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Artículo de Investigación

Studies on the *in vitro* phototoxicity of the antidiabetes drug gliclazide.

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RESUMEN

El fármaco antidiabético gliclazida (**1**) es fototóxico y fotolabil bajo condiciones aeróbicas y de luz UV-B. La irradiación de una solución buffer de **1** bajo atmósfera oxigenada produce dos fotoproductos. La fotoquímica de **1** involucra la ruptura del enlace S-N y C(O)-N de la unidad de urea. La lisis fotosensibilizada de eritrocitos por **1** fue determinada. Sin embargo los fotoproductos de la gliclazida no mostraron este efecto. La inhibición de este proceso en presencia de DABCO, GSH, confirman la posibilidad de la participación de radicales libres en la fotohemólisis. Además, mediante ensayos de peroxidación lipídica con ácido linoleico también se verificó la fototoxicidad *in vitro* de **1**. La generación de oxígeno singlete durante la fotólisis de **1** fue descartada mediante los ensayos de histidina y de NaN_3. En un estudio suplementario la habilidad de **1** de inhibir radicales libres o especies de oxígeno reactivas (OH, $^1O_2$, H_2O_2) en sistemas libres de células fue investigado usando quimioluminescencia y los espectros de absorción. **Palabras claves**: Fototoxicidad, gliclazida, fotohemólisis, antioxidante, quimioluminescencia

ABSTRACT

The phototoxic antidiabetes drug gliclazide (**1**) is photolabile under aerobic conditions and UV-B light. Irradiation of a phosphate buffered solution of **1** under oxygen atmosphere produces two photoproducts. The photochemistry of **1** involves cleavage of the S-N and C(O)-N bonds of the urea unit. Red blood cell lysis photosensitized by **1** was demonstrated. Their photoproducts didn't show this effect. Inhibition of this process by addition of DABCO, GSH, confirmed the possibility of the participation of free radicals in the photoinduced hemolysis. Furthermore, in a lipid-photoperoxidation test with linoleic acid the *in vitro* phototoxicity of gliclazide was also verified. The generation of singlet oxygen during the photolysis of gliclazide was ruled out by means of the histidine and NaN_3 assays. In a supplementary study the ability of **1** to inhibit free radical or reactive oxygen species ($^{\cdot}OH$, $^1O_2$, H_2O_2) generated in cell-free systems was investigated using chemiluminescence and electronic absorption spectra. **Keywords**: Phototoxicity, gliclazide, photohemolysis, anti-oxidant, chemiluminescence.

Introduction

Gliclazide (**1**) belongs to a group of phototoxic antidiabetes drugs[1-3]. The therapeutic use of benzenesulfonamide derived drugs as antidiabetes agents has been associated in some patients with the appearance of phototoxic effects such as erythema, flaring and urticarial weal. In vitro experiments also reveal the potential phototoxicity of these drugs[4]. In clinical tests and in cell culture, photosensitization has been recognized in this family of compounds[5]. The action spectrum of gliclazide seems to be in the UV-B range. Although very useful and almost indispensable, it can produce adverse biological effects such as clinical photosensitization which occurs on the skin of patients[6]. The process of

phototoxicity could occur through a photodynamic mechanism mediated either by singlet oxygen and hydroxyl radical species (an oxygen-dependent mechanism) or directly by the action of photoproducts and free radical intermediate. The sulphonamides in general have been reported to induce this unwanted side-effect[7]. Other phototoxic compounds with a variety of different molecular structures can also produce singlet oxygen by energy transfer[8,9]. The numberand variety of phototoxic compounds is large. Furthermore, for most phototoxic xenobiotics a correlation between structure and photoreactivity is not easily found[10-13]. It is assumed that there must be a relationship between photochemical behavior and phototoxicity. The present study deals with the photodegradation of **1** under UV-B radiation, isolation and identification of the photoproducts, the possible formation of singlet oxygen, and the *in vitro* phototoxicity of the parent compound and that of its photoproducts in order to confirm this hypothesis.

