Photoacoustic assessment of oxidative stress in dialysis and radiotherapy by LPAS system

C. POPA*, C. MATEI
Department of Lasers, National Institute for Laser, Plasma and Radiation Physics, 409 Atomistilor St., PO Box MG-36, 077125, Bucharest, Romania

In this work, a CO_2 laser photoacoustic spectroscopy (LPAS) system was used to detect and monitor traces of ethylene in human breath air resulting from oxidative stress following the radiotherapy and dialysis treatment at patients affected by cancer and patients with renal failure, respectively. The LPAS proved to be a very sensitive and selective gas detection technique. Ethylene is well established as a breath biomarker for free radical induced oxidative stress and cell degradation. Furthermore, anti-tumour radiotherapy and renal dialysis are known to induce the oxidative attack, and our measurements may offer insight into the nature of this assault. We have found out that patients affected by cancer, treated by external radiotherapy and patients affected by renal failure, treated by standard dialysis, suffer increased generation of oxidants. Breath samples were collected before and immediately after treatment. The levels of ethylene in breath samples were measured at 10P (14) CO_2 laser line (where the ethylene absorption coefficient has the largest value of 30.4 $cm^{-1}atm^{-1}$), and compared with breath air contents from healthy humans. Monitoring of breath ethylene may provide guidance for optimal therapy and prevention of abnormality in patients on long-term radiotherapy or hemodialysis therapy.

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1. Introduction

The application of LPAS for rapid measurement of breath biomarkers has emerged in recent years as a very powerful investigation technique for monitoring and diagnostics, able of measuring trace gas concentrations at sub-ppb (parts/billion) level. The technique operates on the principle that the amount of light absorbed by a sample is related to the concentration of the target species in the sample. Light of known intensity is directed through a gas sample cell and the amount of light absorbed by the sample is measured as a sound intensity by a detector, usually a sensitive microphone. During the last years the LPAS techniques has been developed to a high degree of perfection.

Applications of LPAS include concentration measurements and trace gas analysis, accurate determination of thermophysical properties, detection of dynamic processes such as mixing of gases or chemical reactions, relaxation processes, spectroscopic experiments, measurement of aerosols. Trace-gas detection techniques are important for applications such as breath diagnostics, security and workplace surveillance, air-quality measurements, atmospheric monitoring. The laser based instruments can also be used for the detection of a wide variety of industrial gases, a broad range of chemical warfare agents, blistering agents and poisonous gases or explosives [1, 2].

Considering the wide gamut of application areas, the requirements for LPAS are various and the development and implementation of versatile analytical tools is challenging. Important features are multicomponent capability, high sensitivity and selectivity (to be immune to interference), high accuracy and precision, large dynamic range (usually larger than six orders of magnitude, from 100 ppt till 100 ppm – parts/million), none or only minor sample preparation, good temporal resolution, ease of use, versatility, reliability, robustness, and a relative low cost per unit [1-3].

Here, we report on how our recent advances in optical detection of ethylene have enabled us to record breath ethylene for patients treated by anti-tumour radiotherapy and patients treated by hemodialysis. Ethylene is well suited for the estimation of cellular damage, because this species is excreted in breath within minutes of its formation in tissues. Moreover, anti-tumour radiotherapy and hemodialysis may induce such an oxidative attack and could be put in relation to the extent of lipid peroxidation events.

A number of biomarkers can be used for the measurement of oxidative attack. Blood biomarkers include malondialdehyde (MDA), thiobarbituric acid reactive substances (TBARS), glutathione (GSH), and oxidized low density lipoproteins (LDL). Breath biomarkers of oxidative attack such as exhaled hydrocarbons offer the advantage of non-invasive monitoring and analysis of breath is preferred to direct measurement of blood samples, because contamination is easily avoided, and the measurements are much simpler in the gas phase than in a complex biological media such as blood [4, 5].

The quest for non-invasive, real-time monitoring tools is a characteristic of the modern medicine. The technique that is developed in this work complies with this

requirement, ensuring the advantages of health state assessment by monitoring the evolution of gaseous biomarkers in human body, loading a simple equipment for use in clinical practice. We choose to characterize by breath air analysis two treatment conditions: X-ray therapy and hemodialysis, but the method can be easily extended to other pathological issues.

2. Oxidative attack and cellular damage

Reactive oxygen species (ROS) are continuously generated in aerobic organisms both by endogenous factors (as by-products of oxygen metabolism) as well as by exogenous factors.

