WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 8.084
Volume 9, Issue 1, 48-67. Research Article ISSN 2277– 7105

GLYCATION BY GLUCOSE AND KETONE BODIES OF AMINOPHOSPHOLIPIDS FROM HUMAN TISSUES IS INHIBITED BY L-ARGININE AND CREATINE. I. ERYTHROCYTE MEMBRANE

José D. Méndez*

Medical Research Unit in Metabolic Diseases, Cardiology Hospital. National Medical Center, Instituto Mexicano del Seguro Social, 06703 Mexico City, Mexico.

Article Received on
22 Oct. 2019,

Revised on 12 Nov. 2019,
Accepted on 02 Dec. 2019,

DOI: 10.20959/wjpr20201-16399

***Corresponding Author**
Dr. José D. Méndez
Medical Research Unit in Metabolic Diseases, Cardiology Hospital. National Medical Center, Instituto Mexicano del Seguro Social, 06703 Mexico City, Mexico.

ABSTRACT

The aim of this study was to evaluate the *in vitro* effect of L-arginine and creatine on glycation by glucose and ketone bodies of aminophospholipids from human erythrocyte membranes. Aminophospholipids were isolated by selective extraction from human erythrocyte membranes. Purity of aminophospholipids was confirmed by thin layer chromatography. When the effect of L-arginine or creatine was tested, 10 mM concentration of these substances was used. Emulsions were prepared in 0.1 M phosphate buffer, pH 7.4 containing 11 mM glucose, 3 mM β-hydroxybutyrate or 3 mM acetoacetate, respectively and incubated at 37 °C in dark during 61days. Samplings were made at days 0, 12, 19, 26, 48 and 61. Samples were analyzed by thin layer chromatography, fluorescence techniques and ultraviolet spectroscopy. Three intense spots with R_{fs} of 0.097, 0.50, 0.902, respectively were revealed, in addition to other spots with low intensity. Glycation was higher for glucose followed by β-hydroxybutyrate and acetoacetate. Fluorescence analysis registered into two ranks of excitation and emission; 320/460, and 355/460 nm, respectively showed that the fluorescence increased as a function of incubation time. L-arginine and creatine interfere with the glycation process depending on the glycating agent. Relevant spectral changes from 290 to 200 nm were revealed by ultraviolet spectroscopy when aminophospholipids and ketone bodies were combined. Glycation process was affected by L-arginine and creatine. L-arginine effect was better on samples containing β-hydroxybutyrate. The effect of creatine was similar when it was added to aminophospholipids combined with glucose, β-hydroxybutyrate and acetoacetate,

respectively. This study provides evidence that ketone bodies react with aminophospholipids of erythrocyte membranes forming fluorescent advanced glycation end products as do it glucose. Glycation of aminophospholipids was prevented by L-arginine and creatine.

KEYWORDS: Acetoacetate, Aminophospholipids, L-Arginine, Creatine, Glycation, β-hydroxybutyrate, Hyperglycemia.

1. INTRODUCTION

Among the bodily processes that are affected in diabetic patients with poor metabolic control is circulation, blood flows with greater difficulty. This resistance to sliding ability is associated with a property of the blood called thixotropy[1] and describes the contact change action, which increases 30 % with the disease, affecting the microvascular volume and the speed of movement of the erythrocytes,[2] and other cells of blood tissue.[3] On the other hand, the heart decreases its pumping capacity, there is an increase in the left ventricular volume at the end of systole as it has been observed in type 1diabetic patients,[4] but there may be an aggravation of the damage produced by ischemic disease.[5]

Changes in blood flow can have facial manifestations almost from the beginning of diabetes, an aspect that is associated with paralysis of the tone in the blood vessels of the face[6]. Poor blood supply to the skin can cause changes in the collagen and fat underneath. Some alterations have been related to the acid-base balance of the blood, since the hyperventilation associated with ketoacidosis produces hypocapnia[7] and the decrease in carbon dioxide tends to produce vasoconstriction, so that the erythema that has been observed in ketoacidosis seems to be a manifestation of vasomotor paralysis, probably due to alterations in smooth muscle cells in the walls of blood vessels.

In general, the metabolic alterations in diabetic ketoacidosis are accompanied by the mobilization of large amounts of fatty acids from adipose tissue, which are converted in the liver into ketone bodies.[8] The decrease in blood flow can limit the release of fatty acids to the circulation since these are transported both by albumin and by lipoproteins which under hyperglycemia conditions are glycated.[9]

The erythrocytes of the diabetic patient present a reduced capacity of deformation due to an increase in the resistance to the change in the curvature of the membrane.[10,11] It has been observed that there is a resistance twice as great as the change of shape when compared to

normal cells. It has also been found that this resistance correlates with blood glucose levels. The limited control in glucose causes changes in proteins that initially are mild. One of the modifications is the incorporation of glucose to hemoglobin which occurs without the mediation of enzymes and whose measurement is recommended as a routine test for the diagnosis and control of diabetic patients. However, glycation of hemoglobin not only occurs by glucose but also by the ketone bodies; acetone and β-hydroxybutyrate as it has been demonstrated by our group.[12] Previously, we also reported the glycation of rat brain aminophospholipids by acetoacetate and its inhibition by urea.[13] This paper provides evidence that aminophospholipids of human erythrocyte membranes are also glycated by β-hydroxybutyrate and acetoacetate and how this process is inhibited by L-arginine and creatine.

2. MATERIAL AND METHODS

2.1. Chemicals

Acetoacetate (Lithium salt), β-Hydroxybutyrate, Glucose, Creatine and Ninhydrine were purchased form Sigma (Sigma Chemical Co. St. Louis, MO, USA). L-Arginine and silica gel plates (Cat. 5721 DC-Fertigplatten Kieselgel 60) were purchased from Merck (E. Merck, Darmstadt, Germany). Other reagents were of analytical grade.

2.2. Blood sample collection

Blood was used from 20 non-diabetic individuals who attended the Central Laboratory of the Hospital of Specialties, National Medical Center Siglo XXI, IMSS. They received the corresponding consent letters. The confidentiality of the donor was assured and reference was made only to the blood sample with the registration number given by the Central Laboratory. They were: 1. Non - diabetic subjects. 2. Age: Between 20 and 70 years old. 3. Indistinct sex. The exclusion criteria were: 1. Samples of diabetic subjects. 2. Age of donors: Under 20 years and over 70 years.

2.3. Isolation of erythrocyte membranes

The erythrocyte membranes were obtained according to Nigli et al.[14] Briefly, the blood samples were centrifuged 10 minutes at 2000 x g. The plasma was separated. Then, 10 mM solution of Tris (hydroxymethyl)aminomethane hydrochloride (Tris-HCl) pH 7.4 containing 130 mM KCl was added to the erythrocyte package and centrifuged at 5800 x g, 10 minutes at 4^{o}C. The supernatant or "buffy coat" was removed by aspiration. The erythrocytes were lysed with 10 volumes of 1 mM ethylenediaminetetraacetic acid (EDTA) and 10 mM Tris-

HCl and centrifuged at 21000 x g for 50 minutes. The erythrocyte membranes were washed twice with 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) buffer, pH 7.4 centrifuging at 10000 x g for 40 minutes. Finally, the erythrocyte membranes were washed with a 10 mM solution of HEPES, containing 130 mM KCl, 0.5 mM $MgCl_2$ and 50 µM $CaCl_2$ centrifuging under the above conditions. The membranes were resuspended in the same solution and processed to obtain aminophospholipids.