We examined the photolysis of gliclazide under mild conditions, trying to simulate in the laboratory some of those in which the phototoxic effects have been observed *in vivo*, namely aqueous, neutral and oxygenated media. The irradiation was carried out with UV-B light. This study also examine the ability of gliclazide (**1**) to inhibit free radical or reactive oxygen species ($^{\cdot}OH$, $^1O_2$, H_2O_2) generated in cell-free systems using isoluminol and luminol-enhanced chemiluminescence by H_2O_2 and electronic absorption spectra[14-15].

2. Materials and methods

2.1 Chemicals

Gliclazide (N-[[(Hexahydrocyclopenta [c] pyrrol-1(1H)-yl) amino]carbonyl] -4- methyl - benzene-sulfonamide) (**1**) (CAS 21187-98-4) was extracted from the commercial medicament, Gliclazida® (Calox International laboratory, Caracas, Venezuela) by ethanol soxhlet extraction and recrystallized from the same solvent. The purity was 99.5% as determined by ^1H NMR spectroscopy and UV-visible spectrometry. Superoxide dismutase (SOD), galvanoxyl radical, horseradish peroxidase (HRP) and 5,5´dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma (St. Louis, MO, USA), while 3-Aminophthalhydrazide (Luminol), isoluminol, ferrous sulfate heptahydrate, sodium azide (NaN_3), vitamin C, butylated hydroxyanisole (BHA), 1,4-diazabicyclo [2.2.2] octane (DABCO), reduced glutathione, histidine, 2,5-dimethylfuran (DMF), tetraphenyl porphine (TPP) and rose bengal were purchased from Aldrich (Steinheim, Germany). All analytical or HPLC grade solvents were obtained from Merck (Darmstadt, Germany).

2.2 Photolysis

Solutions of **1** were irradiated at room temperature for 72 h in methanol (0.120 g, 0.335 mmol in 100 ml) in an Rayonet photochemical reactor equipped with 16 phosphor lamps with an emission between 275 and 320 nm (23 mW/cm^2 of irradiance) as measured with a model of UVX Digital Radiometer after 1 h continued illumination under oxygen atmosphere. In another experiment the solution was irradiated for 48 h under argon atmosphere. The course of the reaction was followed by UV-Vis spectrophotometry using a Milton-Roy 3000 instrument and also by GC and HPLC until the gliclazide was completely consumed. After irradiation the solvent was evaporated at reduced pressure (14 Torr) at room temperature and the photoproducts **2** and **3** were separated and identified by GC-Mass spectroscopy (Carlo Erba/Kratos MS25RFA). The HPLC used was a Waters Delta Prep 4000 equiped with a 3.9x300 mm, 10 μm Bondapak C18 column using a CH_3CN/H_2O (30:70 vol/vol) gradient as mobile phase at a flow rate of 0.8 ml min^{-1}, with monitoring at 230 nm[16]. The retention time was of 1.20 min for the compound **2** and 3.80 min for the compound **3**.

Compound **2**: MS: m/e (%) = 126 (100, M^+), 110 (32), 96 (42), 82 (24), 44 (36).

Compound **3**: MS: m/e (%) = 199 (4, M^+), 185 (32, M^+-15), 170 (10, M^+- HCO), 155 (53, M^+- HCONH), 108 (46), 92 (100, M^+- $HCONHSO_2$), 91 (25), 77 (34), 45 (32).

Both compounds **2** and **3** were identified as hexahydrocyclopenta [c] pyrrolamine and p-

tolylsulfonylurea respectively showing spectroscopic, physical and chemical properties identical to those of an authentic sample of reported compound in the literature[17-20].

2.3 Singlet Oxygen detection

In a separate experiment, irradiations of **1** were carried out under the same experimental conditions that in the photolysis assay but now in the presence of 2,5-dimethylfuran (2,5-DMF, 5.00 mmol) which is normally used as a trap for singlet oxygen ($^1O_2$)[21]. This process was followed by gas chromatography and by mass spectrometry as in the previous experiment. Rose bengal, a well known $^1O_2$ sensitizer, was used as a reference standard for comparison with 1 as far as $^1O_2$ formation, under identical conditions of photolysis.

To observe a possible quencher effect of the gliclazide on $^1O_2$, **1** was also irradiated in the presence of photosensitizer (specifically generators of singlet oxygen) as rose bengal and tetraphenyl porphirine (TPP) with an Osram HQL 250 W medium pressure Hg lamp using a potassium chromate solution (100 mg/L) as a filter allowing only $\lambda > 400$ nm radiation to reach the reactor and maintaining all other experimental conditions the same.

