Photoacoustic spectroscopy allows to make correlations between blood p450 cytochrome and glycemia in type 1 experimental diabetes

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Abstract. Diabetes is the eight-cause death worldwide. The cause of death of patients with diabetes is mostly the long-term complications, that are not easy to detect opportely. In previous studies, we applied photoacoustic spectroscopy (PAS), a non-destructive technique, to detect several components of blood. The goal of the study was to apply the phase-resolved method (PRM), on blood optical absorption spectra obtained by PAS, to analyse blood components in experimental type 1 diabetes. Diabetes was produced in male Wistar rats through the administrations of streptozotocin (STZ). Venous blood samples were obtained one, two, four and eight weeks after STZ. PRM applied to spectra allowed to detect p450 cytochrome. There was a significant and positive correlation between glycaemia and p450 cytochrome (p=0.001). Since p450 cytochrome participates in detoxification function, results indicate that glycaemia could affect detoxification. It will be important in future studies to study the implications of those results on the development of diabetes complications. The novelty of the study was to use PAS to find out if there was any correlation between spectroscopy variables and glycaemia. It is concluded that PRM applied to PAS is a suitable technology to study p450 cytochrome in diabetes.

1. Introduction
The number of people with diabetes mellitus is increasing at an alarming rate, due to the ageing of the population, obesity, poor diet, and lack of physical activity. In 2019, it is estimated that there are 463 million people with diabetes, \([1]\) the estimates for the year 2030 and due to the pandemic, it is forecast to increase to 578 million and by 2045 it would increase to 700 million. Diabetes has a high mortality, approximately 4.2 million adults die as a result of diabetes complications, placing diabetes as the 9th place of death in the world, the first is ischemic heart disease. \([2]\)

Diabetes mellitus is defined as a chronic disease that occurs when blood glucose levels are \(\geq 126\) mg/dL \([1]\), due to genetic, environmental and autoimmune disorders that lead to alterations in the function of beta cells, causing it to be unable to produce insulin or cannot be used properly. \([3]\) Insulin is a hormone that regulates blood glucose in the body and is produced in the pancreas. Its insulin
functionality allows glucose from the bloodstream to enter the cells of the body, where it is transformed into energy. [3]

There are two types of diabetes: type 1 diabetes or insulin-dependent diabetes is characterized by poor production of insulin in the body. People with this type of diabetes need daily insulin injections to regulate blood sugar. Without it, they cannot survive. [1,4] Whereas hyperglycaemia in type 2 diabetes or non-insulin dependent diabetes, results from the production of inadequate insulin and the inability of the body to respond fully to the hormone, which is defined as resistance to insulin. [1,4] This type of diabetes is seen more frequently in older adults, but nowadays there are younger people, even children, with type 2 diabetes, due to increased levels of obesity, lack of physical activity and deficiency of the diet. [1]

Diabetes is associated with a decreased quality of life which represents an economic and social burden. Patients with diabetes die mostly due to the long-term complications such as cardiovascular disease and nephropathy.

Many physiological mechanisms are affected in diabetes including cytochrome p450 enzymes. [5,6] Those enzymes are involved in the metabolism of endogenous as well as exogenous substrates such as hormones, cholesterol, bile acids, pheromones, vitamins, fatty acids and drugs and diet components. [7]

Cytochrome p450 was initially studied in the 50s in the liver. In 1964 Omura and Sato [7] identified the nature of cytochrome. They found that cytochrome p450 are haemoproteins present in liver microsomes that are reduced by NADPH to bind CO. 450 was added to this group of enzymes because of their characteristic pigment and peak absorbance at 450 nm in the UV-Vis, near Soret's band. [7]

Cytochrome p450 is present in tissues from kidney, lung, skin, particularly in the liver. [8] Cytochromes p450 have an important role in metabolism. [7]

The group heme works as a cofactor in cytochrome p450. Changes on oxidative state and spine of heme iron offer a specific spectral signature that can be detected easily through certain spectroscopic techniques. Such detection depends on the sensitivity and requirements of the instrument like UV-Vis spectroscopy, for instance or photoacoustic spectroscopy that use ch

2. Photoacoustic Spectroscopy

Photoacoustic spectroscopy is a method that allows obtaining optical absorption spectra of solids, semisolids, liquids, and gases. It is a versatile technique that allows the analysis of optically opaque and transparent samples. [10] The technique has an advantage over other optical spectroscopic techniques, it is that the light scattered by the sample does not cause any significant problem for obtaining the optical absorption spectrum, this is because only the light that is absorbed by the sample becomes the desired signal. [10] Another advantage is that in most cases, it does not need a rigorous preparation of the sample or does not require any preparation, and is a non-destructive technique, it allows us to monitor the same sample when it is subjected to another treatments, chemical, physical or other. [11]

The photoacoustic (PA) effect is generated by confining a sample together with gas or air inside a hermetically closed cell, the sample is illuminated with modulated intensity light and absorbs this light; the sample raises its modulated temperature, causing a modulated heat flow from the sample to its surroundings, to the air confined in the cell. The energy supplied to the gas temperature rises so modulated, causing variation of pressure or sound wave which is detected by a microphone. [10]

