Artículo de investigación

In vitro studies of the dual properties of Allopurinol anti- and photo-oxidants Mechanisms

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Summary

The objective of this study was to investigate the ability of allopurinol (1) to inhibit free radical or reactive oxygen species (.OH, $^1O_2$, H_2O_2) as well as the study of its photochemical activity. We investigated the ability of 1 to scavenge oxygen metabolites generated by cell-free systems using luminol enhanced-chemiluminescence and electronic absorption spectra as monitors. Both absorbance and fluorescence scans reveal that 1 is able to react with equimolar quantities of H_2O_2. In the presence of 1 a dose-dependent inhibition period was observed in this system as assayed by isoluminol-enhanced chemiluminescence (ILCL) in the presence of horseradish peroxidase (HRP), as well as by luminol-enhanced chemiluminescence (LCL) in the presence of H_2O_2 or ferrous ion. On the other hand, 1 did not show an efficient scavenging activity of galvanoxyl radical in ethanolic solutions. Furthermore, in a separate experiment, it was not observed trapping of singlet oxygen ($^1O_2$) generated by Rose Bengal, in the presence of 1. The activity of 1 was compared with that of vitamins E and C. *In vitro* experiments of photohemolysis in presence of 1 and cinoxacin, a phototoxic antibacterial quinolone, the photohemolytic effect of cinoxacin was diminished. Allopurinol alone did not show any phototoxic effect under irradiation with UV-A or visible light but was photo-unstable and phototoxic *in vitro* with UV-B light.

Key words: Allopurinol, antioxidant, chemiluminescence, photo-oxidant, reactive oxygen species.

Resumen

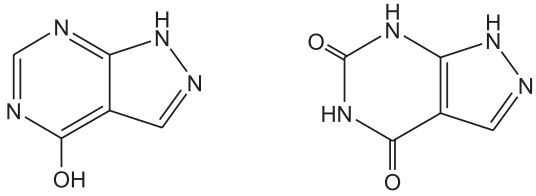
Se estudió la habilidad del alopurinol (1) para inhibir radicales libres o especies reactivas de oxigeno (.OH, $^1O_2$, H_2O_2), igualmente se determinó su actividad fotoquímica. De otro lado se midió la habilidad de 1 para eliminar los metabolitos de oxígeno generados por un sistema libre de células basado en quimioluminicencia aumentada de luminol y se monitoreo el espectro de absorción electrónica. Las dos

determinaciones, absorbancia y fluorescencia, revelan que 1 es capaz de reaccionar con cantidades equimoleculares de H_2O_2. En presencia de alopurinol se observan periodos de inhibición dosis dependiente al usar isoluminol como intensificador de luminiscencia (ILCL) en presencia de peroxidasa de rábano o ión ferroso. Por otro lado, 1 no mostró una eficiente actividad frente a radicales galvanoxil en solución etanólica. En otros experimentos en presencia de 1 no se observó bloqueo de especies de oxígeno singlete ($^1O_2$) generado por rosa bengala. La habilidad de 1 fue comparada con la de vitaminas E y C. En experimentos de fotohemólisis *in vitro* en presencia de 1 y cinoxacin, quinolona fototóxico antibanterial, el efecto fotohemolítico del cinoxacin fue disminuido. El alopurinol no mostró efecto fototóxico por irradiación con luz UV-A o luz visible, sin embargo se mostró foto - inestable y fototóxico *in vitro* bajo irradiación con luz UV-B.

Palabras clave: Alopurinol, antioxidante, quimioluminicencia, foto-oxidante, especies de oxígeno reactivo.

Introduction

Allopurinol (1) (see structure in Figure 1) is a drug that belongs to the group of the xanthine oxidase inhibitors. The potential association between oxidative stress and heart failure (HF) has led to the study of antioxidant interventions that may attenuate the oxidative damage. Promising results have been obtained, mainly from studies using water-soluble antioxidants (such as vitamin C) and factors that inhibit free radical formation such as allopurinol (Reyes and Leary, 2005). The amelioration of oxidative stress in conjunction with pathophysiological abnormalities has been clearly shown in humans, but studies with clinical end-points are scarce. Furthermore, allopurinol, carvedilol and several other cardiovascular drugs, besides their favorable effects on neurohormonal activation in HF, may have additional intrinsic antioxidant properties (Biagi and Abate, 2005). The effect of allopurinol on free radical generation after primary coronary angioplasty for acute myocardial infarction has also been observed (Guan *et al*., 2003). Even though the experimental evidence is promising, many more human clinical trials are needed in order to clarify the exact role of oxidative stress in HF and the potential benefits of antioxidant intervention (Korantzopoulos *et al*., 2003).