Indirectly, singlet oxygen was detected by photosensitized degradation of L-histidine[22]. The latter was measured in the presence of 0.25, 0.50, 1.0 and 1.5 x 10^{-5} M of gliclazide. These solutions were mixed with an equal volume quantity of L-histidine solution in concentrations ranging from 0.60 to 0.74 mM in phosphate buffer 0.01, pH 7.4. Samples of these mixtures were irradiated as above at time intervals from 60 to 180 min. The respective controls were protected from light. The histidine degradation was determined by a colorimetric reaction using the following reagents: phosphate buffer, sulfanilic acid, sodium nitrite, sodium carbonate and ethanol in succession using a modified Pauly reaction[23]. The optical density was read on a spectrophotometer at 530 nm against phosphate buffer as blank reagent.

The irradiation of **1** was also carried out in the presence of 1,4-diazabicyclo [2.2.2] octane (DABCO) and sodium azide (NaN_3) as a singlet oxygen quencher, and superoxide dismutase (SOD) as an oxygen superoxide scavenger as described above.

2.4 Electron transfer mechanism detection by reduction of NBT

Under the same conditions as for the photolysis of gliclazide ([**1**]= 5.1 x 10^{-5} M), the photoreduction of nitro blue tetrazolium ([NBT]= 5.1 x 10^{-5} M) was followed in the presence of NBT in aerobic and anoxic conditions. Photoreduction was followed as a function of the irradiation time by determining the increase in absorbance at 560 nm due to a possible formation of diformazan product[24,25].

2.5 Photoinduced hemolysis of RBC by gliclazide

For the photohemolysis experiments, a red blood cell (RBC) suspension from three different samples of freshly obtained human erythrocytes was prepared by washing the samples of complete blood four times with a tenfold volume of a phosphate-buffered saline solution (PBS) pH 7.4 (0.01 M phosphate buffer and 0.135 M NaCl), centrifuging the cells each time at 2500 *g* for 15 min and carefully removing the supernatant. To carry out the experiments, the RBC's were diluted in PBS containing compound **1** so that the resultant suspension had an optical density (OD) of 0.4 - 0.8 at 650 nm. An OD value of 0.5 corresponds to 3.3 x 10^6 cells ml^{-1} which was read on a Milton-Roy 3000 spectrophotometer.

The rate and percentage of hemolysis were determined by measuring the decreasing OD at 650 nm, since the optical density is proportional to the number of intact RBC[26]. To study photohemolysis, gliclazide (**1**) was dissolved in concentrations of 20 - 80 $\mu g\ ml^{-1}$ in the RBC solution. The RBC solution was irradiated under aerobic conditions in a Rayonet photochemical reactor as described for periods ranging between 10 and 200 min. Similar experiments were carried out with a preirradiated solution of **1** and also with non-irradiated solutions.

The photohemolysis test was repeated in the presence of reduced glutathione (GSH) as a radical scavenger, 1,4-diazabicyclo[2.2.2]octane (DABCO) and sodium azide (NaN_3) as singlet oxygen quenchers, superoxide dismutase as an oxygen

superoxide quencher, and α-tocopherol and vitamin C as antioxidant agents (all at 10^{-5} M).

2.6 Photosensitized peroxidation of linoleic acid

Linoleic acid 10^{-3} M in PBS was irradiated in the presence of compound **1** and in a pre-irradiated solution of **1** (10^{-5} M). The formation of dienic hydroperoxides was monitored by UV-spectrophotometry, through the appearance and progressive increase of a new band at λ = 233 nm[27].

In the antioxidative status, GSH concentration was determined according to Tietze, 1969[28]; and the reduction of DTNB was achieved by the remaining GSH in RBC's.

3. Results

The course of the photolysis of **1**, monitored by the disappearance of 230 nm band gave the data of (Fig. 1).

Irradiation of **1** in PBS produces under oxygen atmosphere photoproducts **2** and **3** (Fig. 2). No formation of singlet oxygen by photolysis of **1** was evidenced by the failure of 2,5-dimethylfuran to trap this species as studied by GC-MS. This result was also evidenced by the histidine assay.