The endogenous production of free radicals derived DNA lesions has been estimated to be up to 10 000 per day per cell in humans. For instance, the yield of superoxide anions has been estimated to be 1-5% of the total consumed oxygen, equaling to 2 kg of superoxide anions per year. The majority of the endogenously produced ROS is derived from the mitochondrial electron transport chain. Even though the predominant sources of ROS in aerobic cells are the mitochondria, substantial amounts of ROS are also generated in peroxisomes. Many chemical and physical agents, including ionizing radiation and dialysis treatment, are known to exert their mutagenic and carcinogenic properties through production of free radicals [5].

Oxidative attack may thus result from: diminished levels of antioxidants, for example caused by mutations affecting the activities of antioxidant defence enzymes such as superoxide dismutase, or glutathione peroxidase, or toxins that deplete antioxidant defences; deficiencies in dietary minerals and/or antioxidants which can also cause oxidative stress; increased production of ROS, for example, by exposure of cells or organism to elevated levels of O_2; physical or chemical agents that generate excess ROS; or excessive activation of natural systems producing such species (e.g. inappropriate activation of phagocytic cells in chronic inflammatory diseases) [6,7].

The biological effects of ROS are intimately related to their tissue concentration. At low concentrations, ROS have many important physiological functions e.g. as secondary messenger of part of the immune defence. On the other hand, moderate and high levels of ROS within cells may lead to oxidative stress. The term oxidative stress, refers to a serious imbalance between production of ROS and antioxidant defence in favour of the oxidants, potentially initiators of cellular damage.

Ethylene from the human breath is an indicator of oxidant stress (in patients on dialysis, in acute myocardial infarction, in inflammatory diseases, ionizing and ultraviolet radiation damage of human skin) and can be directly correlated to physiological events in the patients (or biochemical events surrounding lipid peroxidation) [7].

Lipid peroxidation is the free-radical-induced oxidative degradation of polyunsaturated fatty acids, where biomembranes and cells are thereby disrupted, causing cell damage and cell death. The ultimate step in the peroxidative chain reaction is the formation of different hydrocarbons molecules, depending on the molecular arrangement of the fatty acid involved (Fig. 1). In the human body, the fatty acids inside the membrane lipids are mainly linoleic acid and arachidonic acid. The peroxidation of these fatty acids produces two volatile alkanes: ethylene and pentane respectively. Both of them are considered in literature to be good biomarkers of free radical induced lipid peroxidation in humans [8]. The fact that ethylene is highly volatile, not significantly metabolized by the body and not soluble in body fat, means that this diffuses rapidly into bloodstream after generation and it is transported to the lungs. The membranes separating the air in the lungs from the blood in the capillaries are very thin and are optimized for gas transport, so ethylene is easily emitted in exhaled breath and then collected [9, 10]. This speeds non-invasive patient monitoring applications when an appropriately periodic and rigorous breath sampling regime is used.

Generally speaking, the chain of processes involving any gaseous compound excreted with exhaled breath can be represented in the general form as follows (Fig. 2): production of the biomarker during a particular biochemical reaction or a complex metabolic process; diffusion of biomarker through tissues and input into haematic flow; possible intermediate accumulation; possible trapping of biomarker by utilization and assimilation systems or natural chemical transformation; transport to the lungs; transmembrane diffusion to the air space of lungs; diffusion of biomarker and their mixing with inhaled air in the alveolar space of lungs; release of biomarker in the breathing air; collection of a breath sample and assessment of the biomarker in the breath sample [11].

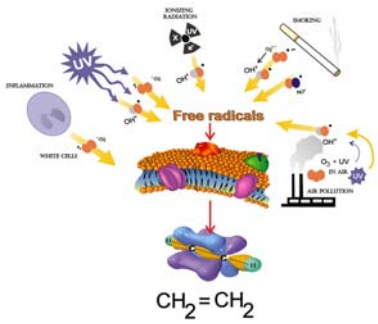

Fig. 1. Oxidative attack and production of ethylene.