2.4. Extraction of aminophospholipids

The biochemical composition of the human erythrocyte membrane is known, it contains 49 % protein, 43 % lipids and 8 % carbohydrates giving a protein to lipid ratio of 1:1.[15] Since the lipid fraction is constituted by a variety of lipids whose concentration expressed as weight percent of total lipids is: 1.5 % phosphatidic acid, 19 % phophatidyl choline, 18 % phophatidylethanolamine, 0 % phophatidylglycerol, 1 % phophatidylinositol, 8.5 % phosphatidylserine, 0 % cardiolipin, 17.5 % sphingomyelin, 10 % glycolipids and 25 % cholesterol, a selective extraction was made following established protocols. [16] First the cholesterol was extracted; acetone was added in a proportion of 6 ml of acetone / 1 gram of fresh tissue (1:6 Weight / Volume). The erythrocyte package was homogenized and the homogenate was allowed to stand with occasional agitation. It was then vacuum filtered using a buchner funnel and filter paper. The supernatant containing cholesterol was stored in a beaker and the precipitate was re-extracted twice more. The cholesterol fraction was not used in the present study and was discarded.

Once the cholesterol was extracted, the precipitate was treated with ether (6 ml / g of tissue) for the extraction of aminophospholipids. The residue was re-suspended and allowed to stand for 10 minutes with occasional agitation. It was then filtered under vacuum separating the supernatant fraction. The residue was re-extracted twice more and the supernatants were pooled. The ether was evaporated in a water bath at 37 ^{o}C. The crystals obtained constituted the fraction of aminophospholipids that was used in this study. The solid residue containing sphingolipids was discarded. Purity of aminophospholipids was confirmed by thin layer chromatography as described below.

2.4.1. Analysis of aminophospholipids by thin layer chromatography

One mg of aminophospholipids from erythrocyte membrane were dissolved in 1 mL of ether, then 4 µL were placed in a silica plate and eluted with a mixture of chloroform-methanol-acetic acid-water (65: 25: 8: 2). The presence of aminophospholipids was visualized using

0.02% ninhydrin in acetone and heating at 80 °C.[16] The separation of the different aminophospholipids was expressed as R_f values, which were calculated by dividing the distance of displacement from the point of application between the distance traveled by the solvent (See Results. Figure 1).

2.4.2. Analysis of the purity of L-arginine and creatine

Before analyzing the effect of both L-arginine and creatine in the reaction of aminophospholipids with glucose and ketone bodies, the purity of both substances was corroborated by thin layer chromatography. Solutions of 1 mg / mL containing L-arginine or creatine were prepared in phosphate buffer 0.1 M pH = 7.4. Then, 4 µL were placed in a silica plate. Chromatography was run and developed as described above for aminophospholipids.

2.5. Experimental assays

2.5.1. Incubation of aminophospholipids with glucose, β-hydroxybutyrate, acetoacetate, L-arginine and creatine.

In order to compare the effect of glucose, β-hydroxybutyrate and acetoacetate on aminophospholipid from erythrocyte membranes, 11 mM glucose, 3 mM β-hydroxybutyrate and 3 mM acetoacetate were used. When the effect of L-arginine or creatine was tested, 10 mM solutions were prepared. The concentration of aminophospholipids was 13.87 mg in each case. All the substances were mixed in 10 ml of 0.1 M phosphate buffer solution, pH = 7.4. The procedure to prepare the vials was as follows: In a glass homogenizer with Teflon plunger, 8 mL of sodium phosphate buffer pH 7.4 was added and the amounts indicated in each of the vials were homogenized. The suspension obtained was poured into the corresponding vial previously labeled and the homogenizer was rinsed with the remaining 2 mL of buffer. This procedure was repeated for each experimental condition. The order of vials was as follow: Vial 1. Aminophospholipids (Control). Vial 2. Aminophospholipids + glucose. Vial 3. Aminophospholipids + β-hydroxybutyrate. Vial 4. Aminophospholipids + acetoacetate. Vial 5. Aminophospholipids + glucose + L-arginine. Vial 6. Aminophospholipids + β-hydroxybutyrate + L-arginine. Vial 7. Aminophospholipids + acetoacetate + L-arginine. Vial 8. Aminophospholipids + glucose + creatine. Vial 9. Aminophospholipids + β-hydroxybutyrate + creatine. Vial 10. Aminophospholipids + acetoacetate + creatine. Duplicate vials were made in all cases. The vials were shaken again. Then emulsified lipids were incubated at 37 ºC in dark conditions for 61 days. A 500 µl

aliquot was taken in duplicate at 0, 12, 19, 26, 48 and 61 days from each vial. Samples were analyzed by thin layer chromatography, fluorescence (Fluoroskan fluorometer ASCENT FL-2.2) and ultraviolet spectroscopy (Lambda 20 spectrophotometer Perkin-Elmer 101N82926). Fluorescence was registered into two ranks of excitation and emission; 320 / 460, and 355 / 460 nm, respectively. The version of the program used by the fluorometer was the Ascent Software 2.4.1. In the case of ultraviolet spectroscopy, the scanning spectrum was recorded from 400 to 200 nm.

2.6. Statistical Analysis

Results of fluorescence were analyzed by one-way ANOVA and Dunnett's test.[17]

3. RESULTS

3.1. Thin layer chromatography

Several aminophospholipids present in the erythrocyte membrane were revealed by thin layer chromatography, however, since some are present in greater proportion than others, some aminophospholipids can be revealed with greater intensity. Three main spots of aminophospholipids (AP1-AP3) with R_f values of 0.097, 0.5 and 0.902, respectively, are shown (Figure 1).

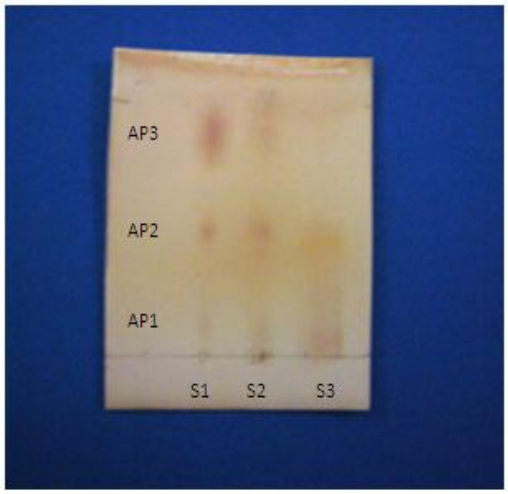

Figure 1: Thin layer chromatography of aminophospholipids (AP1-AP3) obtained from human erythrocyte membranes. In each plate the samples were applied in triplicate (S1-S3).