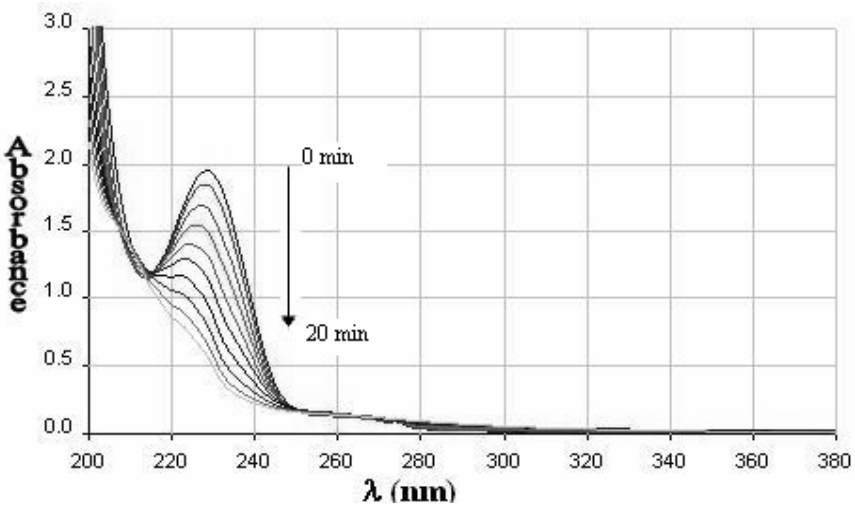

Fig. 1: UV-Vis monitoring of the photodegradation of gliclazide at regular time intervals (2 min) and under UV-B light.

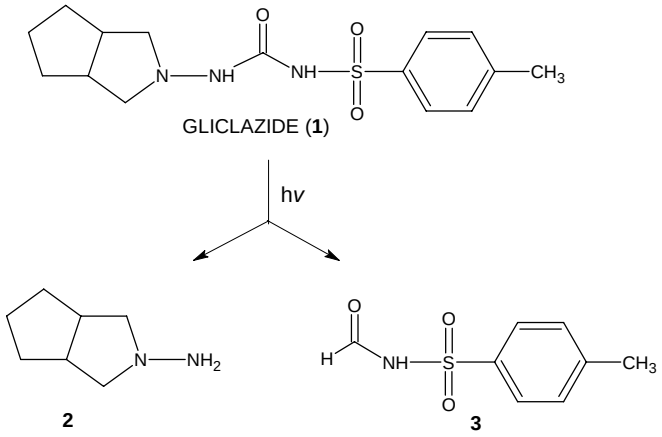

Fig. 2: Proposed photodegradation way of gliclazide.

No degradation of gliclazide was observed when irradiation was carried out in the presence of TPP or rose bengal, using a potassium chromate solution and maintaining all other experimental conditions the same. Therefore, the interaction with or quenching of singlet oxygen by gliclazide was negligible.

An electron transfer mechanism of gliclazide to molecular oxygen was not effective either, as suggested by the reduction assay of NBT[24,25]. Under the same conditions as for the photolysis of gliclazide ([**1**] = 5.1 x 10^{-5} M), the photoreduction of nitro blue tetrazolium ([NBT]= 5.1 x 10^{-5} M) was carried out in the presence and absence of oxygen at 560 nm. No increase in this absorbance band was observed. So the formation of diformazan from the oxidation of the NBT by superoxide anion ($O_2^{\cdot -}$) did not take place. The formation of oxygen superoxide by electron transfer possibly developed during the irradiation of **1** was not detected.

Gliclazide was able to induce photohemolysis of human erythrocytes (Fig. 3). The photohemolysis assay, as well as the *in vitro* phototoxicity test, suggested the involvement of free-radical-mediated skin cellular membrane damage by photo-sensitization due to gliclazide. The efficient inhibition of photohemolysis by the well-established radical scavengers GSH, DABCO and in minor extent by sodium azide suggested a type I mechanism and type II to a minor degree[29]. That addition of sodium azide did not exert an appreciable influence on the photohemolysis agrees with the minor role played by singlet oxygen in hemolysis.

