic diseases, this can produce a decrease in concentration for the specific markers, leading to a false negative diagnostic. Such a case was described for the galactosemia subject, for whom the classical methods indicated a negative result, but through NMR Spectroscopy the proper diagnostic could be made. In another case in which the main marker was missing, the correct NMR monitoring was done using a secondary marker.

Using NMR spectroscopy a unique monitoring in Romania, can be done for cases of rare metabolic diseases. Through this monitoring it is possible to detect the families which do not follow the treatment or the specific diets. If the treatment is not followed, the repercussions are extremely serious, culminating with the death of the patients.

The majority of the cases analyzed in this period were children between 1 and 3 years old. For one of the monitored cases, the quality of the prescribed milk was checked.

Some of the results presented in this chapter were presented at scientific meetings as one conference, 4 oral presentations and 8 posters.

CHAPTER 3. NMR SPECTROSCOPY APPLICATIONS IN THE STUDY OF DIABETES

Diabetes is a chronic metabolic disease characterized by increased blood glucose. There are two types of diabetes - Type I diabetes (typically found in children and adolescents, it requires administration of insulin) and diabetes type II (present in adults over 30 years, it is characterized by insulin resistance and hyperglycemia). According to the World Health Organization (WHO), in 2014, the incidence of diabetes was 9% of the global adult population. According to the same sources by 2030, diabetes will become the 7th leading cause of death.

Classical methods for monitoring of people with diabetes are based on checking the blood glucose concentration concentrations.

The methodology used in this chapter involved two parallel approaches:

1. Determine individual concentrations of metabolites identified in the NMR spectra of urine samples.

2. Statistical analysis of the NMR spectral fingerprint.

The conclusions of these studies were:

1. It has been shown that the study of urine samples by ^{1}H NMR spectroscopy is particularly useful for monitoring the metabolic processes associated with type I diabetes and renal function. Some of the metabolites measured by the NMR spectroscopy can not be analyzed by other conventional techniques commonly used in the clinic.

2. The results obtained in this study contribute to strengthening the hypothesis that increasing of the concentration of metabolites mentioned above is characteristic for type I diabetes by alterations in metabolic pathways or renal function.

3. For several NMR urinary markers associated with type I diabetes, the tendency of concentrations evolution was determined, (the results are reviewed and put into the context of current knowledge of biochemical processes associated with the studied pathology).

4. For this study the NMR spectra from 172 urine samples were processed, converted into digital format (X / Y) and introduced in the statistically analysis. These spectra can be constituted in a reference data base that can be used for other studies involving the NMR spectra of urine samples recorded at 400 MHz.

5. It was developed a statistical model based on NMR spectra, for discrimination between diabetic subjects and normal subjects groups, without resorting to classic glucose marker.

6. A correlation was made between the concentrations of metabolites of subjects involved in this study and literature data for subjects from different geographical areas.

7. The results obtained in this chapter were partially published in two ISI papers and presented at scientific meetings in the form of 2 oral presentations and 3 posters.

CHAPTER 4. NMR CONTRIBUTIONS IN THE ANALYSIS OF CEREBROSPINAL AND AMNIOTIC FLUIDS

In this chapter the results obtained after the NMR analysis of two fluids: cerebrospinal fluid (CSF) and amniotic fluid are presented. The aim was to assess the potential of NMR techniques to study the metabolic profile characteristic for these two fluids. This was achieved by two research directions:

1. Identification and quantification of metabolites present in the canine cerebrospinal fluid.

2. Identification and quantification of metabolites present in human amniotic fluid.

The conclusions of these studies were:

1. The results presented in this chapter can be considered as reference data for normal subjects, since these fluids are very difficult to obtain and the literature data is very scarce in this respect. These data can be used for comparison with pathological values (for which there is more literature data available than for normal samples).

2. The values obtained were of the same order of magnitude as the few existing literature data, confirming the accuracy of the results. These data can also contribute to increase the statistical significance of the existing literature data.

3. The results obtained in this chapter were published in one ISI article and presented at scientific meetings as one poster.