3.2. Purity of L-arginine and creatine

L-arginine showed two spots; one of higher intensity with R_f value of 0.3182 and another with very slight intensity with a R_f value of 0.6136 was considered impurity and was not

taken into account for the purposes of this study. Creatine only showed a spot with R_f value of 0.1932 (Figure 2).

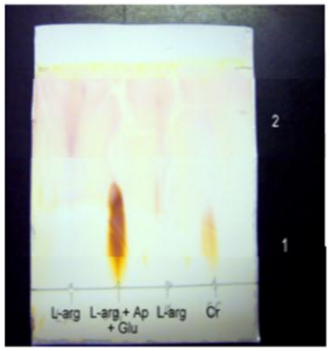

Figure 2: Thin layer chromatography of L-arginine (L-Arg) and creatine (Cr) (4µg / 4µL) dissolved in 0.1 M phosphate buffer pH 7.4. A sample of the mixture of aminophospholipids containing L-Arginine and glucose was run simultaneously for comparative purposes (Day 0).

3.3. Analysis of the aminophospholipid reaction mixtures

3.3.1 Thin layer chromatography

The results of the analysis by thin layer chromatography of the different incubation mixtures of aminophospholipids in the presence of glucose, β-hydroxybutyrate and acetoacetate are presented in Figure 3. Two conditions are compared; Day 0 (Control) and Day 61 incubation, as well as the effect of L-arginine and creatine.

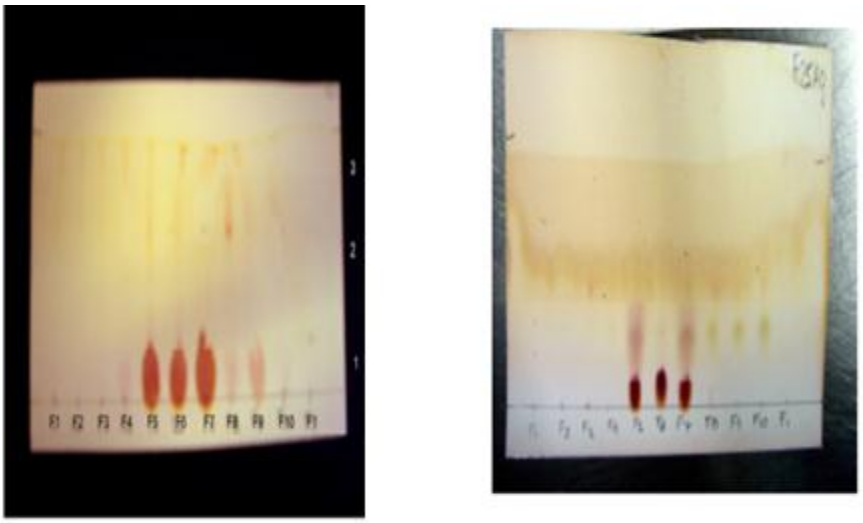

Figure 3: Thin layer chromatography of mixtures of aminophospholipids (AP1-AP3) of erythrocyte membrane with glucose or ketone bodies in the presence or absence of L-arginine or creatine (Day 0, Left and Day 61, Right. F1-F10 = Vial 1-Vial 10).

3.3.2. Fluorescence Analysis

Results of fluorescence analysis registered into two ranks of excitation and emission; 320 / 460, and 355 / 460 nm, respectively, are summarized in Table 2. In both wavelength ranges it was observed that fluorescence increased in all reaction systems as time passed. Fluorescence in the control sample was stabilized after day 19 of incubation. In the systems containing glucose, greater fluorescence intensity was observed. The reaction systems containing either glucose, β-hydroxybutyrate or acetoacetate in the presence of L-arginine showed higher fluorescence compared to the control. In the system containing β-hydroxybutyrate the fluorescence intensity was lower, similar to the fluorescence shown with acetoacetate. In relation to the effect of creatine, in both wavelength ranges greater interference was observed in the system containing β-hydroxybutyrate.

Table 1: R_f values for each aminophospholipid reaction system of erythrocyte membrane at Day 0 and at Day 61 of incubation.

Vial No.	Day Cero			Day 61		
	AP1	AP2	AP3	AP1	AP2	AP3
1	-	-	0.862	-	-	-
2	-	-	0.862	-	-	-
3	-	-	0.862	-	-	-
4	0.106	-	0.840	-	-	-
5	0.106	-	0.872	0.131	-	0.421
6	0.096	-	0.840	0.102	-	-
7	0.128	-	-	0.122	-	0.409
8	0.851	0.596	-	-	0.443	0.796
9	0.106	-	0.915	-	0.489	0.796
10	0.085	-	0.915	-	0.466	0.796

Vial 1. Aminophospholipids (Control). Vial 2. Aminophospholipids + glucose. Vial 3. Aminophospholipids + β-hydroxybutyrate. Vial 4. Aminophospholipids + acetoacetate. Vial 5. Aminophospholipids + glucose + L-arginine. Vial 6. Aminophospholipids + β-hydroxybutyrate + L-arginine. Vial 7. Aminophospholipids + acetoacetate + L-arginine. Vial 8. Aminophospholipids + glucose + creatine. Vial 9. Aminophospholipids + β-hydroxybutyrate + creatine. Vial 10. Aminophospholipids + acetoacetate + creatine. AP=Aminophospholipid.

Table 2: Fluorescence of membrane aminophospholipids of human erythrocytes incubated in different experimental conditions registered into two ranks of excitation and emission; 320 / 460, and 355 / 460 nm, respectively.

Vial No.	Wavelengh (nm) 320 - 460						Wavelengh (nm) 355 - 460					
	Day						Day					
	0	12	19	26	48	61	0	12	19	26	48	61
1	241.0	321.0	391.1	320.5	372.5	382.4	14.44	21.23	33.64	25.06	30.93	34.22
2	246.2	326.8	422.7	408.2	495.9	497.5	14.50	24.64	35.80	34.14	42.76	41.78
3	243.1	389.6	396.2	403.2	473.0	531.3	15.83	23.94	31.76	33.29	39.38	41.17
4	266.7	306.6	372.1	364.5	430.5	481.1	17.71	22.36	29.84	28.47	37.41	42.94
5	263.1	413.9	551.0	583.9	985.3	991.0*	18.12	24.60	43.02	41.28	60.32	69.34*
6	271.0	348.3	423.3	379.6	458.5	534.4*	18.51	23.76	35.06	31.26	39.54	42.24*
7	269.7	349.5	401.9	368.3	501.7	529.6*	19.51	26.24	32.95	28.66	45.02	51.05*
8	283.2	293.8	383.5	329.2	511.2	534.3	20.90	24.54	32.73	26.79	43.90	46.06
9	261.1	329.3	363.1	368.0	414.1	464.2	16.35	20.02	30.83	30.02	35.55	39.66
10	261.7	321.5	405.1	367.3	465.8	493.7	18.24	24.87	34.02	30.62	41.05	43.54

Vial 1. Aminophospholipids (Control). Vial 2. Aminophospholipids + glucose. Vial 3. Aminophospholipids + β-hydroxybutyrate. Vial 4. Aminophospholipids + acetoacetate. Vial 5. Aminophospholipids + glucose + L-arginine. Vial 6. Aminophospholipids + β-hydroxybutyrate + L-arginine. Vial 7. Aminophospholipids + acetoacetate + L-arginine. Vial 8. Aminophospholipids + glucose + creatine. Vial 9. Aminophospholipids + β-hydroxybutyrate + creatine. Vial 10. Aminophospholipids + acetoacetate + creatine. Relative fluorescence (a.u). The values are the average of the values recorded in both measurements.*(P <05).