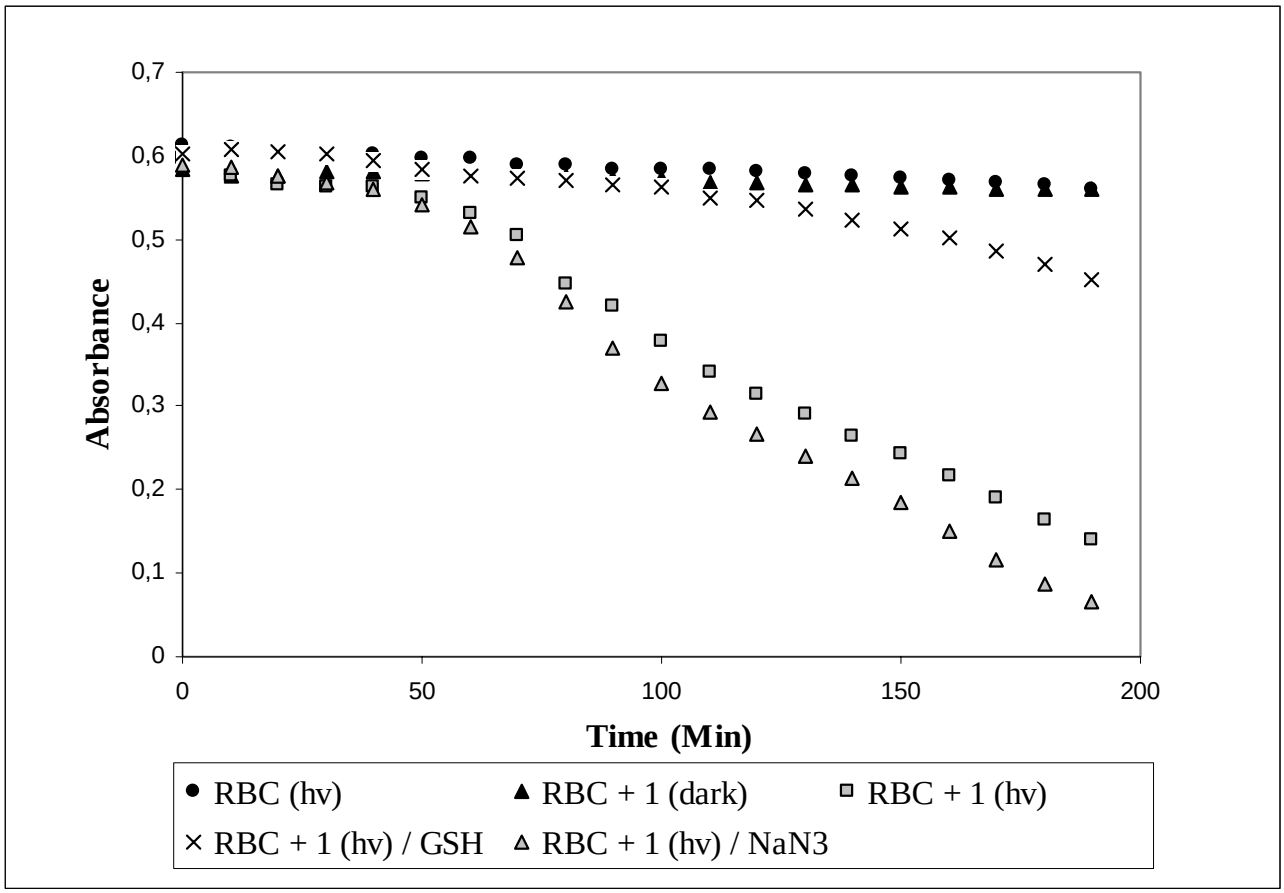

Fig. 3: Effects of additives on the photohemolysis of RBC induced by gliclazide (**1**) (at 650 nm).

The observed photohemolysis on red blood cells (RBC) induced by gliclazide might reflect extensive photoperoxidation of the membrane lipids (Fig. 4). When solutions of linoleic acid in PBS were irradiated in the presence of gliclazide (**1**) significant amounts of dienic hydroperoxides were formed, as evidenced spectrophotometrically by the appearance of a new UV-absorption band at 233 nm. Lipid photoperoxidation certainly correlates with damage produced in the cell membranes and therefore with the photohemolysis observed. Hydrogen abstraction by the generated intermediate radicals in the gliclazide photodegradation leads to the linoleic acid peroxidations. Figure 4 shows the increment of oxidized linoleic acid when it is irradiated in presence of gliclazide.