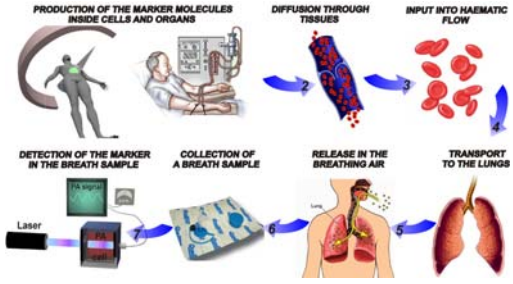

Fig. 2. Circuit of the biomarkers in human organism.

Breath analysis (breath tests) offers many and unique benefits compared with existing serum or urine tests: safe, rapid, simple to perform, non-invasive and frequently repeatable sampling; potential for real-time analyses.

3. Materials and methods

Hemodialysed patients were recruited from a Renal Dialysis Clinics (International Healthcare Systems), while the radiotherapy patients were recruited from Coltea Hospital (Radiotherapy and Oncology Clinic) in Bucharest. Breath samples were taken from patients before and immediately after the X-ray therapy/ hemodialysis treatment.

To get an efficient breath air sample, we used aluminized multi-patient collection bags (750 mL aluminum-coated bags-QuinTron), composed of a disposable mouthpiece and a tee-mouthpiece assembly [12]. After an approximately normal inspiration (avoiding filling the lungs at maximum), the subject places the mouthpiece in his/her mouth, forming a tight seal around it with the lips. A normal expiration is then made through the mouth, in order to empty the lungs of as much air as required to provide the alveolar sample. The first portion of the expired air goes out, after which the valve (from the tee-piece) is opened, the remaining expired air being redirected into the collection bag. When a suitable sample is collected, the patient stops exhaling and removes the mouthpiece [13].

After the alveolar air sample is collected (can be analyzed immediately or later), the sample gas is able to be transferred at any time into the photoacoustic cell (PA cell).

The experimental setup (Fig. 3) consists of a line-tunable CO_2 laser emitting radiation in the 9.2 – 10.8 μm region on 73 different vibrational-rotational lines and a PA cell, where the gas content is detected. The requirement for gases to be detected with this sensitive laser is that they should possess high absorption strength and a characteristic absorption pattern in the wavelength range of the CO_2 laser (e.g. 10.53 μm for ethylene) [14, 15].

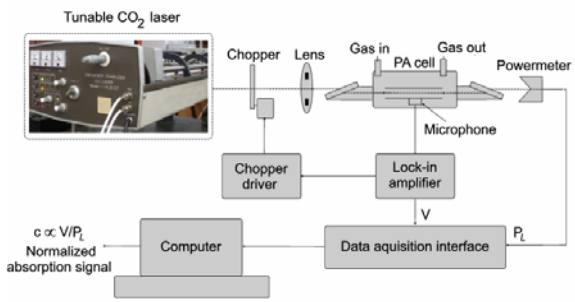

Fig. 3. General scheme of the LPAS technique.

Inside the PA, traces of ethylene can absorb the laser radiation and the absorbed energy is released into heat, which creates an increase in pressure inside a closed volume. By modulating the laser beam with a mechanical chopper, pressure waves are generated and detected with four sensitive miniature microphones mounted in the cell wall. There electric signal is fed into a dual-phase, digital lock-in amplifier and its filtered output signal is introduced in the data acquisition interface. All experimental data are processed in real time and stored by a computer. Another important element of the system is the gas handling system, due to its role in ensuring gas purity in the PA cell. It can be used to pump out the cell, to introduce the sample gas in the PA cell at a controlled flow rate, and to monitor the total and partial pressures of gas mixtures.

To increase the accuracy of LPAS method for measurements of biomarkers in exhaled air of subjects, we took several supplementary measures, such as aluminium-coated plastic bags for preserving the sample gas, or traps filled with potassium hydroxide (KOH) for retention of the CO_2, and $CaCl_2$ or $CaSO_4$ (known as Drierite) for filtration of water vapors [16, 17].

The measurements were performed for one specific CO_2 laser line where we have the maximum absorption coefficient for ethylene: 10P(14) , λ= 949.479 cm^{-1} , α= 30.4 $cm^{-1}atm^{-1}$.

