"BABEŞ-BOLYAI" UNIVERSITY
FACULTY OF PHYSICS

STUDIES OF BIOLOGICAL SAMPLES USING ATOMIC AND MOLECULAR METHODS

PhD Thesis
Summary

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INTRODUCTION

Spectroscopy is the study of the interaction between electromagnetic radiations and matter. At the same time, spectroscopy represents a generic name for a category of experimental techniques and procedures used in order to qualitatively and quantitatively analyze solid, liquid or gas samples. As a result of the energetic interferences between the electromagnetic radiation and matter, the spectrum of the substance to be analyzed appears, offering exact information on the qualitative and quantitative composition of the matter.

Historically, spectroscopy referred only to the analysis of the visible light spectrum. Nowadays, spectroscopy also covers, along with the visible light spectral area, the remaining of the electromagnetic radiation spectrum, starting with the gamma radiation area and ending with the radio waves area.

From all the spectroscopic techniques, mass spectroscopy is maybe the one offering the most probabilities, due to the large variety of spectrum types that it can offer. At the beginning of the 20^{th} century, mass spectrometry developed as a technique mainly used by the physicians, in order to determine the structure of the atom. At the end of the 30s and the beginning of the 40s, mass spectrometry played an important part in the development of the atomic energy. In the 40s, when mass spectrometry was used in order to identify and quantify the organic substances, the commercial instruments started to appear, which led to its being used in various areas such as: nuclear physics, biology, medicine, geology, the study of the environment.

Mass spectrometry is a powerful tool for the study of all substances, as using only an insignificant quantity it provides more data about the structure and the composition of a substance than any other analytical technique. At the same time, it is also a powerful quantification tool. Taking a stained fruit, one can identify and quantify traces (10^{-15} g) of pesticides; one needs only zeptomole quantities (10^{-21} mol) of proteins in order to characterize certain generic anomalies, or one can detect iron picograms (10^{-12}g) in the silicon crystal, before using it as a raw material in the expensive semiconductor manufacture process.

At its turn, chromatography has the most powerful impact in the qualitative and quantitative analysis area. This is the method used in order to analyze mixtures of hundreds of compounds in only a few minutes time.

By coupling gas chromatography to mass spectrometry, it is possible to detect and identify compounds of an unknown mixture. The sample quantity necessary for the analysis may be extremely small, due to the high performance detectors. The use of computers allowed an important analysis cost reduction and placed the GC-MS technique among the top techniques with applications in many areas, such as: petrochemistry, chemical and pharmaceutical industry, in the

area of air, water, soil, food pollutant analysis, of flavor and volatile oil analysis, clinical studies, criminology, etc.

In this paper, we present the analysis of biological samples using various spectroscopic methods (especially mass spectrometry and gas chromatography) in order to come to specific determinations in medical and pharmacological areas.

The first chapter shortly presents a few theoretical aspects related to the main spectroscopic methods and their applications in biology studies.

In chapters II and III we are describing in detail the mass spectrometry and the gas chromatography, as these are the techniques the most used for the analyses performed in the experimental part.

Chapter IV comprises the experimental results related to the quantitative and qualitative analyses of the biological samples from the above mentioned areas.

The last chapter synthesizes the conclusions drawn from the obtained results.

1. SPECTROSCOPIC ANALYSIS METHODS

1.1 General Information

Spectroscopy is the name given to a category of experimental procedures and techniques by which one follows and quantifies the effect of energy absorption or emission by a sample subjected to qualitative and / or quantitative chemical analysis.

The spectroscopy's purpose is to get, from one spectrum, information on the analyzed sample, such as: internal structure, composition, dynamics. The analytical spectroscopy allows us to recognize the nature of atoms and molecules according to the characteristic shape of their spectra. The high-precision spectroscopy's purpose is to determine certain physical constancies or to test various hypotheses related to natural laws.

Spectroscopic analyses are based on the interaction between the electromagnetic waves and the matter.

The origin of the spectral lines from the *atomic spectroscopy* is given by the variation of one atom's energy as a result of the electronic transitions occurring when a photon is created or absorbed. The atomic spectra are *line spectra*.

The origin of the spectral lines from the *molecular spectroscopy* is given by a photon's creation or absorption, when a molecule's energy varies. A molecule's energy may vary not only as a result of the electronic transitions, but also because the molecule is subject to transformations in

the rotation and vibration status. It results that the molecular spectra are more complex than the atomic ones, they are *band spectra*.

Molecular spectra contain information used to determine a series of molecular properties: molecular dimension and shape, values of the dipole moments, values of the molecule strength and length and of the angle between the connections.

1.2. UV - Visible Spectroscopy

UV - visible spectroscopy is based on the interaction of the electromagnetic waves from the visible and UV area with the substance and it uses the Lambert-Beer Law applied to the waves belonging to this area. This law shows the global absorption effect produced when the electromagnetic radiation travels through a layer of substance whose thickness is x.

$$I = I_0 \cdot e^{-\alpha x} \tag{1}$$

where: I_0 stands for the intensity of the radiation falling on the sample,

I stands for the intensity of the radiation leaving the sample,

x stands for the thickness of the sample layer,

α stands for the attenuation (absorbance) coefficient.

The spectrum is actually a graphic representation of the absorbance according to the wave number $\tilde{\nu}$ ($\tilde{\nu} = 1/\lambda$). The absorbance is defines by the formula:

$$A = \lg \frac{I_0}{I} \tag{2}$$

1.3 IR Spectroscopy

IR Spectroscopy is based on the interaction between the IR area electromagnetic radiation and the molecules of a given substance. It consists in measuring the wavelength and the infrared light absorption intensity by a sample.

If the electromagnetic radiation interacts with the molecules, along with the electron excitement, the molecule may also take energy as molecule rotation energy and molecule vibration energy. The molecule energy consists of:

$$E=E_{tr}+E_r+E_v+E_e$$

where: E_{tr} – translation energy

E_r – rotation energy

E_v – vibration energy

E_e – electronic energy

$E_{tr}<E_r<E_v<E_e$

In spectroscopy it is important to find the expressions for the molecule energy levels, so that to be able to subsequently calculate the transition frequencies by applying the selection rules. The spectrum shape is made taking into account the state populations.

1.4 Electron Spin Resonance (ESR) Spectroscopy

Any particle that has an electric charge and that is moving along a closed path is equivalent with a circular current and it generated a *magnetic dipole*.

The magnetic moment of an electron having an electric charge (e) moving on a circular path with the radius (r) and with the rotation period (T) is:

$$\mu = \frac{e}{Tc}\pi r^2 = \frac{e\nu\pi r^2}{c} = e\frac{\omega}{2\pi c}\pi r^2 = \frac{e\omega r^2}{2c} \tag{3}$$

where: ω - angular velocity of the electron, ν - electron's frequency.

The electron spin resonance (RES) is an analysis physical method based on the energy absorption from the microwaves area by a system of paramagnetic ions placed in a static magnetic field. The paramagnetic ions have incomplete electronic layers, with unpaired electrons having the spin $s = 1/2$ and a dipolar magnetic moment of:

$$\vec{\mu} = \frac{e}{2mc}|\vec{s}| \tag{4}$$

The interaction energy between the paramagnetic ions (magnetic dipoles) and a stationary magnetic field ($\vec{H}$) is expressed by the formula:

$$E = -sg\mu_B H \tag{5}$$

where g – is the Landé factor (or the spectroscopic splitting factor).

If, perpendicularly on the direction of the static magnetic field $\vec{H}$, one applies an alternative high frequency magnetic field $H_Z\ (\nu)$ satisfying the resonance condition:

$$\Delta E = h\nu = g\beta H \Rightarrow H_{rezonanta} = \frac{h\nu}{g\beta} \tag{6}$$

the magnetic dipole shall absorb the energy of the H_z field and it shall modify its orientation towards the $\vec{H}$ field from parallel to anti-parallel, namely it shall pass from the inferior energy condition to the superior energy condition. This represents the very essence of the electron spin resonance.