CHAPTER 5. SIGNAL ASSIGNMENT STRATEGIES AND FACTORS AFFECTING THE NMR FINGERPRINT OF BIOFLUIDS

The NMR spectra of biological fluids are very complex, containing many signals with different intensities and shapes, many of them overlapping. In this situation, the interpretation of such a spectrum is very difficult. This complexity is due to the large number of metabolites and biochemical composition variation.

In the analysis of biological samples, each stage from taking the sample to obtaining the NMR spectrum, must be carefully prepared and be perfectly reproducible for the whole lot of samples in the study. To obtain more reproducible results and fewer errors is necessary to identify the factors that may influence the NMR profile of the biological sample. The profile can be influenced by many factors. Biological factors (age, diet, gender, medication, etc.) are more difficult to control, but the technical ones (sample handling, NMR parameters used in the analysis, etc.) can be standardized, at least in the laboratory analyzing such samples.

Given the above facts, this chapter presents the following studies:

1. Creating a spectral database of metabolites. Information obtained from the implementation of this database is later used in confirming the assignments made in the case studies included in Chapters 2-4.

2. Description of the way in which the signals are assigned in the ^{1}H NMR spectra of biological fluids.

3. Studying the influence of two common painkillers on the spectral profile of urine samples.

4. Studying the influence of pH on the spectral profile of urine samples.

The conclusions of these studies were:

1. A spectral database was developed, consisting of different types of one and two-dimensional NMR spectra in aqueous solution, for several individual metabolites

2. It was conducted an extensive study on the influence of pH upon the chemical shifts of signals of metabolites.

3. The change of NMR spectral fingerprint of urine samples following administration of analgesics was studied.

4. Several metabolites of aspirin and acetaminophen were identified in the NMR spectra of urine samples and it was determined the period for which these signals are present in urine, after administration of the therapeutic doses.

5. The results obtained in this chapter were used to support the studies presented in Chapters 2-4.

6. Some of the results were partially published in 2 ISI journals and 1 BDI+ journal, and presented as a poster at a scientific conference.

CHAPTER 6. APPLICATIONS OF ^{15}N NMR SPECTROSCOPY IN STRUCTURAL CHARACTERIZATION OF METABOLITES AND BIOLOGICAL ACTIVE COMPOUNDS

This chapter presents the technical aspects influencing the NMR spectroscopy detection of nitrogen isotop, at natural abundance. Although this isotop is very difficult to record by liquid state NMR spectroscopy, the potential of this technique for biomedical sciences is very interesting, which is why the subject was presented in the thesis. The usefulness of the analysis of this isotop is exemplified on several classes of biologically active compounds.

The conclusions of these studies were:

1. The ^{15}N chemical shifts for a large number of biologically important compounds (metabolites, compounds with biological activity) have been obtained.

2. The ^{15}N NMR data were obtained from two-dimensional techniques with indirect detection which have been optimized for each individual case.

3. The influence of *N*-oxidation on the chemical shift of the ^{15}N isotop in a series of amines was studied. *N*-oxidation is a process of great importance in biochemical reactions.

4. The results helped to elucidate the reaction mechanisms in the synthesis pyrrolobenzimidazoles and pyrroloqyinoxaline derivatives.

5. The results were partially published in 8 articles and 3 posters.

CHAPTER 7. EXPERIMENTAL PART

This chapter discusses in detail the handling and storage of samples until NMR analysis, the protocol for samples preparation for NMR analysis, the experimental conditions for recording NMR spectra and the important parameters used in the pulse sequences.

CHAPTER 8. CONCLUSIONS

1. 18 cases of rare metabolic diseases in children and newborns have been diagnosed by NMR spectroscopy, and this was vital for patients and doctors.

2. For all the studied cases, the diagnostic obtained by NMR spectroscopy was subsequently confirmed by conventional tests abroad.

3. NMR spectroscopy allowed a unique monitoring in Romania for cases of rare metabolic diseases.

4. A comparative study between patients with type I diabetes and a control group was performed and concentrations correlations for a number of metabolites, others that classic one (glucose) were obtained.

5. A statistical model that allows the NMR diagnosis of patients with type II diabetes, without using the glucose marker, was developed, with an accuracy of over 80%. (If the glucose marker is included in the model the accuracy is 100%).