The phototoxicity mechanism for gliclazide probably involves reaction of free radicals intermediates with cellular components.

Also, a GSH-depletion of 35% in relation to the normal cellular level was induced by gliclazide under UV irradiation[28]. Convergent results were obtained from the optical density at 412 nm. A yellow compound identified as reduced DTNB by the remaining GSH in RBC's was responsible for this.

Screening for phototoxicity *in vitro* is necessary before introducing drugs into clinical therapy. This may help prevent unwanted drug reactions in humans. This fact makes relevant new investigations to protect from photooxidation to this family of drugs and also to find new derivatives with minor phototoxic character.

Contrary to the phototoxic character of **1**, this drug is in the darkness able to produce anti-oxidant effects. The chemiluminescence (CL) observed both in the processes induced by H_2O_2 -ferrous ion in luminol or by HRP in isoluminol were used to evaluate the scavenging capacity of gliclazide (**1**) on reactive oxygen substances (ROS). In the presence of **1** a dose-dependent inhibition period of CL was observed. The following figure shows the inhibitory effect of the luminescence (measure in intensity of relative light units (rlu) vs time) in presence of **1** on the system peroxide-luminol.

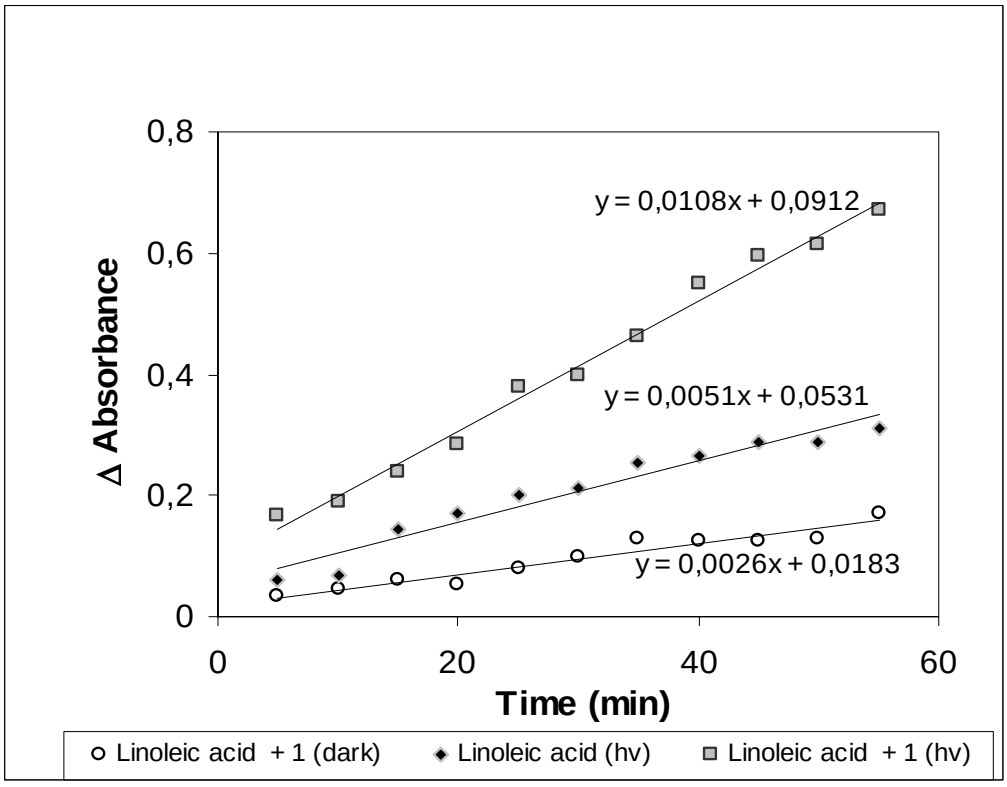

Fig. 4: Photoperoxidation of linoleic acid (10^{-3} M) sensitized by gliclazide (**1**).