The electron spin resonance shall apply to molecules containing unpaired electrons. One shall use a (monochromatic) fix frequency electromagnetic radiation (ν) and shall vary the intensity of the *H* magnetic field.

1.5 NMR Spectrometry

The study of the atomic spectra also showed, along with the fine structure, a hyper-fine structure in the spectral lines, which could have been explained based on the *nuclear spin motion* leading to the occurrence of the nuclear magnetic moment. The nuclear spin is coupling with the electron spin and leads to the supplementary splitting of the energetic levels, thus explaining the emergence of the hyper-fine structure in spectral lines.

The nuclear magnetic resonance phenomenon is based on the attribute of certain nuclei of having a magnetic moment.

The NMR signal provides information on:

- The number of nuclei,
- The number of neighboring nuclei with whom a certain nucleus is coupled,
- The chemical neighboring of the studied nucleus, determining the chemical motion.

2. MASS SPECTROMETRY APPLIED IN BIOLOGY STUDIES

Usually, a mass spectrometer consists of the following elements:

- The sample injection system, where the sample is injected according to the appropriate shape and quantity.
- A ion source, vaporizing the ions from the analysis substance.
- The analyzer, which separates the ions according to the (m/z) fraction
- A detector, recording the relative abundance or the intensity of the mass function.

Depending on the purpose set, these elements may very much vary constructively and functionally. The ion sources may adapt to solid, vaporous or gas samples, while the analyzer may have time constant or variable fields.

Also, one must notice that both in the injection system as well as in the ion source and in the analyzer, a vacuum must be created so that the sample analysis process may not be influenced by the presence of air molecules, if some of the analyzed compounds are present in very low concentrations (10^{-9}g).

The separation power of a mass spectrometer is called resolution, defined by the fraction:

$$R = \frac{m}{\Delta m} \tag{7}$$

where: *m*-ion mass, *Δm*-mass difference between two distinct consecutive peaks of the mass spectrum.

The mass spectrum represents the record of the ion abundance in a compound, according to the mass (m/e fraction) and it is specific to the substance, characterizing it. The mass spectrum represents the "fingerprint" of a substance, and this is why the mass spectrometer represents an ideal detector for the compounds separated of gases by chromatography. The GC/MS coupling is applied in many science and medicine-related fields.

The EI spectra contain, together with the $[M]^+$ molecular ion (there are cases when it is absent) the fragment ions, that can be logically explained by losses of functional groups from the molecular ion. The mass spectrum may be used in order to identify an unknown compound. In this case, usually the unknown mass spectrum is compared with that belonging to known compounds. The peaks of the mass spectrum belonging to the unknown compound may be used in order to determine its structure. The study of the fragmenting ways of the compound using the measuring techniques of the exact masses in high resolution and the metastable ion measurements offer more exact information on the structure and strength of the connections of the compound to be analyzed.

Mass Scanning. The mass spectrum is obtained by scanning the magnetic field. The computer controls the scanning and optimizes the conditions in the ion source. The signals coming from the SEM pass through the analogous filter before passing to the digital to analogous converter (DAC). The digitalization velocity is selected in order to obtain a sufficient number of point data along each signal ion in order to define the peak and to accurately determine its position and intensity. The scanning velocity and the resolution dictate the necessary digitalization speed, and for rapid scan tools (0.1 seconds / decade) a conversion speed of up to 250 kHz is required. The interface between the MS and the computer houses the DAC and other operating devices.

Selected Ion Mode (SIM). In SIM, the individual masses of the ions are continuously selected, or when one wishes to measure several ions, each one is detected in sequence for a given period of time. In this mode the sensitivity is much higher than in the case of scanning, where each ion is recorded for a short period of time (0.05-1 ms).

The first step in processing the acquisition data is to identify the calibration mass. The computer processes a reference mass list, scanning a calibration compound used for establishing the values of the sample ion masses. One usually uses a time scale. For the quadrupole tools, the reference masses are connected to a tension scale derived from the tension of the quadrupole's bars. Similarly, a Hall rod placed in the magnetic field may give a tension to be correlated with the

reference signals. One uses *perflorokerosen* or *perflortributilamina* as reference calibration standards. The adjustments of the mass spectrometer concerning the focusing of the ion fascicle are made in order to obtain a signal which is symmetric and reproducible in intensity from one scanning to another. In case of quadrupole mass spectrometers, the computer has to conventionally focus the tool using the reference substance until the intensities of the selected ions are within certain limits. The reference compounds must give ions that should not interfere with the sample's ions. The reference perfluorinated compounds, due to the fluorine's mass effect (M=18.9984) and to the mass deficiency resulted for the ions containing fluorine, fulfill this condition.

The graphic presentation of the mass spectrum is the representation on abscissa of the m/z fraction and on the ordinate of the relative abundance of ions towards the most intense ion of the spectrum considered 100%. This procedure is called *spectrum normalization*. There are programs representing the mass spectrum and automatically marking the most intense ions.

Spectrum Decrease. The presence of the mass spectrum, of the background or of some other undesired component, may be removed by decreasing the interference ions from the compound spectrum. This program is used in order to clean the spectrum in case of partially solved components.

Selected Ion Monitoring Data. The SIM data, acquired for quantitative detection sensitive to specific components, are graphically represented as a chromatogram containing the marking of each ion according to the time. In the SIM mode, the sensitivity is about 10^3 times.

The Spectra Library is used in order to identify the compounds by comparing them with the mass spectra of known components. The identification of an unknown component may be assisted by exact mass measurements, metastable measurements, use of derivatizations, incorporation of stable isotopes, alternative ionization methods, MS/MS, etc.

Any quantitative analysis must be specific, precise and sensitive. In order to make such an analysis, one must consider several aspects: the operation way of the spectrometer and the use of the internal and external reference standards. One may record data using the mass spectrometer in several operation ways. Usually, the mass spectrometer is adjusted in order to scan a certain mass area. This area may be quite large, so as to comprise the entire mass area of the device, but it can also be quite narrow, as it is the case when monitoring a specific ion. One may monitor a specific ion anywhere in the mass area. The most usual ways of operating a mass spectrometer are: recording the mass spectrum on the entire available area and monitoring a specific ion.

In the first case one obtains the representation of the total ion current according to time. It is difficult to identify a component based on this, as there may be several ions having the same mass. The molar mass is not a unique identifier in case of the organic compounds.

When monitoring a specific ion, the spectrometer is adjusted to scan a mass area as narrow as possible. The narrower the area, the more specific the monitoring. The chart of the ionic current results from the contribution of the masses from this narrow area. This chart may also present several peaks. The chart obtained by monitoring a certain ion is more specific than the chart obtain by scanning the entire spectrum (the chart of the total ion current). Consequently, for quantitative analyses the spectrometer is operated in SIM mode.

For analyses, one must build the calibration curve employing a certified substance used as a reference standard.

3. GAS CHROMATOGRAPHY AND ITS APPLICATIONS

Due to the relative simplicity, to the increased sensitivity and to the efficiency in separating the components of a mixture, gas chromatography has lately become a very important chemistry "instrument", both for the quantitative as well as for the qualitative analysis of mixtures; it can also be used in order to purify the compounds, in order to determine the thermal and chemical constancies of certain solutions in heating and vaporizing conditions, in order to determine the vapor pressure and the activity coefficients or to automatically monitor certain industrial processes.

On the other hand, using gas chromatography, one can rapidly perform many usual environmental analyses as well as others. As a result, many countries established monitoring points for the continuous measurement (using the gas chromatographic method) of the level of emissions such as nitric oxides, carbon dioxide and monoxide. At the same time, gas chromatography is helpful for analyzing the pharmaceutical products, the alcohol level from the blood, the essential oils, the food products, etc.