To improve the measurement of ethylene absorption coefficients, a special procedure was followed. Prior to each run, the gas mixtures was flowed at 100 sccm (standard cubic centimeters per minute) for several minutes to stabilize the boundary layer on the cell walls, since a certain amount of adsorption would occur and possibly influence background signals; after this conditioning period, the cell was closed off and used in measurement. For a gas fill with 0.96 ppm ethylene, buffered in pure nitrogen, the responsivity of the cell was determined supposing an absorption coefficient of 30.4 $cm^{-1}atm^{-1}$ for ethylene at 10P(14) laser transition. The trace gas concentration at each laser line was obtained from Eq. (1) by using the measured PA signal and the laser power, and knowing precisely the responsivity of the PA cell:

$$V = \alpha R P_L c \tag{1}$$

where: V (V) is the photoacoustic signal; α (cm^{-1} atm^{-1}), the gas absorption coefficient at a given wavelength; $R = CS_M$ is the cell responsivity, where C (Pa cm W^{-1}) is the cell constant and S_M (V Pa^{-1}) is the microphone responsivity; P_L (W), the cw laser power; and c (atm), the trace gas concentration (usually given in units of per cent, ppmV, ppbV or pptV). An average over several independent measurements at each line was used to improve the overall accuracy of the results [14, 18].

4. Analysis of the exhaled ethylene from patients treated by anti-tumour radiotherapy

The first objective was to measure the exhaled ethylene from patients receiving radiation treatment and to compare the results with healthy subjects in order to

correlate the ethylene concentrations with the level of oxidative stress.

Radiation therapy uses ionizing radiation (e.g. X-rays) to kill cancer cells and shrink tumours. When considering ionizing radiations, a substantial part of the total interactions concerns water molecules, water being the major component of living tissue present in all biological systems. Consequently, water ions and radicals are mainly generated inside tissues as primary reactive species. Those reactive species (free radicals) interact with biomolecules and damage them (indirect effect of radiation); in particular, they can start lipid peroxidation events on cell membranes [19-21].

We analyzed the exhaled breath air from cancer patients, subjected to radiation treatment, based on X-ray external beam, after tumor surgery.

To analyze the bag contents, firstly we evacuated thoroughly the previous gas mixture from all the handling system, including the PA cell, traps, pipes etc., and then we flushed the system with pure nitrogen at atmospheric pressure for 10-15 minutes. After a second vacuum cleaning, the exhaled air samples were transferred in the PA cell and analyzed.

Fig. 4 presents the levels of ethylene experimentally measured for a healthy subject (female, 28 years old) and three patients (females, aged 32, 53, 77 years) with mammary cancer treated by X-ray radiation with a dose of 8 Gy. The concentration level of ethylene for the first patient before and immediately after the X-ray therapy shows an increase from ~ 0.018 ppm to ~ 0.023 ppm, for the second patient the concentration level of ethylene before X-ray therapy was ~ 0.021 ppm and immediately after the X-ray therapy ~ 0.03 ppm, for the third patient the concentration level of ethylene before and after the X-ray therapy was ~ 0.017 ppm and ~ 0.023 ppm, whereas for a healthy subject they are ~ 0.006 ppm. After X-ray irradiation we observed that the ethylene concentration increases, showing that lipid peroxidation took place and it is already possible to detect the process in the very first minute after irradiation.

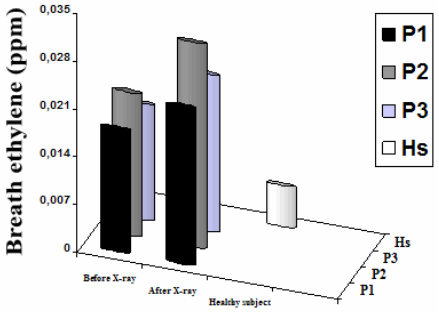

Fig. 4. The level of ethylene for a healthy subject and for three patients treated by anti-tumour radiotherapy.

The interaction of X-rays with the human body modifies the oxidative stress status through an increase in the peroxidation processes initiated by the free water radicals which were generated by the indirect radiation effect in the living tissue. A consistent part of these peroxidation events produces ethylene by lipid peroxidation. The fraction of the produced ethylene present in the exhaled breath reveals us the scale of tissue damage in the body following each X-ray session.

5. Analysis of the exhaled ethylene from patients treated by hemodialysis

The second objective of the work was to detect and measure the exhaled ethylene from patients with renal failure receiving haemodialysis. The analysis of ethylene traces from human breath would provide the necessary insight into severity of oxidative stress and metabolic disturbances and assure optimal therapy and prevention of pathology at patients on continuous haemodialysis.