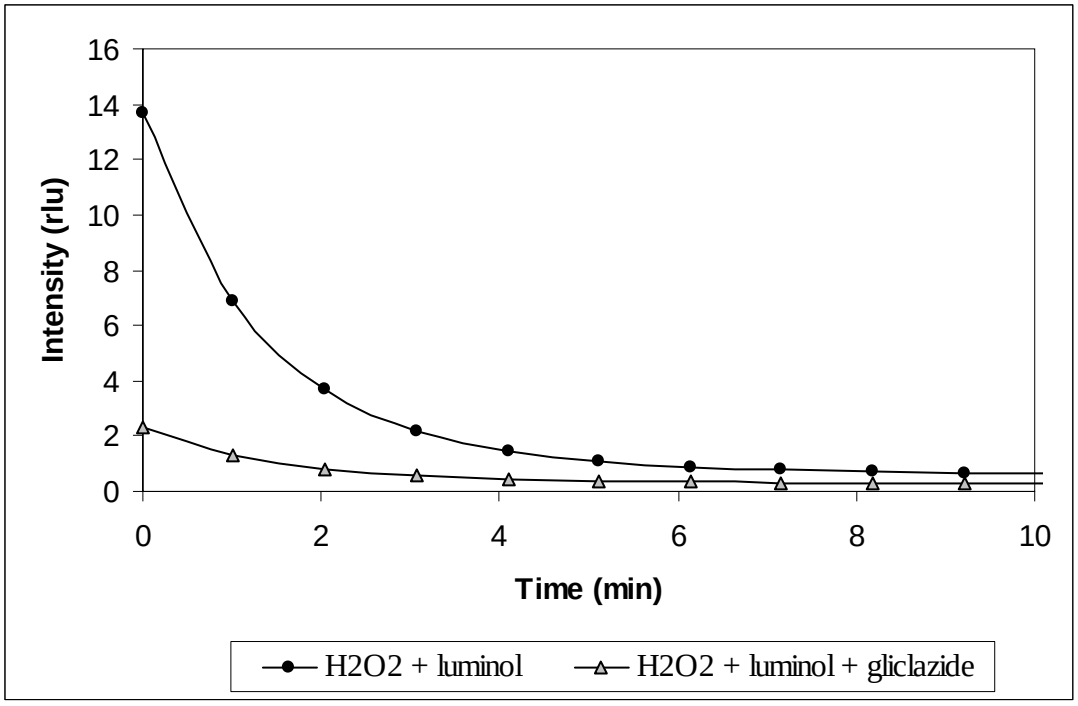

Fig. 5: Quenching effect of the luminol chemiluminescence (H_2O_2) taken place for gliclazide. Data are the mean and SEM, (n=4, $p < 0.05$ vs. control; analysis of variance).