3.3.3. Ultraviolet spectroscopy

3.3.3.1. Aminophospholipids, glucose, β-hydroxybutyrate and acetoacetate

Figure 4 shows the absorption spectra of aminophospholipids incubated 61 days with glucose and ketone bodies (Vials 1-4). Difference is observed in the absorption patterns at 270 and 230 nm that show the formation of products between aminophospholipids, glucose, β-hydroxybutyrate and acetoacetate. The absorption pattern slightly greater than the control is similar between the three substances.

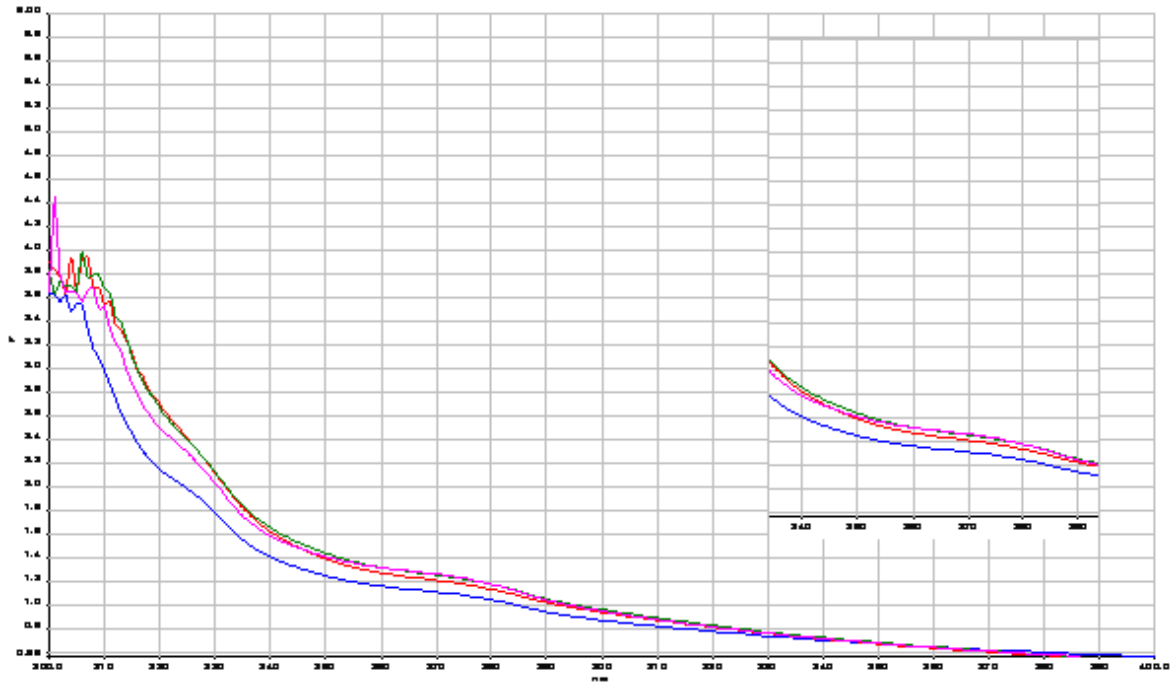

Figure 4: Scanning spectrum 400 to 200 nm (from right to left) of aminophospholipids at day 61 of incubation (Each division corresponds to 10 nm) (Insert: 290-240 nm). Color blue: Vial 1. Aminophospholipids (Control). Changes in some regions of the spectrum similar to changes that occur in the presence of glucose and ketone bodies can be seen. Red color: Vial 2. Aminophospholipids + Glucose. Green color: Vial 3. Aminophospholipids + β-hydroxybutyrate. Pink color: Vial 4. Aminophospholipids + Acetoacetate. Absorbance vs. λ (nm).

3.3.3.2. Aminophospholipids, glucose, L-arginine and creatine

The effect of L-arginine and creatine on the mixture of aminophospholipids with glucose compared with their respective controls is shown in Figure 5 (Vials 1, 2, 5 and 8). The addition of L-arginine to the system containing aminophospholipids and glucose (Vial 5) caused a decrease in absorption (Figure 4), while creatine not only produced an increase in absorption but also a change in the absorption pattern.