Allopurinol (1) Oxypurinol (2)

Figure 1. Structure of allopurinol (4-hydroxypyrazolo[3,4-d]pyrimidine, **1**) and oxypurinol (**2**).

On the other hand, the therapeutic use of allopurinol has been associated in some patients with the appearance of phototoxic effects (Mauget-Faysse *et al*., 2001; Escousse *et al*., 2002; Zurcher and Krebs, 1970). Although controversially some of these effects like those of photosensitization of ocular tissues by allopurinol, bringing out any positive results (Rudy *et al*. 1981).

Materials and methods

Chemicals

Allopurinol (4-hydroxypyrazolo[3,4-d]pyrimidine, **1**) (CAS 315-30-0) was extracted from the commercial medicament Aluprol® (Isern products, *Tecno-químicas* laboratory, Caracas, Venezuela) with a soxhlet extractor using methanol as the solvent, purified by TLC and recrystallized from the same solvent. The purity was 99% as determined by mass spectrometry ^{1}H-NMR and by comparison with an authentic pure commercial sample. Superoxide dismutase (SOD) and 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), human serum albumin (HSA), tetraphenyl-porphine (TPP) were purchased from Sigma (St. Louis, MO, USA), while vitamins E and C, reduced glutathione (GSH), cysteine, sodium azide (NaN_3), histidine, 2,5-dimethylfuran (DMF) and Rose Bengal were purchased from Aldrich (Steinheim, Germany). All analytical or HPLC grade solvents were obtained from Merck (Darmstadt, Germany).

Photolysis

Photolysis of **1** was carried out in methanol solution (1.50 mmol in 50 ml) at 20°C during 6 hours in a Rayonet photochemical chamber reactor (model RPR-100, Southern New England Ultraviolet Company-USA) equipped with 16 phosphorus lamps with a maxima emission in the UV-A between 320 and 400 nm and in UV-B between 290 and 320 nm (23 mW/cm^2 of irradiance as measured with a UVX Digital Radiometer, Melles Griot, USA). The distance between the light sources and the test aliquots was 10 cm. The temperatures detected in the cuvette during a standard 1 h irradiation were no higher than 28°C. In the determination of quantum yields the photolysis was limited to less than 10% to minimize light absorption and the formation of sideproducts. The photon flux incident on 3 mL of solution in quartz cuvettes of 1 cm optical path was measured by means of a ferric oxalate actinometer and was of the order of 10^{15} - 10^{16} quanta s^{-1} (Vargas *et al*., 2002).

In separate experiments either oxygen or argon was bubbled through the reaction mixture throughout the whole irradiation process. The photodegradation reaction was followed using a Perkin Elmer 559 UV-visible spectrophotometer, a Milton-Roy Spectronic 3000 array instrument (Milton Roy Company-USA) and also by means of a Lambda 650 spectrophotometer Perkin Elmer and ^{1}H NMR as well as by thin layer chromatography and HPLC (Water Delta Prep 4000 equipped with a 3.9 x 300 mm,

10 µm Bondapak C18 column using a CH_2Cl_2/MeOH gradient as mobile phase at a flow rate of 0.6 mL min^{-1}, with monitoring at 240 nm). After the irradiation was finished the solvent was evaporated under reduced pressure (14 Torr) and the residue was purified by chromatography on a silica gel (230 mesh) column. The elution was carried out by means of solvent mixtures (dichloromethane/methanol) (3:1 vol/vol). The structure of the isolated products was elucidated by ^{1}H NMR and ^{13}C NMR (Brucker Aspect 3000, 300 and 100 MHz respectively), I.R. (Nicolet DX V 5.07) and mass spectrometry (Varian Saturn, 2000) in connection with a Varian chromatograph equipped with a 30-m capillary (CP-Sil, 8CB-MS).

Photoinduced hemolysis of RBC by allopurinol

A red blood cell (RBC) suspension from three different samples of freshly obtained human erythrocytes was prepared by washing them 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.

For the photohemolysis experiments RBC were diluted in PBS containing the compounds **1** or its photoproduct **2** (Figure 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 corresponded to 3.3 x 10^6 cell ml^{-1}. The photon flux incident on the cuvettes (measured as before) was 2 x 10^{16} photon s^{-1}. Samples received, on average, 12.9 J cm^{-2} in an hour.