Chromatography's Principle

Basically, a chromatograph consists of a column and a detector, as well as the following supplementary parts: a carrier (eluent) gas cylinder or a gas generator, a pressure adjustment device, a sample injection device, a recorder and the computerized data processing, temperature programming and gas debit programming systems.

The chromatograph's functioning system consists in injecting the sample through the sample injection device, where it takes the sample to be analyzed and injects it in the chromatographic column, which is the headquarters of the separation process. Due to the interactions between the sample's molecules with the stationary phase, the compounds from the mixture to be analyzed remain behind the eluent, migrating through the column with different velocities. Thus, when they exit the column, the compounds shall be separated and carried by the eluent to the detector.

Usually, the detector transforms a physical and chemical property of a component from the eluent into an electric signal, proportionally with the component's concentration. The graphic record of the signal provided by the detector according to the time, for all the sample components, is called chromatogram, and in the case of a component, it is called peak. The peak's surface may be integrated using a computer.

The retention factor (R) is the ratio between the migration velocity (u_z) of a component and the moving velocity (u) through the column of the eluent:

$$R = \frac{u_z}{u} \tag{8}$$

On the other hand, the value of R is a balance property and it depends on the repartition coefficient of that component, as the area retention, measure setting their position in time and space, is determined by the component distribution in the area, between the two phases: the mobile one and the stationary one.

The retention volume (V_R) is the necessary volume of eluent in order to carry a component from the moment of injection until the occurrence of the maximum concentration. In gas chromatography, the eluent's volume is specified by the exit pressure and the column's temperature.

The total volume or the retained volume (V_M), is the necessary volume in order to carry a component whose concentration in the stationary phase is negligible compared to the concentration from the mobile phase until the occurrence of the maximum concentration. This volume is included in the retention volume (V_R), also called the total retention volume. The retention volume is a qualitative characteristic for that component.

Gas chromatography usually operates with retention measures expressed in time units:

$$t_R = V_R / F_C \qquad t_M = V_M / F_C \tag{9}$$

where F_C is the volumetric flow measured at the exit point from the column and according to the column's temperature.

The gas phase chromatography is a technique used in order to separate and identify the chemical substances, by which the mixture to be separated is injected in a stationary phase and it is subjected to an important number of sorptions, desorptions and resorptions in the stationary phase, while it is carried through the mobile phase system. The migration speeds of the mixture's components depend on their distribution coefficients between the two phases and they are determined by their physical and chemical properties.

The component parts of a chromatograph are:

- The mobile phase and the gas flow control,
- The sample injection system – injectors (classical ones, splitting ones, split-splitless ones, on column, PTV, other types),
- Stationary phase deposited in the chromatographic columns,
- The chromatographic detector,
- The signal transmission system

Qualitative and Quantitative Analysis through Gas Phase Chromatography

Qualitative analysis ensures the identification of certain unknown organic compounds from a sample subjected to analysis. The qualitative identification may be done in two ways:

- By comparing the retention time (the time between the moment of sample injection and the moment of maximum elution) or the retention volume of the unknown compound with that of a standard known substance or of a synthetic mixture of compounds;
- By analyzing the chromatographically separated components using a mass spectrometer or an IR spectrometer.

In order to perform a qualitative analysis it is necessary to take the following working steps:

- Preliminarily processing the samples;
- Establishing the operation parameters;
- Calibrating the equipment;
- Calculating the results;
- Applying the quality insurance measures.

The most used calibration and compound concentration determination methods are:

- The standard curve method (area normalization). The unknown component concentration (X) is calculated using the peaks' area, according to the formula:

$$\%X = \left(\frac{A_x}{\sum_i A_i} \right) \cdot 100 \tag{10}$$

- The area normalization with response factors. The components' concentration is calculated with response factors (f_i) experimentally determined with gauges or theoretically calculated or taken from the specialized literature, according to the formula:

$$\%X = \left[\frac{(A_x f_x)}{\sum_i (A_i f_i)} \right] \cdot 100 \tag{11}$$

- The internal standard method

- The standard additions method
- The external standard method.

The calibration curve method. This method consists of previously accomplishing a calibration curve, $A = f(C_i)$, and afterwards determining the equation of the most probable straight line. Using this equation, after determining the peak's area, one may calculate its concentration. By this technique, one may obtain a precision of ±0.5%.

The internal standard method. This method consists of adding a C_S (% weight) known concentration reference substance (called standard substance) to the mixture to be analyzed. In this case the *I* component concentration is calculated using the formula:

$$C_i = \left(A_i f_i / A_S f_S\right) C_S \cdot 100\% \quad (12)$$

The standard substance must fulfill the following conditions: it must not be contained by the mixture to be analyzed, it must be completely separated from the other components to be determined and its concentration must not be very different that that of the component to be determined.

Using this method one may obtain a precision of ±0.1%.

The standard addition method. With this method, for determining the C_i concentration of the *I* component, one shall determine its are A_i. Then, a known quantity having the standard concentration C_S of this component is added to the sample and the A'_i area is determined (composed of A_i+A_S) from the new chromatogram. The C_i concentration is determined from the formula:

$$C_i/(C_i+C_S) = A_i/A'_I \text{ from where it results } C_i=C_S A_i/(A'_i-A_i) \quad (13)$$

The external standard method. At the unknown C_i concentration of a compound *I* from the mixture to be analyzed it corresponds the area of A_i peak. Then, a sample from the same component but having a known C_S concentration is injected in the chromatograph. The following observation must be made: the concentration of the external standard must not differ very much from the concentration of the component to be determined. One may easily notice that this method does basically not differs from the calibration method, only that in this case one makes a single determination for the known concentration component. The concentration of the unknown component is calculated using the formula:

$$C_i = A_i C_S / A_S \quad (14)$$

4. GC-MS APPLICATIONS IN BIOLOGY AND MEDICINE STUDIES

4.1. Quantitative Analysis of Bioactive Compounds Using the ID-MS Method

Irrespectively of the quantitative analysis method used, it must be validated according to the following **validation parameters**:

- ***Linearity*** **–** capability of an analytical method of allowing, in a pre-established field, to obtain variable test results, directly proportional with the concentration of the item to be tested. The linearity is proved by raising the calibration curves for the considered tested items and by calculating the corresponding correlation coefficient. We recommend the reference value 0.997 for the correlation coefficient, but smaller values are allowed, if the deviation of the calculated value as compared to the reference value, for all the calibration points, does not exceed 5% of the effective value of the considered item.
- ***The limit of detection (LOD), the limit of quantitation (LOQ)*** **–** the limit of detection is represented by the smallest quantity of tested substance that may be detected using the considered method. The quantity of substance within the detection limit must be bigger than the error associated with the measurement (the signal / noise ratio = 2 or 3). The quantity limit is represented by the smalles quantity of tested substance offering precise measurements, having a signal / noise ratio =10.
- ***Accuracy (exactness)*** **–** represents the degree of closeness between the results obtained with the analytical method and the values accepted as reference values or conventionally true values. It is determined by analyzing a laboratory generated sample, using reference materials. The exactness estimates *the systematical errors.* One may compare the results of this method with a reference method or one may use a known concentration sample (CRM = certified reference material). One uses completely trustful matrixes with a known quantity of substance to be analyzed, etalons or standards. The error is the difference between the true value and the measured one.