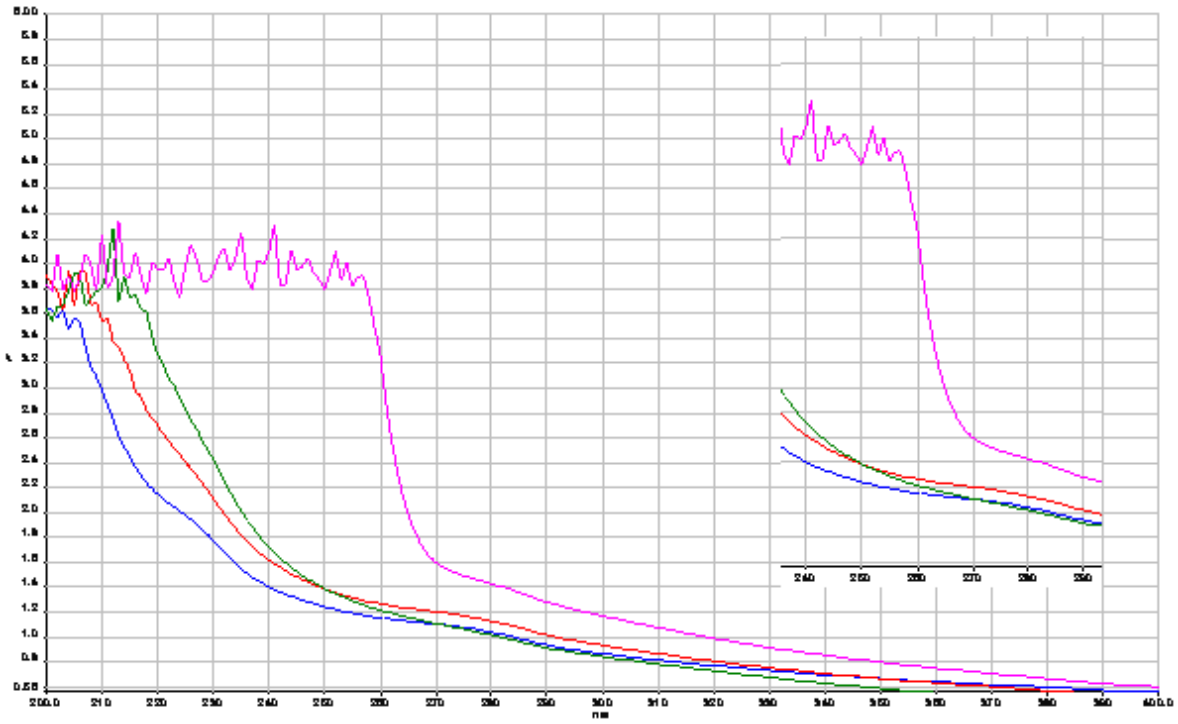

Figure 5: Aminophospholipids incubated for 61 days with glucose and L-arginine or creatine (Insert: 290-240 nm). Color blue: Vial 1. Aminophospholipids (Control). Red color: Vial 2. Aminophospholipids + Glucose. Green color: Vial 5. Aminophospholipids + Glucose + L-arginine. Pink color: Vial 8. Aminophospholipids + glucose + creatine. Absorbance vs. λ (nm).

3.3.3.3. Aminophospholipids, β-hydroxybutyrate and L-arginine

The effect of L-arginine on the incubation mixture containing aminophospholipids and β-hydroxybutyrate is presented in Figure 6 (Vials 1, 3 and 6). The addition of β-hydroxybutyrate caused a slight increase in the absorption pattern from 310 nm. Similar effect was caused by L-arginine, being marked at 270 nm.

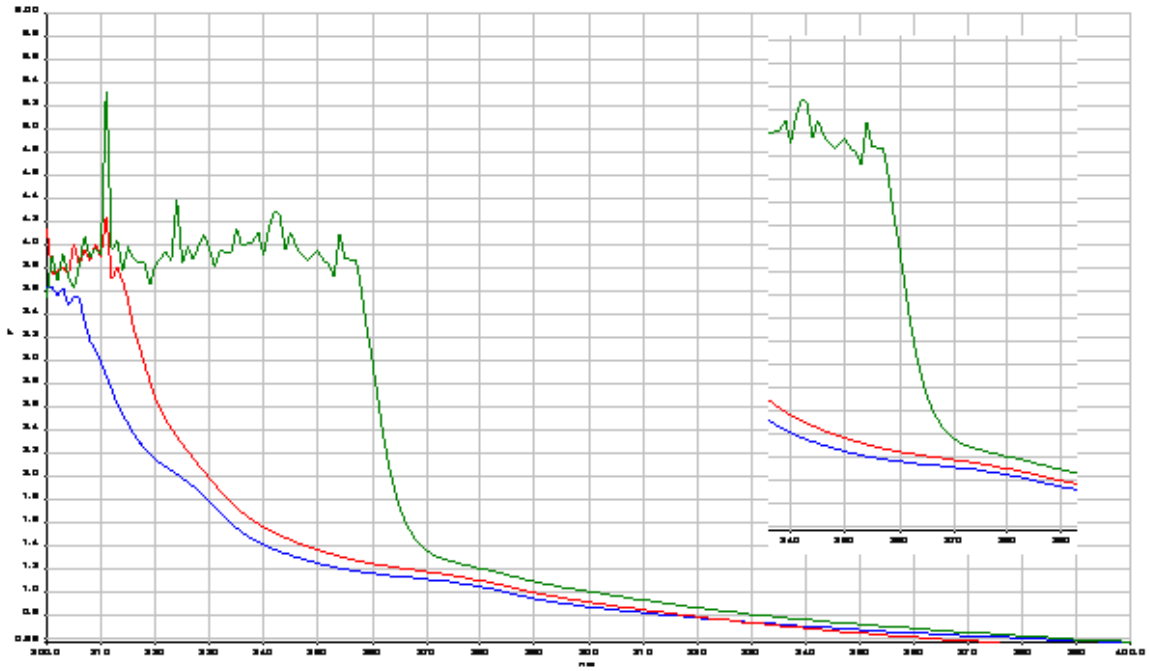

Figure 6: Aminophospholipids at day 61 of incubation with β-hydroxybutyrate and L-arginine (Insert: 290-240 nm). Blue color: Vial 1. Aminophospholipids (Control). Red color: Vial 3. Aminophospholipids + β-hydroxybutyrate. Green color: Vial 6. Aminophospholipids + β-hydroxybutyrate + L-arginine. Absorbance vs. λ (nm).

3.3.3.4. Aminophospholipids, acetoacetate, L-arginine and creatine

The effect of L-arginine and creatine on the mixture of aminophospholipids and acetoacetate is presented in Figure 7 (Vials 1, 4, 7 and 10). Seen by the increase in absorbance between 280 and 270 nm in the absorption pattern, there is interaction between aminophospholipids and acetoacetate. While L-arginine caused a slight increase in absorption, creatine caused a significant increase in absorbance from 270 nm, similar to that shown by L-arginine with β-hydroxybutyrate (Figure 6).