The hemolysis rate was determined by measuring the decreasing optical density (OD) at 650 nm, since the optical density is proportional to the number of intact RBC (Valenzeno, 1985). Compound **1** and the isolated photoproduct **2** were added to the RBC solutions and irradiated at concentrations of 20 - 80 mg ml^{-1} under aerobic (oxygen) as well as under anaerobic (argon) conditions in a Rayonet photochemical reactor equipped with 16 phosphor lamps with an emission maximum in the UV-A and in a separate experiment in the UV-B for periods ranging between 10-100 min. The photohemolysis experiments were carried out also in the presence of [SOD] and [NaN_3] = 1.0 x 10^{-5} M as singlet oxygen quenchers, and [GSH] = 1.0 x 10^{-4} M as radical scavengers. The hemolysis rate and the hemolysis percentage were determined by measuring the decreasing OD at 650 nm. Control experiments performed in the dark did not show OD changes. All of the data shown are the averages (mean arithmetic) of the values obtained repeating the experiments three times.

Photosensitized oxidation of glutathione and cysteine by allopurinol

To determine the photoinduced oxidation of glutathione in the presence of allopurinol (**1**), it was irradiated in the presence of the latter. Detection of glutathione depletion was carried out with 5,5′-dithiobis [2-nitrobenzoic acid] (DTNB) and performed by means of similar assays as those described by Beutler (1984). However, the red cells used as a blank were hemolyzed with a "lysis buffer" (NH_4Cl, $KHCO_3$, EDTA, pH 7.5).

The same experiment as the one described was used to determine the photoinduced oxidation of cysteine in the presence of allopurinol. Similar control experiments were carried out without irradiation of the samples.

In a separate experiment, in order to detect the probable formation of a radical intermediate, allopurinol (**1**) (0.5 mmol in 50 ml H_2O) was irradiated under the same conditions as described above, in the presence of equimolar quantities of either reduced glutathione (GSH) or cysteine as radical scavengers. The GSH concentration was determined according to the method of Tietze (Tietze, 1969), and the reduction of DTNB was achieved by the remaining GSH in RBC's.

Photosensitized peroxidation of linoleic acid

Linoleic acid 10^{-3} M in PBS was irradiated with UV-B and as well as with UV-A light in the presence of compound **1** and also 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 233 nm (Recknagel and Glende, 1984).

For studies of the possible antioxidant effects of the allopurinol in darkness the following assay was carried out: Linoleic acid 10^{-3} M in PBS was oxidized by using the method described by Yen (Yen and Hsieh, 1997) in the presence of allopurinol (**1**), and of vitamins C or E (10^{-4} M) respectively, and monitored by UV-spectrophotometry, reading continuosly the absorbance at l = 500 nm on a Milton-Roy 3000 spectrophotometer after colouring it with $FeCl_3$ and thiocyanate at intervals during incubation at 37º C. This test was also carried out under an argon atmosphere.

Chemiluminescence experiments

Chemiluminescence (CL) was generated in cell-free systems; H_2O_2-induced CL (as a blank): H_2O_2 (3.5 mM) was added to a phosphate buffered saline solution (PBS, 10 mM KH_2PO_4 and 150 mM NaCl, pH 7.4) and luminol (250 mM, prepared daily in 2 M NaOH and diluted with PBS). Allopurinol was irradiated at different concentrations with 2 phosphorus lamps with emission in UV-B (290-320 nm) in the presence of NADH. The generated CL at 37ºC was measured continuously for 10 min in a Luminoskan Ascent luminometer (ThermoLabsystems, Finland) in a 96-well ThermoLabsystems Microtiter plate. (Lundqvist and Dahlgren, 1996; Vargas *et al*., 2003; Yildiz *et al.,* 1998).

Determination of the radical scavenger allopurinol ability

a) Chemiluminescence (CL) generated in cell-free systems; H_2O_2-induced CL

H_2O_2 (3.5 mM in PBS, 10 mM KH_2PO_4 and 150 mM NaCl, pH 7.4) was added to a luminol solution (250 mM, prepared daily in 2 M NaOH and diluted with PBS) and allopurinol at different concentrations. The generated CL at 37 ºC was continuously

measured for 10 min in a Luminoskan Ascent luminometer (Thermo Labsystems, Finland) in a 96-well Thermo Labsystems Microtiter plate.

b) CL generated in cell-free systems; Ferrous ion)-induced CL

Hydroxyl radical was generated by addition of ferrous iron to the buffer solution as described previously (Yildiz and Demiyürek, 1998). Freshly prepared $FeSO_4$ (40 nM) was added to the PBS plus luminol (250 mM) mixture and CL was recorded continuously for 2 min.

c) Isoluminol amplified chemiluminescence

Chemiluminescence was measured in a Luminoskan Ascent (ThermoLabsystems, Finland) using 96-well Thermo Labsystems Microtiter plates, containing 56 mM isoluminol, 4 U (HRP) and allopurinol at different concentrations or PBS as a blank (0.5%). The emitted light was recorded as luminescent units at 20 s intervals during 20 min. All results were expressed as percentages of the control (relative light units (rlu) vs. time curves).