  In order to assess the error, the relative standard deviation (RSD) is calculated, using the formula:

$$R.S.D(\%) = \frac{|measuredvalue - truevalue|}{truevalue} \cdot 100 \tag{15}$$

  where the measured value for *n* measurements represents $\bar{x}$:

$$\bar{x} = \frac{\sum_{i=1}^{n} x_i}{n} \tag{16}$$

- ***Precision*** **–** represents the degree of closeness between the results obtained by measuring a series of samples made of the same homogenous sample, according to the conditions required by the method. The precision is investigated taking into account three levels:

- ***repeatability*** (short term precision) – it is determined by reading the sample in a repeatable manner, on the short term, in the same operating conditions,
- ***intermediary precision*** (precision within the laboratory) – one determined the result variability on the longer term, usually in different days.
- ***reproducibility*** (inter-laboratory precision) – it supposes the analysis, in several laboratories, of the same homogenous sample, using the same method (direct method transfer).

The precision and the reproducibility characterize the conformity between the results of the individual measurements or those of the multiple series of measurements. In other words, the precision is the *statistical error*.

The precision is expressed with the standard relative deviation or with the variation coefficient (C.V.) expressed in percents:

$$C.V.\% = R.S.D.(\%) = S.D \cdot 100 / M \tag{17}$$

where *S.D.* is the standard deviation for *n* measurements, given by the formula:

$$S.D. = \sqrt{\frac{\sum \left(x_i - \bar{x}\right)^2}{n(n+1)}} \tag{18}$$

where $\bar{x}$ is the average of individual measurements *I* given by the formula (16).

- ***Robustness*** – represents the method stability, in terms of analytical results, in case of variations of the operational parameters. It is checked by inducing, in a controlled manner, certain deviations from the operational parameters (in a realistically chosen area) and by checking the effect on the results.
- ***Selectivity*** – the capacity of an analytical method of accurately measuring a substance to be tested in the presence of other substances to be tested and of possible interference substances. In case of chromatographic processes, it is insured by correctly establishing the chromatographic separation parameters, and in case of ICP, by correctly choosing the spectral lines, free of interferences in the concentration field chosen, as compared to other spectral lines. In many specialized journals, the selectivity and the specificity are used as synonyms.

4.1.1. Quantitative Analysis by Isotopic Dilution. Calibration Curve

The isotopic dilution analysis (ID) is a quantitative analysis method where one adds a known amount of tracer to an unknown amount of tracee. After acquiring the isotopic balance (where the relative isotopic abundance is the same in the entire system, and the initial isotopic abundance of the added tracer is diluted), the result of the isotopic abundance to be measured is a measure of the unknown amount to be traced.

The unmarked compound contains an amount of stable compound and the marking signifies only the exceeding amount over this natural amount. In order to calculate the unknown amount to be traced, one uses the following isotopic balance formula:

$$n_x a_0 + n_1 a_1 = (n_x + n_1)a \tag{19}$$

where: n_x – (unknown) amount to be traced, in moles,

a_0 – the (natural) abundance of the tracee, in percents,

n_1 – amount of tracer, in moles,

a – average relative abundance of the mixture $(n_x + n_1)$.

$$n_x = \frac{n_1(a_1 - a)}{a - a_0} \tag{20}$$

The isotopic dilution method is useful in order to calculate the amount of a given compound "in vitro". In the "in vivo" tracer's kinetics, this principle is superposed with speeds of chemical reactions, under-layer flows, reservoir formations, etc.

Calibration Curve

Irrespectively of the analytical technique chosen, one measures the value of a *y* amount for the given values of a *x* amount. In the mass spectrometry area, *x* may be the concentration of a solution that was extracted as a sample to be determined, while *y* may be the response to a particular *m/z* value. A *y* representation according to *x* shall show a distribution, obtaining a curve or a straight line. If the relation is a linear one, the straight line may be built by the least squares method. The result, called linear *y/x* regression, is the straight line minimizing the sum of squares of the vertical deviations towards this straight line.

The equation for the linear regression is:

$$y - \bar{y} = b(x - \bar{x}) \tag{21}$$

where *b* is the slope or the regression coefficient.

One must establish whether there is a significant relation between the two sets of results. This may be done by calculating the *r* correlation coefficient, given by:

$$r = \frac{\sum(xy) - \sum(x)\sum(y)/n}{\sqrt{\left\{\sum(x^2) - \sum^2(x)/n\right\}\left\{\sum(y^2) - \sum^2(y)/n\right\}}} \tag{22}$$

The perfect correlation means that $r = \pm 1$. If $r = 0$, it means that *x* and *y* are completely independent.

4.1.2 Theophylline Analysis in Biological Fluids through ID-MS

Theophylline is a very important drug used for treating asthma and premature apnea in children. Both the daily dose as well as the frequent dose must be individualized, especially in case of preschoolers, to whom excessive fluctuations of theophylline concentration in serum have been reported. The therapeutic level of theophylline in plasma ranges between 5-15 μg/ml. The doses exceeding this range cause toxic effects and the smaller ones are inefficient.

In this study we have compared two calculation methods (a matric one and one using the regression straight line) in order to determine the theophylline in biological fluids. If a strict technique is being observed, the concentration of theophylline in the stimulated saliva is just as relevant as the plasma measurements. The method is a non-invasive one and it was applied in order to optimize the treatment in the clinic.

Material and method

We have used a Hewlett Packard 5989B mass spectrometer coupled with a HP-5890 gas chromatograph, in the following conditions: electron energy 70 eV, electronic emission 300 μA and ion source temperature 200^{o}C, selected ion mode (SIM). The gas chromatograph used a capillary column HP-5MS, 30m x 0,25mm, 0,25μm filament thickness, programmed from 200^{o}C to 270^{o}C with 10^{o}C/min, the flow 1 ml/min, using helium as carrying gas.

As an internal standard, we have used the theophylline marked with ^{15}N (74,2 atom% theophylline-^{15}N) synthesized by INCDTIM Cluj-Napoca. The purity of the internal standard was checked by IR spectroscopy, mass spectrometry and melting point. The chloroform and the isopropanol used for the extraction were purified by distillation.

The analysis was performed on the molecular ions m/z 180 and m/z 181 of the substance to be analyzed and of the internal standard, employing the SIM mode for the quantitative analysis.

Extraction Procedure

An amount of 0.5 ml of plasma containing theophylline was injected in a 5 ml ampoule with cover and thread and then we added 5 μl of internal standard ^{15}N-theophylline, 1 ml of extraction solvent chloroform:isopropanol 20:1 v/v with 0.2 g NaCl addition. After one minute of mechanically mixing, the sample was centrifugedfor 3 minutes and then the inferior (organic) layer was injected into the GC.

Study Groups:

We have chosen two different study groups: *the A group* formed of 27 children with asthma, aged between 2-16 years and *the B group*, formed of 13 new-born babies with premature apnea, aged between 2-10 weeks. We have measured the theophylline concentration for the 40 hospitalized and theophylline-treated children. For the A Group, we have used a 15 mg·kg^{-1}·24h^{-1} dose. For the

B Group, the administered dose of aminophylline IV was of 5 mg/kg and the maintenance dose was of 3 mg/kg every 8 hours.

Results:

The method was validated in the 0-40 μg/ml area. We have analyzed the standard samples, two extractions of each sample, containing 5, 10, 20, 30, 40 μg/ml theophylline and10 μg/ml internal standard. The regression curve obtained was: *y = 0,103x - 0,336* with a regression coefficient of 0.998.

The design matrix was built firstly from the molar fractions of the mass spectrum of the natural theophylline, of the tracer and of the internal standard, and when it was necessary, by synthetically building the mass spectrum by computerization. It was necessary to solve a series of simultaneous linear equations, each one describing the isotopic contributions like:

$$I_x = \sum_{x=i,j} A_i X_j \tag{23}$$

where I_x represents the relative abundance of the x ion and X_j is the unknown fractional abundance. The relative abundance of the contributing ions (A_i) was calculated for two of the most intense ions, forming the simultaneous equations in matric notation:

$$I = AX \tag{24}$$

The solution of the least squares of X may be obtained using the pseudo-inversed matrix:

$$X = (A^T A)^{-1} A^T I \tag{25}$$

where A^T is the transposed matrix and X is estimated by minimizing the sum of the squares.