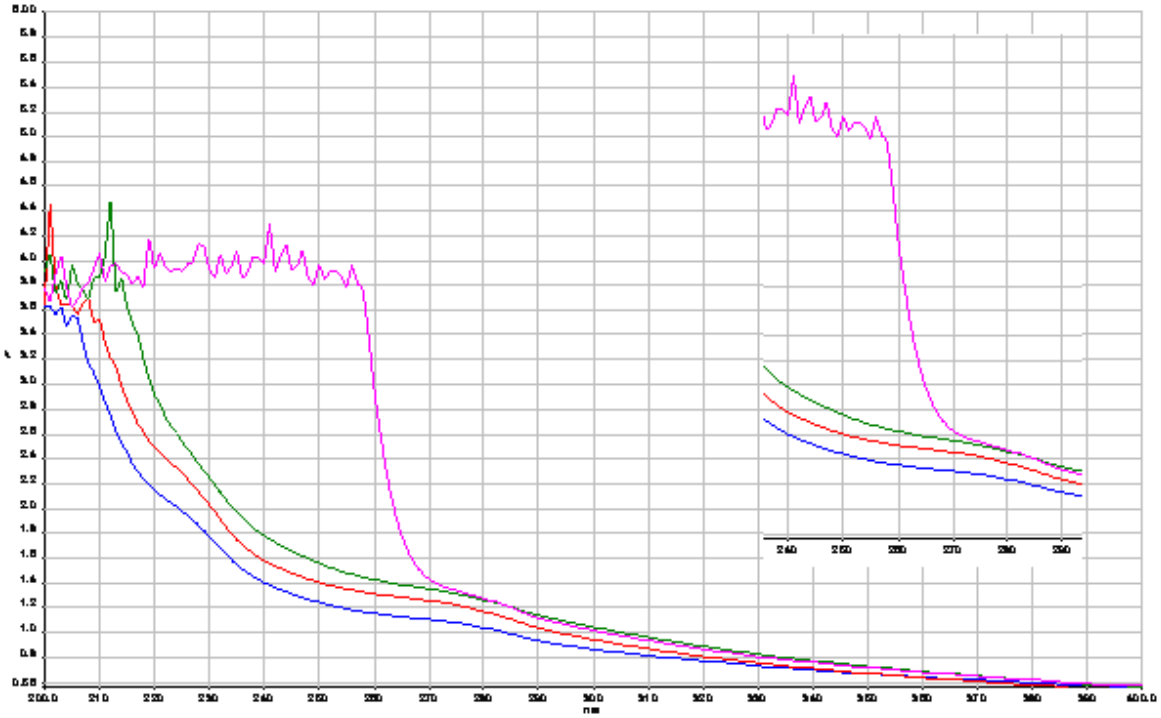

Figure 7: Aminophospholipids at day 61 of incubation with acetoacetate, L-arginine or creatine (Insert: 290-240 nm). Blue color: Vial 1. Aminophospholipids (Control). Red color: Vial 4. Aminophospholipids + acetoacetate. Green color: Vial 7. Aminophospholipids + acetoacetate + L-arginine. Pink color: Vial 10. Aminophospholipids + acetoacetate + creatine. Absorbance vs. λ (nm).

3.3.3.5. Aminophospholipids, glucose, β-hydroxybutyrate, acetoacetate and L-arginine

The effect of L-arginine on mixtures of aminophospholipids with glucose, β-hydroxybutyrate and acetoacetate is shown in Figure 8 (Vials 1, 5, 6 and 7). The addition of L-arginine to the acetoacetate-containing mixture caused an increase in absorption at 270 nm which decreased at 235 nm similar to that shown for β-hydroxybutyrate, while with glucose it increased around 240 nm presenting two points of intersection with the spectra of both ketone bodies at 260 nm for β-hydroxybutyrate and 235 nm for acetoacetate (Insert: 290-240 nm). This could be due to the formation of different reaction products.

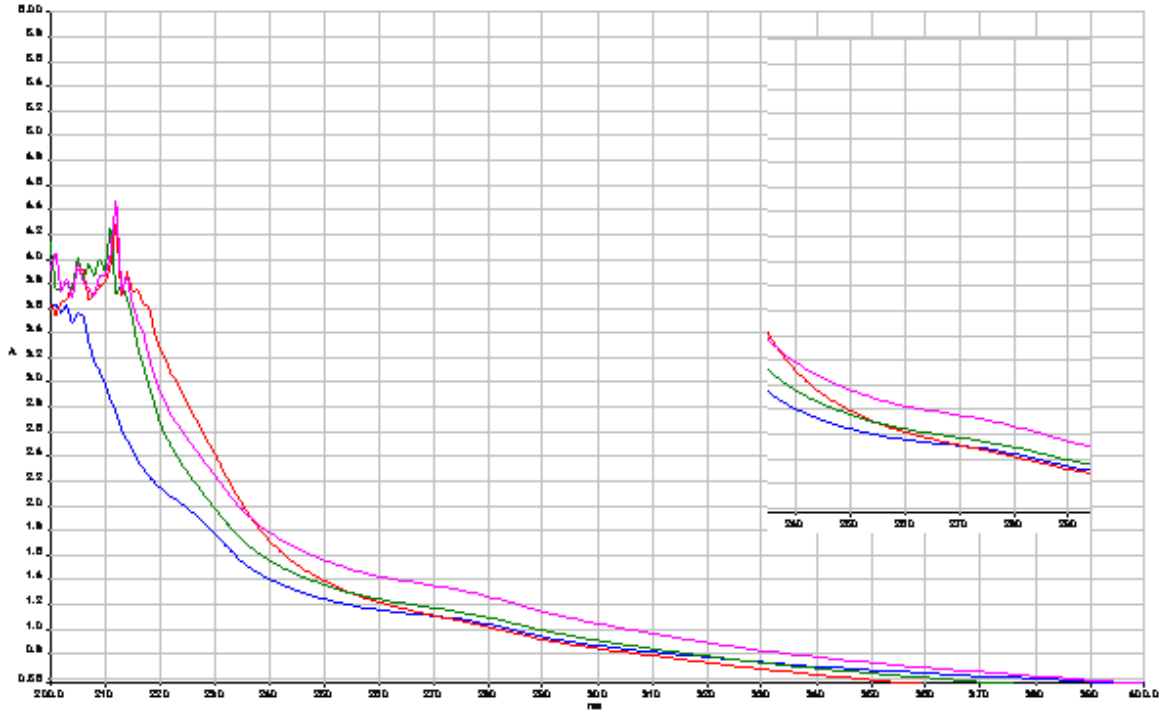

Figure 8: Aminophospholipids at day 61 of incubation (Insert: 290-240 nm). Blue color: Vial 1. Aminophospholipids (Control). Red color: Vial 5. Aminophospholipids + glucose + L-arginine. Green color: Vial 6. Aminophospholipids + β-hydroxybutyrate + L-arginine. Pink color: Vial 7. Aminophospholipids + acetoacetate + L-arginine. Absorbance vs. λ (nm).

3.3.3.6. Aminophospholipids, glucose, β-hydroxybutyrate, acetoacetate and creatine

The effect of creatine on the mixture of aminophospholipids with glucose, β-hydroxybutyrate and acetoacetate is presented in Figure 9 (Vials 1, 8, 9 and 10). The effect was similar, taking the wavelength of 280 nm as reference, the absorption from highest to lowest was: aminophospholipids + Glucose > aminophospholipids + acetoacetate ≥ aminophospholipids + β-hydroxybutyrate. In all three cases there was a series of peaks in the region of 260 to 200 nm that do not appear in the spectrum of the control sample.