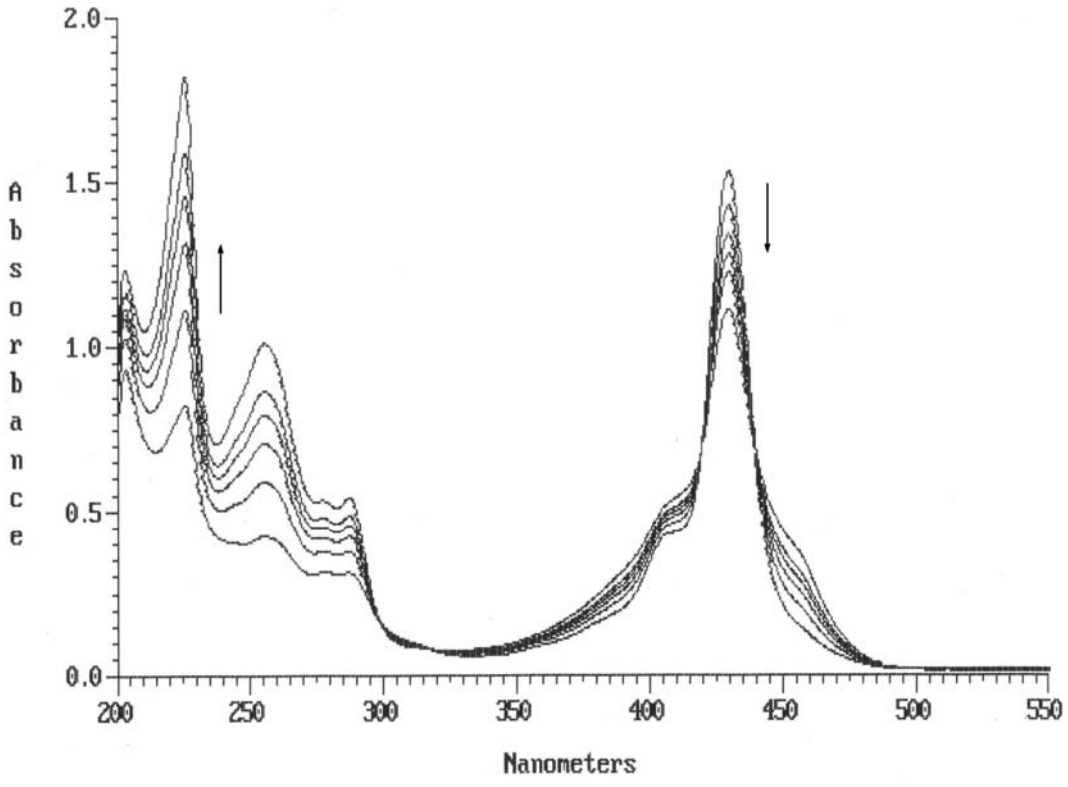

Fig. 6: UV monitoring of the galvanoxyl radical quenching by addition of gliclazide (without irradiation).

On the other hand, the reactivity of the gliclazide toward galvanoxyl (a model phenoxyl radical) in ethanol solution was also investigated. Gliclazide (**1**) showed an efficient scavenging activity in darkness of galvanoxyl radical in ethanolic solutions. Figure 6 shows the absorption spectrum of the galvanoxyl radical and its progressive disappearance when it is then titrated with **1**. A noticeable decrease in the absorption band at 435 nm was observed.

The rates of the antioxidant activity (scavenging of galvanoxyl radical) of the compounds **1** was directly proportional to its concentration (data not show).

4. Discussion

The degree of phototoxicity induced by gliclazide could depend on the balance between the generation of free radical species and the effectiveness of the defense systems against toxic radicals. Although gliclazide presented a phototoxic character when being irradiated with light UV-B, as was demonstrated by means of the photohemolysis experiments and lipid peroxidation, this drug presents in the darkness a character anti-oxidant, specifically radical scavenger. This last process can be of great analytic utility for the determination of the scavenging capacity of free radicals by drugs with suspected anti-oxidant properties.

The studies of phototoxicity carried out in this work may help explain the damage produced in proteins and organs. Radical-mediated damage to protein may be initiated by electron leakage and photoinduced oxidation of lipids and aminoacids. This fact was demonstrated in the *in vitro* experiments where gliclazide photoinduced lipid peroxidation and oxidation of glutathione occurred, and in consequence of protein[30]. These observations may contribute to elucidate the observed accumulation and damaging activity of oxidized proteins during aging and in pathologies such as diabetes, atherosclerosis and neurodegenerative diseases such as Alzheimer's[31]. Lipid photo-peroxidation certainly correlates with the damage produced in cell membranes and thus with the observed photohemolysis. The phototoxicity mechanism for gliclazide most probably involves reaction of free radical species than singlet oxygen, superoxide anion or photoproducts with cellular components.

The use of human erythrocytes as cell systems in this investigation, combined with other *in vitro* tests employing linoleic acid for lipid photoperoxidation and histidine for singlet oxygen detection confirmed an important methodology for the study of the phototoxicity of gliclazide. Studies on peripheral blood mononuclear and polymorphonuclear cells (lymphocytes and neutrophils) are in progress.

The results obtained may also be very useful from the medical standpoint in the elucidation of the biological action of many pharmaceutical products *in vitro* and *in vivo*. It is necessary to perform the appropriate screening for phototoxicity *in vitro* before introducing drugs and chemicals into clinical therapy. At present the photodegradation of other biologically active compounds utilizing *in vitro* tests are in progress in this laboratory.

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