It is preferable to determine the isotopic abundance matrix from mass spectra experimentally measured for samples of pure compounds and internal standards.

In table 1 we are presenting an example of matrix built in this metabolic experimental study. The calculation was made using Excel.

Table.1 Built matrix (left) and pseudo-inversed matrix (right) used for calculating the theophylline

theophylline	**[M]**	**[M+1]**
n.a.	0.95	0.05
^{15}N	0.27	0.73

theophylline	**[M]**	**[M+1]**
n.a.	1.07	-0.07
^{15}N	-0.40	1.40

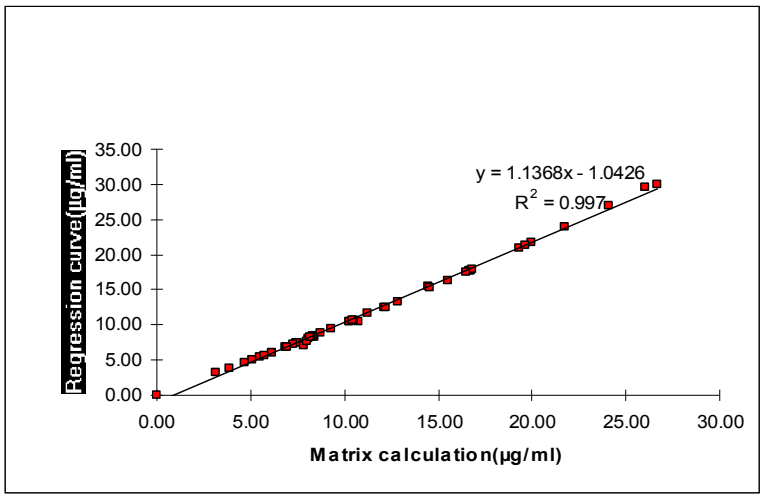

Figure 1. Comparing the two methods (the regression curve and the matrix)

The results obtained for the saliva and plasma samples gave a good correlation using the two calculation methods. Picture 1 represents the comparison of the two calculation methods. The calculated correlation coefficient was 0.9985.

Picture 2 presents the very good correlation between the drug values obtained from the plasma and saliva for the two groups of patients. The drug levels from the plasma and from the saliva are presented in table 2.

Table 2. Comparative values of the drug levels in plasma and saliva for the two groups of patients

Population	**area, µg/ ml**	**average± SD, µg/ml**
Plasma levels		
Group A	1.98 – 21.96	7.98 ± 5.25
Group B	1.62 – 27.90	7.76 ± 5.85
Saliva levels		
Group A	1.41 – 15.06	5.12 ± 3.45
Group B	1.02 – 18.23	5.51 ± 4.61
Saliva/ plasma ratio		
Group A	0.47 – 0.71	0.60 ± 0.09
Group B	0.43 – 0.88	0.69 ± 0.13

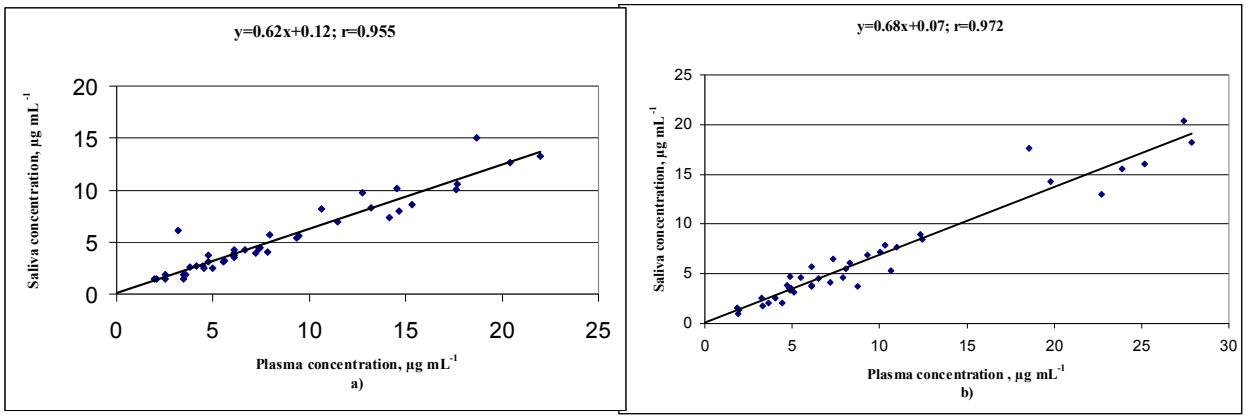

Figure 2 The correlation between the theophylline concentration in plasma and in saliva in the two groups: a) Group A, b) Group B.

Conclusions

The isotopic dilution method by mass spectrometry (ID-MS) is simple, precise and fast. The regression curve method calculation leaded to similar results with the matrix calculation method. We obtained a good correlation between the drug levels measured in plasma and saliva.

4.2.2 Caffeine Test – Diagnosing Method for the Hepatic Disorder

Caffeine may be used in order to measure the metabolic capacity of the liver. It has been noticed that caffeine metabolism is low in patients with different types of liver disorders, depending on the status of the disease. Caffeine has the advantage of being well tolerated when it is orally administered, the levels in saliva being according with the serum concentrations, and thus non-invasive tests are possible.

Reactives: As an internal standard, we have used the ^{15}N-theophylline, 74.2 atom% ^{15}N, synthesized by INCDTIM Cluj-Napoca. The purity of the internal standard was checked by IR spectroscopy, mass spectrometry and melting point. Caffeine was orally administered to children with various hepatic dysfunctions. A sterile caffeine solution - sodium benzoate in water containing 125 mg caffeine and 125 mg sodium benzoate per 1 ml pharmacy ampoule was used for injection. All the other reactives used were taken from Merck (Germany).

Equipment: We used a Hewlett Packard (Palo Alto, CA, USA) HP 5989B mass spectrometer coupled with a HP 5890 gas chromatograph in the following conditions: EI (electronic impact), electron energy 70 eV, electron emission 300μA and ion source temperature 200^{o}C, selected ion monitoring mode (SIM). For the GC/MS measurements we used a capillary column HP-5MS, 30m x 0,25mm, 0,25μm film thickness, programmed from 200^{o}C to 270^{o}C with 10^{o}C/min, the flow 1 ml/min, using helium as carrying gas. The injector's temperature was 200^{o}C. The caffeine retention and the internal standard - ^{15}N-theophylline time was 3.5 min, and 2.8 min. respectively. We injected 3 μl of sample. In order to perform the quantitative analysis in SIM mode, we measured the m/z 194 caffeine molecular ion and the internal standard m/z 181 molecular ion.

Extraction procedure: We used a very simple extraction procedure. An amount of 1 ml of plasma containing caffeine was injected in a 5 ml ampoule with cover and thread and then we added 10 μl of internal standard ^{15}N-theophylline, 2 ml of extraction solvent chloroform: isopropanol 20:1 v/v and 0.5 g NaCl. After one minute of mechanically mixing, the sample was centrifuged for 3 minutes. The (inferior) organic layer was transferred in another ampoule and then evaporated in

argon flow. The residue was dissolved in 100 µl solvent and 3 µl were injected into the GC. As the method's sensitivity is very good, we were able to work without concentrating the extract.

Method validation: The method was validated in the area 0-20µg/ml caffeine. Distilled water standard samples (aliquots) containing known amounts of caffeine 3, 5, 10, 15, 20 µg/ml and 10 µg ^{15}N-theophylline were processed according to the above mentioned procedure. Each sample was prepared twice and was measured twice. The regression straight line, represented as the ratio of the peak's area, m/z 194 per m/z181, depending on the caffeine concentration, resulted in the following linearity parameters: slope 0.1208, ordinate at the origin 0.0926, and the correlation coefficient r = 0.98.