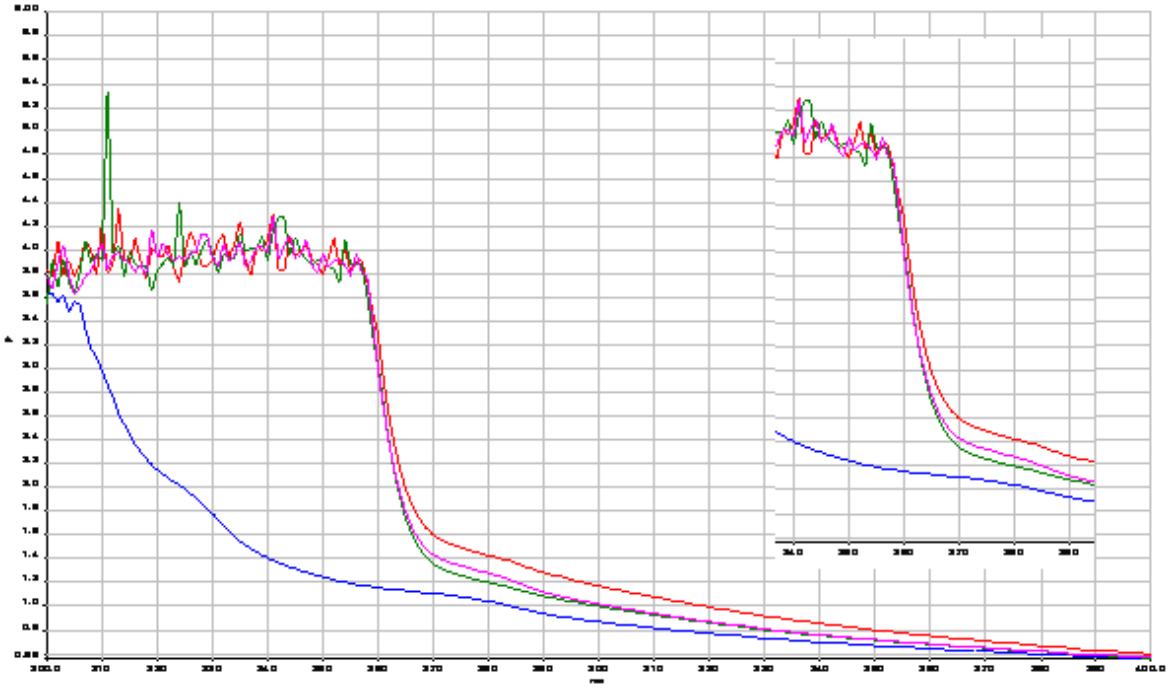

Figure 9: Aminophospholipids at day 61 of incubation with creatine (Insert: 290-240 nm). Blue color: Vial 1. Aminophospholipids (Control). Red color: Vial 8. Aminophospholipids + glucose + creatine. Green color: Vial 9. Aminophospholipids + β-hydroxybutyrate + creatine. Pink color: Vial 10. Aminophospholipids + acetoacetate + creatine. Absorbance vs. λ (nm).

4. DISCUSSION

The metabolism of ketone bodies has been studied in health and disease[8] as well as the treatment of patients affected by elevated levels of ketone bodies.[18] However, little is known about the molecular mechanisms that lead to the development of complications associated with high concentrations of ketone bodies.

Based on previous observations on glycation of human hemoglobin by acetone and β-hydroxybutyrate [13] and brain aminophospholipids by acetoacetate.[14] we designed this study to investigate the effect of high concentrations of glucose, β-hydroxybutyrate and acetoacetate on aminophospholipids isolated from erythrocyte membranes of non-diabetic subjects, under the hypothesis that these substances could carry out glycation reactions. The effect of L-arginine whose antiglycant action had been previously demonstrated[19] and creatine, a metabolite of L-arginine, was tested. For this, mixtures of aminophospholipids, ketone bodies and antiglycant agents were prepared and incubated at different times and analyzed by three methods; Thin layer chromatography, fluorescence and ultaraviolet

spectroscopy. The results obtained directly or indirectly demonstrate the formation of glycation products.

4.1. Thin layer chromatography

The aminophospholipids were selectively isolated from erythrocyte membranes (Figure 1). When they were incubated for 61 days with or without glycant and antiglycant substances, changes occurred not only in the intensity of the spots but also in the displacement expressed in terms of R_f (Figure 3). Samples marked F_5, F_6 and F_7 correspond to aminophospholipids incubated with glucose, β-hydroxybutyrate and acetoacetate in the presence of L-arginine, respectively, compared to a control sample (vial 1). Samples F_8, F_9 and F_{10} correspond to aminophospholipids incubated with glucose, β-hydroxybutyrate and acetoacetate in the presence of creatine. The R_f values of the spots that showed greater intensity are presented in Table 1. It is important to note that in samples F_2, F_3 and F_4 the intensity of the spots is not very clear, suggesting that the aminophospholipids reacted with glucose and ketone bodies.

The addition of L-arginine or creatine caused modification in the R_f values and the appearance of compounds whose formation was dependent on the incubation time. L-arginine showed greater intensity in some components and variation in R_f values, compared with the effect of creatine, suggesting that L-arginine protects more efficiently than creatine.

In the case of the control sample, the spots were not intense which could be attributed to degradation of lipids by oxidation, which occurs from day 12 of incubation. However, it is interesting to note that both L-arginine and creatine appear to exert a protective effect since on day 61 of incubation intense stains suggesting the preservation of aminophospholipids are still observed.

4.2. Fluorescence Analysis

The different incubation mixtures were analyzed in the excitation and emission ranges of 320/460 and 355/460 nm (Table 2). Similar to that observed by thin layer chromatography, the control sample showed a behavior suggesting that with a prolonged incubation time the aminophospholipids degrade. It was observed that both glucose and ketone bodies reacted with the aminophospholipids, which corroborates the results obtained by thin layer chromatography.

Using the average values obtained from the reaction systems that did not contain L-arginine or creatine, the slope of the line was calculated from time zero to day 12 of incubation. This slope indicates the speed with which the substances present in the incubation system react. The reaction with glucose showed a higher speed in the emission range 355/460 nm, while for β-hydroxybutyrate it was in the emission range of 320/460 nm. When these results were analyzed using the single-factor ANOVA test, it was confirmed that there is a significant difference in both cases ($P < 0.05$). Using the Dunett test, a comparison was made between all incubation and control systems. The result of this test demonstrates that in both cases L-arginine presented a significant difference ($P < 0.05$), which is consistent with the graphic analysis of fluorescence means, thus demonstrating the inhibitory effect of L-arginine (Table 2).

4.3. Ultraviolet spectroscopy

Ultraviolet spectroscopy analysis confirmed that the aminophospholipids isolated from the erythrocyte membrane reacted with glucose and ketone bodies; β-hydroxybutyrate and acetoacetate giving similar absorption patterns (Figure 4). Both L-arginine and creatine interfere with the glycation reaction. The effect of these antiglycant agents varies depending on the ketone body.

Seen by absorption patterns, the addition of L-arginine to the system containing glucose and aminophospholipids inhibited glycation while maintaining virtually the same absorption pattern (Figure 6). The addition of creatine caused a marked increase in absorbance, an effect that can be associated with its greater reactivity and the formation of alternative compounds that contribute to absorption in the ultraviolet region. This may suggest greater inhibition potential (Figure 5). A similar absorption pattern was presented when the aminophospholipids were incubated with β-hydroxybutyrate and L-arginine (Figure 6) and aminophospholipids with acetoacetate and creatine (Figure 7). The systems containing L-arginine or creatine showed greater absorbance and a similar amount of peaks, both substances compete for the carbonyl group and can favor the formation of other compounds. Figure 9 summarizes the effect of creatine on the action of glucose and ketone bodies when they are combined and incubated for long periods of time such as 61 days.