6. A correlation was performed between the concentrations of urinary metabolites detected by NMR spectroscopy for groups in Romania, and literature data for populations in other countries located at great geographical distance.

7. A NMR study was conducted on samples of cerebrospinal fluid from dogs and it was showed that this data can be extrapolated to human patients, for whom it is very difficult to obtain cerebrospinal fluid samples from healthy persons. The data are useful for the study of central nervous system disorders.

8. Several amniotic fluid samples were studied by NMR spectroscopy, the obtained concentrations can contribute to very little existing literature data.

9. A database was developed with ^{1}H / ^{13}C one and two-dimensional NMR spectra for a large number of metabolites. These spectra have proved very useful in the assignment of signals in the studied biofluids.

10. The influence of the pH on the NMR spectra of the metabolites of interest was studied.

11. The aspirin and acetaminophen metabolism in the human body was studied by NMR.

12. It was conducted an extensive study on the isotop ^{15}N NMR spectroscopy detection at natural abundance, for a number of metabolites and compounds with biological activity.

CHAPTER 9. DISSEMINATION OF RESULTS

The results obtained in during the thesis period were partially published in 12 ISI and 2 BD+ scientific papers.

Some of the results were presented at 28 scientific meetings as: 2 conferences, 10 oral presentations and 16 posters

Scientific papers published with the results obtained in the thesis:

1. E. Georgescu, **A. Nicolescu**, F. Georgescu, F. Teodorescu, S. Shova, A. T. Marinoiu, F. Dumitrascu, C. Deleanu, "Fine tuning the outcome of 1,3-dipolar cycloaddition reactions of benzimidazolium ylides to activated alkynes", *Tetrahedron*, **2016**, 1-14, publicat online. (**F.I. 2,641**).

2. E. Georgescu, **A. Nicolescu**, F. Georgescu, S. Shova, B. C. Simionescu, C. Deleanu, "Contributions to synthesis of pyrrolo[1,2-*a*]benzimidazole derivatives *via* 1,3-dipolar cycloaddition reactions", *Rev. Roum. Chim.*, **2016**, acceptat. (**F.I. 0,311**).

3. G. L. Ailiesei, M. Ciobanu, M. Balan, C. Stavarache, L. Barbes, **A. Nicolescu**, C. Deleanu, "NMR detected metabolites in complex natural fluids. Quinic acid in apple juice", *Ovidius Univ. Annals Chem.*, **2015**, *26* (*2*), 51-56. (**BDI+**).

4. E. Georgescu, **A. Nicolescu**, F. Georgescu, S. Shova, F. Teodorescu, A.-M. Macsim, C. Deleanu, "Novel one-pot multicomponent strategy for the synthesis of pyrrolo[1,2-*a*]benzimidazole and pyrrolo[1,2-*a*]quinoxaline derivatives", *Synthesis*, **2015**, *47*, 1643-1655. (**F.I. 2,689**).

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10. E. Georgescu, F. Georgescu, M. R. Caira, **A. Nicolescu**, C. Deleanu, M. G. Danila, P. Filip, F. Dumitrascu, "A new synthesis of pyrrolo[1,2-*c*]quinazoline from quinazolinium *N*-ylides: a re-investigation", *Arkivoc*, **2009**, (*xii*), 232-241. (**F.I. 1,165**).

11. F. Potmischil, M. Marinescu, **A. Nicolescu**, C. Deleanu, "Hydroacridines: ^{1}H and ^{13}C NMR spectra of 9-substituted 1,2,3,4,5,6,7,8-octahydroacridines and of their *N*-oxides", *Magn. Reson. Chem.*, **2009**, *47*, 1031-1035. (**F.I. 1,179**).

12. F. Potmischil, M. Marinescu, **A. Nicolescu**, C. Deleanu, M. Hillebrand, "Hydroacridines: ^{15}N NMR chemical shifts of 9-substituted 1,2,3,4,5,6,7,8-octahydroacridines and their *N*-oxides – Taft, Swain–Lupton, and other types of linear correlations", *Magn. Reson. Chem.*, **2008**, *46*, 1141-1147. (**F.I. 1,179**).

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