Table. 3. Method's precision and accuracy

Added concentration (µg ml^{-1})	n	Measured concentration (µg ml^{-1}))	DSR (%)	Accuracy (%)
3	5	3.1	2.96	3.36
5	7	5.5	5.06	10

Study groups: We studied three different groups: *group **A***, formed of 19 children with hepatitis aged between 3-19 years, *group **B***, consisting of 5 children with cirrhosis, aged between 5-12 years, and group **C**, 10 witness children aged between 5-15 years. The average dose was of 4 mg/kg, p.o., for all groups. We took blood samples every 0, 30 min, 1, 3, 6, 9 and 12 h. Blood samples were transferred in plastic tubes with heparin and immediately centrifuged. The plasma was preserved at -20 ^{o}C. Before starting this study, we obtained the written authorization of the parents of each subject.

Way of calculating: The regression straight lines obtained using the GC/MS method in SIM mode were used for the study of the analyzed pharma-kinetic parameters. The caffeine elimination constancy was calculated as follows:

$$k_{el} = (\ln C_1 - \ln C_2) / \Delta t \tag{26}$$

where: C_1 – high concentration of caffeine in the blood,

C_2 – low concentration of caffeine in the blood

Δt – time between two blood sample collection.

The clearance between two points was calculated using a distribution volume constancy (V_d) of 0.6 liters per kg body:

$$Cl = k_{el} \cdot V_d \tag{27}$$

and the half-time

$$t_{1/2} = \ln 2 / k_{el} \tag{28}$$

The values calculated for clearance as a ratio dose/area under the curve were comparable with the two points.

Results:

The caffeine clearance, measured in patients with cirrhosis and chronic hepatitis, was reduced, and the half-time was increased in ill children as compared to healthy children. The metabolism decrease noticed in patients with various forms of hepatic disease was correlated with the status of the illness.

The average values of the two pharma-kinetic parameters studied, *Cl* and $t_{1/2}$, for patients with hepatic diseases and for witnesses, show important differences especially between the witnesses and the cirrhosis cases. The values of the average life times decreased and the values of the clearance (elimination speed) increased in patients with hepatic dysfunctions as compared to the witnesses. *Picture 3* presents the high values of caffeine concentrations of patients as compared to the average value of caffeine concentration of the witnesses (n=10).

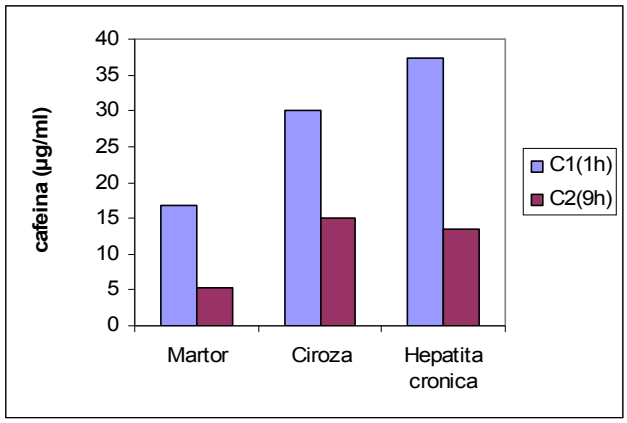

Figure 3. Caffeine level after one hour and after 9 hours since the administration, as compared to the witness sample

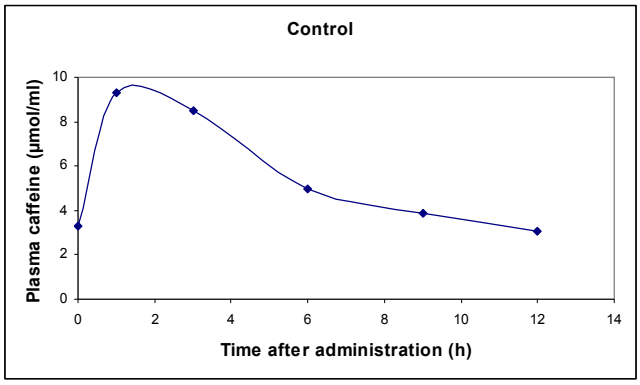

Figure 4. Caffeine elimination curve of the subjects belonging to the control group

The presented method is simple, precise and rapid, useful for analyzing the xanthines. The use of the isotope-marked internal standard avoids the superposition with various contaminants. For this drug, in the interest area 0-20μg ml^{-1}, we obtained good linearity, precision, accuracy and detection limit.

We noticed significant changes (test T-Student, $p<0.01$) in the caffeine metabolism in case of children with decompensated cirrhosis. The clearance values 0.74±0.49 ml min^{-1} kg^{-1} and half time 14.73±12.36 h are different than those of the witness due to the decrease of the "functioning hepatocyte mass".

The patients with non-cirrhotic hepatic diseases (hepatitis) have intermediary values (Cl = 1.23 ± 0.45 ml min^{-1} kg^{-1} and $t_{1/2}$ = 6.32± 2.17 h), but higher values of caffeine concentrations especially during the first hours after the dose.

In the specialized literature, the witness clearance levels and half time were 1.3±0.4 ml min^{-1} kg^{-1} and $t_{1/2}$=4.4±1.9 h and our data were 1.28±0.31 ml min^{-1} kg^{-1} and $t_{1/2}$=5.73±1.58 h (n=10).

The plasmatic caffeine concentrations were measured in 18 patients with chronic hepatitis, in 5 patients with cirrhosis and in 10 healthy subjects after administering caffeine (4 mg/kg p. o.). The values obtained for the caffeine clearance (elimination speed from the body) measured with the two points method (assay times 1h and 9 h) or the seven-points method (assay times 0, 0.5, 1, 3, 6, 9, 12 h) correlated very good ($r = 0.94$, $p<0.001$), so that it is necessary to collect blood only 1 hour and 9 hours after administering the caffeine dose in order to perform the caffeine test for diagnosing the hepatic dysfunction. The caffeine half time ($t_{1/2}$) was significantly higher for patients with cirrhosis as compared to the other study groups, and the clearance was substantially reduced in case of these patients.

Conclusions:

The presented method is simple, precise and rapid, useful for analyzing the xanthines. The use of the isotope-marked internal standard avoids the superposition with various contaminants. Good validation parameters were obtained in the interest area.

We noticed significant changes in the caffeine metabolism in case of children with decompensated cirrhosis ($p<0.01$). The clearance values and the half times are different due to the "functioning hepatocyte mass". The patients with non-cirrhotic hepatic diseases presented intermediary values, but higher values of the plasmatic caffeine concentration.

These results suggest that the pharma-kinetic parameters of caffeine may be determined using the two points sample collection procedure and the GC-MS determination, after only one dose. The caffeine clearance test could not make the difference between the liver functioning in case of witness subjects and those with hepatitis ($p>0.05$). The high caffeine concentration noticed within the first hour after the dose in subjects suffering with hepatitis as compared to the witness

cases may be used as a rapid hepatitis test when we are using highly precise and exact analysis methods.

4.3. Diagnosing Innate Metabolic Disorders through GC-MS

4.3.3. Monitoring Amino-Acids Profiles for Diagnosing Phenylketonuria and "Maple Syrup Urine Disease"

Phenylketonuria (PKU) is an inherited disorder caused by the deficiency of phenylalanine-hydroxylase. The normal phenylalanine catabolism (Phe) in case of animals requires its initial conversion into tyrosine (Tyr) in the liver. The enzyme's deficiency leads to specific plasma amino-acids levels, with the abnormal increase of Phe or decrease of Tyr. The disease appears within the first weeks of life. The mental deficit becomes obvious four to six months later. The oldest test is the urine reaction with iron polychlorure, resulting in a characteristic dark green color. The recommended treatment is a low phenylalanine diet. The plasmatic phenylalanine must be maintained under 2 mg% .