To the normal buildup of urea in the body, a particularly increase in uremia at dialyzed patients should be added due to the oxidative stress. The oxidative stress is a persistent manifestation at patients with renal failure, where the loss of balance between free radical or ROS production and antioxidant systems is more pregnant, with strong negative effects on carbohydrates, lipids and proteins. In order to make the distinction between the level of uremia due to the normal physiological processes in the body and those induced by the stress of undergoing the dialysis, the determination of ethylene concentration is required [22-24].

There are two main types of dialysis: hemodialysis and peritoneal dialysis. Hemodialysis uses a special type of filter to remove excess waste products and water from the body [25, 26]. During hemodialysis, blood passes from the patient body through a filter into the dialysis machine provided with a dialysis membrane. In this procedure, the patient has a specialized plastic tube (gortex graft) placed between an artery and a vein in the arm or leg.

We have measured the levels of ethylene in the case of a healthy subject (a female, 28 years old) and three patients with renal failure (males, aged 20, 66, 80 years), before and after the hemodialysis procedure (Fig. 5).

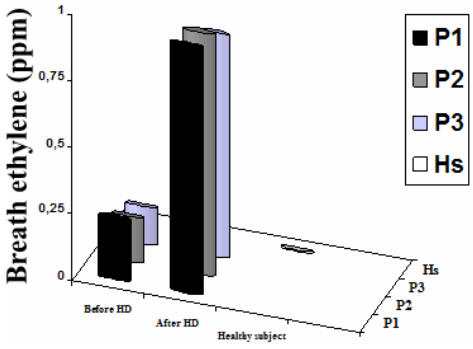

Fig. 5. The level of ethylene for a healthy subject and for three patients treated by hemodialysis.

The ethylene concentration for the healthy subject was ~ 0.006 ppm, whereas for the first patient before hemodialysis it was ~ 0.23 ppm and after hemodialysis treatment increased to ~ 0.93 ppm, for the second patient the concentration level of ethylene before hemodialysis was ~ 0.17 ppm and immediately after hemodialysis it was ~ 0.91 ppm, while for the third patient the concentration level of ethylene before and after hemodialysis was ~ 0.14 ppm and ~ 0.84 ppm, respectively.

As a first observation, we see that, immediately after hemodialysis treatment, the ethylene concentration increases, suggesting the presence of oxidative attack. Oxidative stress is a persistent manifestation at patients with renal failure, proving an imbalance between oxidant and antioxidant systems.

Hemodialysis is associated with increased oxidative stress and all treated patients are exposed to this stress. This observation appears to be due to an increased production of free radicals immediately after hemodialysis and a net reduction of many antioxidants. This factor can be added significantly to the risk for morbidity and mortality in these patients, and further studies are required.

6. Conclusions

Noninvasive medical diagnosis using breath analysis method is a topic of great interest because of its ability to distinguish more than 200 compounds in human breath. Many of these compounds, if measured accurately at very low concentrations levels, typically in the range of few ppb, can be used to identify particular medical conditions [27, 28].

In the present work, we have monitored changes in the oxidative attack during hemodialysis and ionizing radiation treatment using exhaled ethylene as a biomarker and LPAS as a method to detect this biomarker.

Our data show that both categories of patients treated by hemodialysis or radiotherapy are exposed to oxidative stress and consequently these methods treatment determine a large increase of the ethylene concentration in the exhaled breath.

In the case of X-ray treatment we observed a slight increase in the ethylene concentration values; however, in accordance with the clinical practice, this increase is sufficient to assess the body response to the treatment: the smaller the increase, the higher the radio-resistivity developed by the patient.

In the case of hemodialysis we had a relevant increase (3-5 times higher) of the ethylene levels measured after hemodialysis session compared to those before. Though the ethylene level is not a stand-alone indicator, by the correlation with the ammonia level it is possible to evaluate the efficiency and the required duration of the hemodialysis treatment.

These measurements were only possible because of the high sensitivity of our LPAS system, sensitivity that was obtained through successively improvements in optics, laser source and electronics (faster response, low noise equipment) [29].

With the relevant characteristics of high sensitivity and specificity, laser photoacoustic spectroscopy holds a great potential for medical diagnostics and future steps are to be taken in order to bring this method near the patient bad, that is more comprehensive studies to establish the exact biomarker correlation with the disease status, considering age, sex, and treatment received by the patients, and technological improvement of the system in order to increase compactness and ease of maneuverability.

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*Corresponding authors: cristina.achim@inflpr.ro