The photoacoustic spectroscopy (PAS) has proven useful in the determination of optical absorption spectra of different types of samples, powders, gels, liquids, solids. [12] It also has a great advantage in solving spectra in a system composed of several absorbing centres. This capacity is due to the possibility of analysing both the amplitude and its phase, to each wavelength, a method defined as phase-resolved. [13]
2.1. Phase-resolved Method (PRM)

Optical absorption spectra give us important information about molecular energy bands and allow the assignment of transitions. [13] The signals obtained by photoacoustic spectroscopy, amplitude and phase, can be used to perform the Phase-resolved Method (PRM). PRM is a numeric method used in PAS, which can characterize and distinguish two absorption centres (A and B) that are present in the same spectrum.

If there are two optical absorption centres A and B, with \( S_A(\lambda) \) and \( S_B(\lambda) \) PA signals, with superimposed bands and being centred on two wavelengths so close \( \lambda_1 \approx \lambda_2 \). On the other hand, the PA signal phase difference must be different from zero \( \Delta \psi \neq 0 \). Also the observed signal \( S_f(\lambda) \) can be considered as the resultant of two components with a PA signal phase \( \phi \). The in-phase \( S_0(\lambda) \) and the quadrature \( S_{90}(\lambda) \) components can be obtained, as showed in Figure 1A. Figure 1B shows the projection of \( S_f(\lambda) \) signal at some \( \theta \) angle. If the PA signal \( S_f(\lambda) \) is the resultant of two PA signals, \( S_A(\lambda) \) and \( S_B(\lambda) \), corresponding to PA signals of absorption centers of A and B, it is possible to project \( S_f(\lambda) \) at different \( \theta \) angles in order to obtain \( S_A(\lambda) \) and \( S_B(\lambda) \) components, with \( \phi_a \) and \( \phi_b \) corresponding to absorption centers A and B, and \( \Delta \psi = \phi_b - \phi_a \), as showed in figure 1C [14,15]

\[
S_{\theta} = S_0(\lambda)\cos\theta + S_{90}(\lambda)\sin\theta
\]  

(1)

Figure 1. Phasorial diagram for phase-resolved photoacoustic method.

In this method, the PA signal is represented in a phasor image, where it must be separated into two components: The in-phase \( S_0(\lambda) \) and the quadrature \( S_{90}(\lambda) \) components, with which it is possible to perform the composition of spectra for several \( \theta \) phases, starting from the following equation:

If the spectra of the two peaks are known, corresponding to the optical absorption centres A and B, then by projecting the PA signal to several \( \phi \) angles, it is possible to reproduce the spectra of A and B centres to some defined angles \( \phi \). When the absorption peak A is minimized by angle \( \theta_a \) and maximized by \( \phi_a = \theta_a \pm 90^\circ \). Consequently, the projection of the peak B spectrum can be found at \( \theta_b \) angle. If the centre of peak B lies at \( \phi_b = \theta_b \pm 90^\circ \) then the A peak will be isolated at \( \theta_b \). For absorption peaks, A and B, located at different wavelengths, the determination of \( \phi_a \) and \( \phi_b \), can be made by isolating the spectrum of each centre, by obtaining the projection of the PA signal at different angles [14] (See Figure 1C).

Phase-resolved method was applied in the present study because it is a method that distinguishes cytochrome p450 in photoacoustic spectroscopy and the goal of the study was precisely to analyse cytochrome p450 in blood samples obtained from rats with induce type 1 diabetes, looking for any correlation with glycaemia.
3. Material and methods

3.1. Experimental animals
The study complied with the ARRIVE Guidelines [16] and was conducted by the National Institutes of Health guidelines for the care and use of laboratory animals [17]. The study was approved by the Institutional Ethical Committee to work on animals (CICUAL committee).

Male Wistar rats, six weeks old, weighing between 150 to 180 gr, were kept at the animal house of the Escuela Superior de Medicina (ESM), subjected to standard photoperiod conditions (12h light/12h dark) at room temperature (22 ° C), with fed with water and standard Purina food ad libitum.

Animals were randomly divided into two groups: control and experimental. The experimental group was treated with streptozotocin (STZ, 65 mg/kg), intraperitoneal (ip), to produce type 1 diabetes. Both groups continued under the same environment and diet. Glycaemia was measured 72 hours after STZ treatment (glucometer, one drop of blood taken from the tail). Those animals that reached glycaemia >200 mg / dL were considered diabetic. Animals that did not reach such glycaemia were discarded from the study.

Thereafter, animals (control and diabetic) were divided in 4 groups (n=3) depending on the time after STZ treatment, 1, 3, 4, 8 or 10 weeks. At the end of the time after STZ administration, animals were anesthetized (pentobarbital, 45 mg/kg ip) to place a PET50 catheter in the right jugular vein to obtain blood samples. Two ml of blood were anticoagulated with heparin, 100 µl were stored on dry to be analysed by with photoacoustic spectrometry.