The *Maple syrup urine disease* - MSUD appears due to the activity decrease of alpha-ketoacid-decarboxylase (referring to isoleucine, leucine, valine). It is a metabolic disease caused by a genetic defect, where the body cannot break certain ramified amino-acids. The disease leads to the appearance of certain proteins in the blood and it is characterized by cerebral symptoms and by eliminating maple syrup smelling urine. If one does not gives a diagnosis, the child shall die within a few months. The treatment consists in diets avoiding the three amino-acids.

The purpose of this study was to develop a precise and rapid quantitative and screening analysis method through SIM-GC/MS in order to certainly diagnose Phenylketonuria (PKU) and MSUD.

Experimental Part

Reactives and Samples

We compared two different extraction and derivation methods.

The subjects from whom we collected samples were aged between 3 and 10 years. We collected blood using two methods. The first one was the *venous puncture*, and the second one, more largely used, was the *capillary blood collection,* applying the blood drop collected on a filter paper with spots delimitated at *8 mm* diameter for an amount of 20 µl of blood respectively. The extraction from the blood spots was made after cutting them in cap bottles, initially 1 hour at $4^{o}C$,

and then 1 min in a microwave bath, with methanol 0.1% HCl, the results being identical. We added the internal standard ^{15}N-Ile (25µg/ml or 0.5µg/blood spot) for the isotopic dilution method.

Method 1. The amino-acids were purified on a Dowex 50W-X8 ion-exchange resin, on a 2x40mm column and they were eluted with 4M NH_4OH. We applied a two step derivation procedure: *esterification* with butanol – acetyl chloride (4:1 v/v) for 1 h at 110^{o}C for the esterification of the carboxyl group, and *trifluoroacetylation* with 200 µl trifluoroacetic anhydride at 60^{o}C for 30 min, for the acetylation of the amino group.

Method 2. The blood was put in a container with lid and thread with 200 µl methanol/HCl 0.1% and the extraction was obtained either 1h later at 4^{o}C or by sonication for 1 min. 100 µl of extract were placed in another container and derived after adding the internal standard. The amino-acids from the blood samples or from the standard samples were derived as butyl trifluoroacetic esters. The derivation was made in two steps, in containers with cover and thread. The dry samples were esterified with 100µl ml butanol: acetyl chlorine, 4:1, (v/v) for 30 min at 100 °C. The excess reactive was removed in nitrogen flow. The amino group of the amino-acids was trifluoracetylated with 100 µl trifluoroacetic anhydride (TFAA) at 60 °C for 30 min. After cooling, the excess reactive was removed with ice temperature nitrogen and we added ethyl acetate.

As biomarkers we used the amino-acids presented in the following table:

Table.4. Amino-acids used as biomarkers

Amino-acid	***Symbol***	***Ions (SIM)***
Valine	Val	m/z 168
Leucine	Leu	m/z 182
^{15}N-Glycine	^{15}N-Gly	m/z 155
^{15}N-Isoleucine	^{15}N-Ile	m/z 183
Proline	Pro	m/z 166
Phenylalanine	Phe	m/z 91,148
Tirozin	Tyr	m/z 203, 260,316, m/z 107,164,220

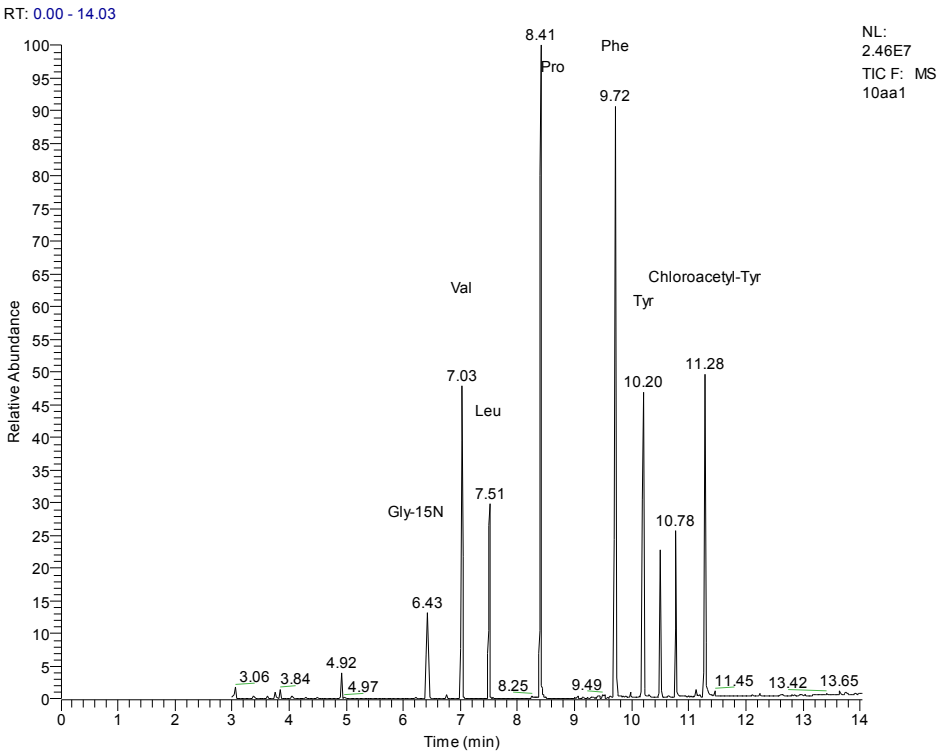

Figure 5. SIM-GC-MS interest amino-acid separation chromatogram used for diagnosing PKU and MSUD

In SIM mode we used the most important ions from the mass spectra of amino-acids derived as trifluoroacetyl butyl esters: m/z 168 for Val, m/z 182 for Leu, m/z 166 for Pro, m/z 91, 148 for Phe, m/z 203,260,316 for Tyr completely derived (Tyr-di-trifluoroacetyl butyl ester) and m/z 107, 164, 220 for Tyr-mono-trifluoroacetyl butyl ester.

Results:

The quantitative analysis of the five amino-acids (valine, leucine, proline, phenylalanine, tirozin) in blood samples through two different extraction, derivation and analysis methods led to similar results. The regression coefficient for comparing the amino-acid values through the two extraction methods gave r = 0.91 (n = 4).

The methods were validated using 15 standard amino-acids, and 5 amino-acids respectively. The standards followed the same extraction, derivation and analysis procedure (n = 3) as the samples. The precision value was smaller than 19.81% for the relative standard deviation (R.S.D.), except for the Arg, Cys and Met amino-acids and the sensitivity value was under 10 ng for the injected amino-acid. The regression straight lines were obtained by injecting standard solutions containing amino-acids in concentrations of 5, 10, 15, 20 and 40 µg/ml with 20 µg/ml ^{15}N-Gly added at each standard solution (method 1).

Using the second method, the linearity was calculated representing the amino-acid selected peak area ratio per internal standard according to the concentration of the standard amino-acid (in µg/ml). The regression straight lines were obtained by injecting standard solutions containing

amino-acids in concentrations of 1, 5, 10, 20, 30 and 40 µg/ml with 25 µg/ml ^{15}N-Ile added at each standard solution, and per ml blood sample respectively.