5. COMMENTS

In this study, fatty acids of aminophospholipids were not characterized, but it is known that several carbonyl compounds such as malondialdhyde, 4-hydroxy-2-alkenals and acrolein

among others are formed by lipid peroxidation of unsaturated fatty acids.[20-23] This could partly explain the changes observed in the control sample both by fluorescence and by ultraviolet spectroscopy.

We can hypothesize that the damage caused by high concentrations of ketone bodies can be enhanced by carbonyl compounds produced by lipid peroxidation. Several unsaturated fatty acids are structural components of aminophospholipids of the human erythrocyte membrane. Among them; Oleic acid (18: 1), Linoleic acid (18: 2), Arachidonic acid (20: 4), Nervonic acid (24: 1) and Docosahexanoic acid (22: 6), of which oleic acid and arachidonic acid they constitute 18.1 and 23.7%, respectively.[24, 25] Other biological membranes such as human myelin have high phosphatyldylethanolamine content (15%).[25] (The data are expressed as weight % of total lipids).

Studies in rat liver have revealed that internal and external mitochondrial membranes are high in phosphatidylethanolamine; 38.8 and 34.9%, respectively. Smooth and rough endoplasmic tissue 22 and 20%, respectively. The lysosomal, nuclear, Golgi and plasma membranes contain 20.5, 25.1, 17.0 and 20.5 of phosphatyldylethanolamine, respectively.[25] Since ketone bodies are formed in the liver, liver function may be affected by excessive production of ketone bodies in chronic hyperglycemia.

6. CONCLUSION

In conclusion, these results shown that ketone bodies; β-hydroxybutyrate and acetoacetate react with aminophospholipids from erythrocyte membranes forming glycation products similar to those formed by glucose, which partly explains the effect of high concentrations of ketone bodies in the development of hematological complications observed in the diabetic patient with poor metabolic control.

Glycation reactions can be partially prevented by L-arginine and creatine, which is clinically relevant since the ability of erythrocyte deformation is related not only to the improvement of microcirculation but also to circulation in the large arteries.

7. ACKNOWLEDGEMENTS

The author thanks to Paulina Hernández Sánchez López, B. Pharm. for her technical assistance.

8. REFERENCES

1. Meadows R. Mystery matters. Chem Matters. 1993; 12-15.
2. Rendell M, Bergman T, O´Donnell G, Drobny E, Borgos J, Bonner RF. Microvascular blood flow, volume and velocity measured by lasser Doppler techniques in IDDM. Diabetes. 1989; 38: 819-824.
3. Vague P, Juhan I. Red cell deformability, platelet aggregation, and insulin action. Diabetes. 1983; 32(Suppl. 2): 88-91.
4. Fisher BM, Guillen G, Lindop GBP, Dargie HJ, Frier BM. Cardiac function and coronary arteriography in asymptomatic type I insulin-dependent diabetic patients: Evidence for a specific diabetic heart disease. Diabetologia.1986; 29: 706-712.
5. McMillan DE.Monitoring the apperance and progress of blood and vascular abnormalities. In: Clinical Diabetes Mellitus. Davidson JK, (Eds).2nd edition, Thieme Medical Publishers, Inc., New York, USA, 1991, pp. 414-426.
6. Crepaldi G, Nosadini R. Diabetic cardiopathy: Is it a real entity? Diabetes Metab Rev. 1986; 4: 273-288.
7. Tasker RC, Lutman D, Peters MJ. Hyperventilation in severe diabetic ketoacidosis. Pediatr Crit Care Med. 2005; 6(4): 405-411.
8. Laffel L. Ketone bodies: a review of physiology, pathophysiology and application of monitoring to diabetes. Diabetes Metab Res Rev. 1999; 15: 412-426.
9. Soaita I, Yin W, Rubenstein DA. Glycated albumin modifies platelet adhesion and aggregation responses. Platelets. 2017; 28(7): 682-690.
10. McMillan DE, Utterback NG, La Puma J. Reduced erythrocyte deformability in diabetes. Diabetes. 1978; 27(9): 895-901.
11. Shin S, Ku Y, Babu N, Singh M. Erythrocyte deformability and its variation in diabetes mellitus. Indian J Exp Biol. 2007; 45(1): 121-128.
12. Xie J, García-Pérez E, Méndez JD. *In vitro* glycation of hemoglobin by acetone and β-hydroxibutirate. World Appl Sci J. 2007; 2(2): 99-106.
13. Aguilar-Hernández M, Méndez JD. *In vitro* glycation of brain aminophospholipids by acetoacetate and its inhibition by urea. Biomed Pharmacother. 2007; 61(10): 693-697.
14. Nigli V, Zurini M, Carafoli E, Purification, reconstitution and characterization of the Ca^{2+} pump of plasma membranes. Methods in Enzymology. 1987; 139: 791-708.
15. Voet D, Voet JG. Lipids and Membranes. In: Biochemistry. 2nd ed. John Wiley & Sons, Inc. New York 1995. p. 290.
16. Méndez JD. Experimentos Básicos de Bioquímica. Ed. Prado. México, D. F. 2001; 89-96.

17. Ratkowski DA, Evans MA, Alldredge JR. Cross-over experiments. Design, analysis and application. Statistics Textbooks and Monographs, 135, Marcel Dekker, New York, 1993.
18. Davidson, JK. Diabetic ketoacidosis and the hyperglycemic hyperosmolar state.In: Clinical Diabetes Mellitus. A Problem-Oriented Approach.Third edition. Davidson, J. K. (Ed). Thieme. New York, USA. 2000. pp. 479-498.
19. Méndez JD, Balderas FL. Inhibition by L-arginine and spermidine of hemoglobin glycation and lipid peroxidation in rats with induced diabetes. Biomed Pharmacother, 2006; 60(1): 26-31.
20. Gardner HW. Oxygen radical chemistry of polyunsaturated fatty acids. Free Radic Biol Med. 1989; 7: 65-86.
21. Pryor AW, Stanley PA. A suggested mechanism for the production of malonaldehyde during the autoxidation of polyunsaturated fatty acids. Nonenzymatic production of prostaglandin endoperoxides during autoxidation. J Org Chem. 1975; 40: 3615-3617.
22. Uchida K. Current status of acrolein as a lipid peroxidation product. Trends Cardiovasc Med. 1999; 9(5): 109-113.
23. Naudí A, Jové M, Ayala V, Cabré R, Portero-Otín M, Pamplona R. Non-enzymatic modification of aminophospholipids by carbonyl-amine reactions. Int J Mol Sci. 2013; 14(2): 3285-3313.
24. Cullis PR, Hope MJ. Physical properties and functional roles of lipids in membranes. In: Biochemistry of Lipids, Lipoproteins and Membranes. Vance DE, Vance J (Eds.). Elsevier Science Publishers. Amsterdam 1991. Chapter 1. Pages 1-41.
25. Voelker DR. Lipids assembly into cell membranes. In: Biochemistry of Lipids, Lipoproteins and Membranes. Vance DE, Vance J(Eds.). Elsevier Science Publishers. Amsterdam 1991. Chapter 17. p. 489-523.