3.2. Experimental PAS setup
The optical absorption spectra of blood samples were obtained by using a PA spectrometer (see Figure 2). The experimental PAS setup consists of a Xenon lamp, 700W power, as a light source. This light pass through a monochromator, to obtain a monochromatic light beam which is focused to a mechanical optical chopper, which modulates the monochromatic light at fixed 17Hz frequency. For the PA signal detection an electret microphone was used, coupled to the closed photoacoustic cell. The microphone detects the pressure variations and gives an electrical signal which is sent to a Lock-in amplifier. A personal computer is used to data acquisition of the PA amplitude and phase signals [10,18]. The spectra were normalized to the emission spectrum of the Xe lamp. PAS spectra were obtained in the ultraviolet-visible region (300-800 nm) (See Figure 3)

Figure 2. Photoacoustic spectrometer configuration

Figure 3. Optical absorption spectrum (proportional to PA signal amplitude) of blood, on the left y-axis (black circles) and PA signal phase, on the right y-axis (red circles), obtained from the lock-in amplifier as a function of the incident light wavelength
To obtain each optical absorption spectrum, the same quantity of sample was used under the same conditions and optical arrangement. When processing a photoacoustic spectrum, the raw photoacoustic data should be divided (normalized) by the emission spectrum of the Xe lamp (obtained from the PA signal of graphite powder as the sample), to avoid any discrepancies in the related optical absorption spectra with the differential intensity of the light in the entire spectrum of the lamp.

3.3. Statistical methods.
The distribution of the variables was analyzed using the Kolmogorov-Smirnov test. Comparison of the variables (control vs diabetic group) was analyzed with non-paired t test or Mann-Whitney test, depending on the distribution. Spearman test was used to analyze correlation of variables. Differences were considered significant at p<0.05. Prism 5 was the software used for statistical analysis.

4. Results and discussion
Spectra data were processed using MATLAB software. The Soret peak was detected at 420 nm (see Figure 4). It is noticeable that the group with diabetes has a higher absorption than the control. The finding indicates that there is more heme group in the blood of rats with diabetes. The Q band shows two peaks, α (580 nm) and β (550 nm), which indicate the presence of oxyhaemoglobin. [19]

![Figure 4. Optical absorption spectrum of control group (black circles) and diabetic group (red circles)](image)

The Soret band, from 400 nm to 450 nm, is associated with a sum of the absorption spectra and represents the porphyrin group. [13]. Figure 5 shows that the Soret band is reduced, and the spectra are spliced after the application of the separation method. Cytochrome p450 is shown peaking at 450 nm. The presence of cytochrome p450 has been associated with the production of reactive oxygen species. [13,15]

![Figure 5. PRM signal of control group (black circles) and diabetic group (red circles)](image)
To analyse differences related to cytochrome p450, the results were divided depending on the glucose concentration. As mentioned above, diabetes was considered with glycaemia ≥200 mg/dL. Figure 6 shows the results after applying the phase resolved method. Peak of the spectra at 450 nm as well as area under the curve from 440 to 600 nm are shown. It is important to note that glycaemia and spectra variables from each animal were analysed using the Spearman test to find any correlation.

Figure 6. The figure shows the results after applying the Phase Resolved Method, in control (glycaemia < 200 mg/dL) as well as diabetic (≥200 mg/dL) animals. Maximum absorption peak at 450 nm (A) was higher in animals with diabetes, although there was not a significant difference (p=0.06). However, the peak significantly correlated with glycaemia (p=0.02). Area under the curve (AUC) from 440-600 nm (B) was significantly higher in diabetic compared with control animals (p=0.01). AUC correlated significantly with glycaemia (p<0.001)

Cytochrome p450 enzymes participate in the metabolism of many physiological substrates as well as drugs and exogenous toxins. Cytochrome p450, as a detoxifying system, has an important role in diabetes. Cytochrome p450 is increased in liver from type 1 diabetic rats (STZ model [20]. Such increment is inhibited by insulin treatment. [21] However, it has not been detected in whole blood and that is the novelty of the present study. Moreover, the volume of the sample used for photoacoustic spectroscopy is small (around 60 μl), it is not destroyed, it is used without any previous treatment, and it is possible to detect other components of blood addition, which gives many advantages over the current technology to measure cytochrome p450. It is important to mention that the metabolism mediated by cytochrome p450 enzymes originate reactive oxygen species as a by-product. The increment of cytochrome p450 metabolism could contribute to the oxidative stress in diabetes. It has been shown that free radicals are important in the pathophysiology of diabetes and its complications. [6] Therefore, the increment of cytochrome p450 in diabetes could be associated to diabetic morbidity and mortality. It is necessary to continue the study and look for any relation between blood cytochrome p450 and the evolution of diabetic complications.

5. Conclusion
It is concluded that photoacoustic spectroscopy constitutes a suitable technology to evaluate changes on systemic cytochrome p450 in diabetes. Such technology is non-destructive and requires small quantity of blood, which makes is a suitable technology to be applied on the evaluation of patients with diabetes. Future studies will reveal if there is any relation between the increments of cytochrome p450 enzymes in blood and the development of long-term diabetes complications.

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