Table.5. RSD Values (%) for precision and accuracy (method 2)

Amino acid (n=4)	Precision RSD(%) 30 µg/ml	Precision RSD(%) 40 µg/ml	Acc. RSD(%) 30 µg/ml	Acc. RSD(%) 40 µg/ml
Val	9.02	12.82	3.75	0.65
Leu	12.90	6.73	0.15	1.66
Pro	11.73	8.68	13.33	2.11
Phe	9.70	18.60	24.67	1.76
Tyr	7.92	8.22	30.44	5.54

A good precision was obtained for the same child (R.S.D. smaller than 10.4 %). The results obtained from only 20 µl blood spot showed that the PKU diagnosis could be tested by calculating the Phe/Tyr ratio. The MSUD diagnosis shall be obtained by calculating the ration between the aliphatic and aromatic amino-acids in blood samples. The results of some of the PKU patients using method 2 are presented in *Table 4.13*.

The values of the Phe/Tyr ratio obtained for witnesses and patients diagnosed with PKU in some of the studied cases are presented in *Picture 6.*

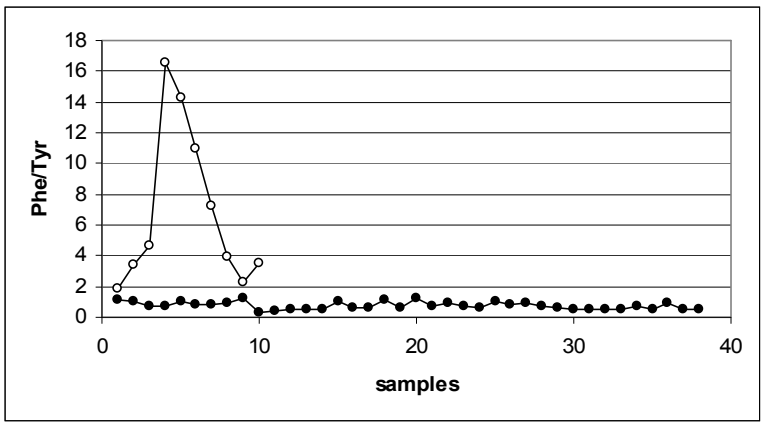

Figure 6. Values of the Phe/Tyr ratio for blood spots for PKU (o) and witnesses (●) through SIM/GC/MS.

Conclusions:

GC/MS is a sensitive and rapid method for quantitatively determining the amino-acids from sanguine plasma samples.

The method employed is useful for diagnosing metabolic disorders by quantitatively determining certain amino-acids.

It is necessary to monitor all new-born babies using this method.

The diagnosis of Maple Syrup Urine Disorder **(MSUD)** within the first three months of life and its treatment is vital, and it is the same in the case of Phenylketonuria **(PKU).**

The measurements performed on plasma amino-acids showed that GC-MS is an appropriate method for diagnosing PKU in blood samples taken from new-born babies, either by screening or by the quantitative analysis of certain amino-acids (from the Phe/Tyr ratio).

This method is a minimum invasive one, as it uses very small amounts of blood.

The ratio of aliphatic and aromatic amino-acids may indicate other metabolic disorders such as MSUD.

5. CONCLUSIONS

The most important conclusions that arise from the experimental results obtained are, as follows:

1. GC-MS coupling aggregates the special features of the two devices, ideal separation using the gas-chromatograph and ideal identification using the mass spectrometer.

2. The very good selectivity and specificity of the mass spectrometer ensure the very high precision and safety of the analyses. The mass spectrometry method is a unique, extremely precious and irreplaceable method, as it allows the identification with a high degree of precision of the components at the chromatographic elution time of the substance(s) analyzed and also the continuous testing during the analysis of the components' identity.

3. It is necessary to perform a continuous and objective control of the analytic results by validating the quantitative analysis methods, both to prove that the method chosen is correct and complies with the purpose and also to verify if the analyst performed his work appropriately.

4. We determined a quantitative analysis method by using the isotopic dilution – mass spectrometry for analyzing the theophylline. In order to correlate the drug's levels in plasma and saliva, two calculation methods were compared: the matrix and the regression curve. The ^{15}N marked theophylline, synthesized at INCDTIM Cluj-Napoca, was used as an internal standard. The method was validated for the range 0-40 μg/ml. A good linearity r = 0.99 was obtained, while the precision, accuracy and reproducibility were having very good variation coefficients.

- The results obtained for the saliva and plasma samples gave a good correlation using the two calculation methods, r = 0.997.
- It was obtained a very good correlation between the drug levels measured in the plasma and saliva for the two groups of study chosen, with r = 0.955, respectively r = 0.972.
- The method is useful in case of testing new drugs containing theophylline with retarded action, that are intensively studied worldwide, having good effects in case of crises.
- This method is also useful in pharmacokinetic studies and it can be used as a control method of the regular methods, as it has a high degree of precision.

5. By using CG-MS a very fast and precise method was determined for the assessment of the caffeine level and pharmacokinetic parameters in blood for the children suffering with hepatic disorders. The ^{15}N marked theophylline, synthesized at INCDTIM Cluj-Napoca, was used as an internal standard. The method was validated for the range 0-20 μg/ml of caffeine. The regression curve obtained had a correlation coefficient of r = 0.98. The relative standard deviation values of the accuracy were below 10%. The detection limit was of 0.1 μg/ml caffeine in the blood samples, for a signal / noise ratio of 4:1. The values obtained for the caffeine clearance, measured using the two points method (1 h and 9 h) or the seven points method (0, 1/2, 1, 3, 6, 9, 12 h) were very well correlated ($r = 0.94$, $p < 0.001$), thus proving that in order to simplify the method a two points sampling is sufficient for the caffeine test as a diagnosing method for hepatic disorder. The caffeine half time ($t_{1/2}$) was significantly higher and the clearance was substantially reduced for patients with cirrhosis as compared to the other study groups. The caffeine test could not make the difference between the liver functioning in case of witness subjects and those with hepatitis ($p > 0.05$), but it is possible that the high value of the blood caffeine after an hour since the dose was medicated to be a sign of hepatitis diagnosis. We noticed significant changes in the caffeine metabolism in case of children with decompensated cirrhosis ($p < 0.01$). The clearance values and the half times are different due to the "functioning hepatocyte mass".

6. We elaborated the *minimum invasive analyze method of the aminoacids* from a minimum quantity of blood (20 μl), monitoring the witness children and the patients diagnosed with phenylketonuria, tests using comparative spectroscopic methods for diagnosis. 20 μl blood spots were collected (n = 6) from 63 children, 53 witness samples and 10 samples from patients that were suspected with Phenylketonuria (PKU). Minimum invasive analysis method was elaborated, using blood spots taken from the finger, on a special paper. As an internal standard, ^{15}N glycine and ^{15}N isoleucine were used for the isotopic dilution. The minimum invasive

analysis method was applied for a high number of cases (n = 53), thus obtaining a phenylalanine / tyrosine ratio of 0.70 (n = 53) as compared to the average of the values for patients diagnosed with PKU that was higher than 2 (n = 10). The results obtained using SIM GC/MS for samples of the ill patients also produced positive values using the semi-quantitative classic method (BIA). The method can be used for monitoring the new-born babies in order to diagnose two diseases of metabolic disorder, phenylketonuria (PKU) and the maple syrup urine disorder (MSUD).

- During the second stage of the study, the minimum invasive method (20 µl blood spots) was compared with the GC-MS method by isotopic dilution, using higher quantities of blood (1 ml), collected by venous puncture from witnesses aged between 3 and 10 years. By comparing the values of amino acids assessed using the two methods, closed values were noticed, obtaining a regression coefficient of r = 0.91.
- The linearity was calculated by comparing the report of the concentration of each amino acid to the internal standard. Good regression coefficients were obtained for the two marked amino acids (^{15}N-Gly and ^{15}N-Ile) used as an internal standard.

7. The ID-MS and ID-GC/MS, using compounds marked with stable isotopes, are advanced physical techniques for quantitative analysis with high precision tracks, having a large number of interdisciplinary applicative possibilities. The use of compounds marked with stable isotopes allows us to avoid the contaminants existing into the samples.

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