Reactions with galvanoxyl radical

Titrations of an ethanol solution of galvanoxyl radical (1.0×10^{-4} M) were carried out with aliquots of 10 mL of the corresponding ethanolic solutions of allopurinol. The course of the reaction was followed by UV-Vis spectrophotometry (for 10^{-4} M solutions) using a Milton-Roy Spectronic 3000 array instrument (Milton Roy Company-USA).

Titration of solution of 1 with HSA

Allopurinol solutions titration (1.0×10^{-4} M) with HSA was performed directly to the absorbance or fluorescence cell by addition of appropriate aliquots of an aqueous-buffered HSA stock solution (1.0 mM, pH 7.4). Therefore, the final protein concentration was from 0 up to 5.0×10^{-4} M. The solutions were allowed to incubate in the dark for 20 min. Then, samples placed in 1-cm^2 Suprasil quartz cells were irradiated under the above mentioned conditions with varying time periods. Controls included drug protein mixtures kept in the dark and HSA solutions irradiated for the same periods of time. The drug was separated from the protein using a Sephadex G-25 column equilibrated with PBS. The photobinding was monitored by fluorescence spectroscopy (Moreno *et al*., 1999; Vargas *et al*., 2003).

Statistical treatment of results

At least three independent experiments were performed except where indicated. The results are expressed as a mean ± S.E.M. derived from 3-4 observations. The level of significant acceptance was $p \leq 0.05$.

Results and discussion

The drug allopurinol (**1**), a pyrimidine derivative, is photolabile under aerobic conditions and UV-B light. As stated before, the photolysis of **1** was followed by monitoring the disappearance of the 240 nm band and appearance of the 278 band (Figure 2).

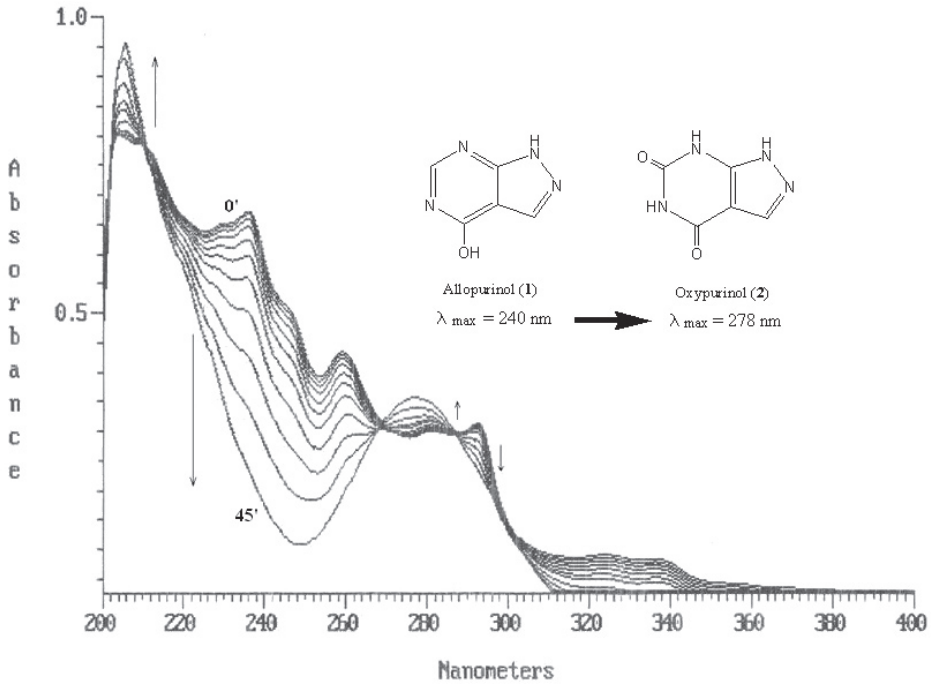


Figure 2. UV-Vis monitoring photodegradation of allopurinol at regular time intervals (5 min) and under UV-B light.

Irradiation of **1** in PBS yields under oxygen atmosphere one photoproduct, the known compound oxypurinol showed in Figure 1 (Moorhouse *et al*., 1987). In fact, oxypurinol is a product resulting from attack of the hydroxyl radical upon allopurinol, and which is also a major metabolite of allopurinol.

Phototoxic effects of allopurinol

Formation of singlet oxygen was not detected in the photolysis of **1**, as evidenced by not being trapped with 2.5-dimethylfuran, which was confirmed by GC-MS. This result was also evidenced by means of the histidine assay. As a result, it may be inferred that allopurinol is not a photosensitizer of type II (Foote, 1991).

On the other hand, the generation of hydroxyl radical and hydrogen peroxide could be detected after irradiation of allopurinol with UV-B light in the presence of NADH (Figure 3). This was determined by means of the chemiluminescence assays.

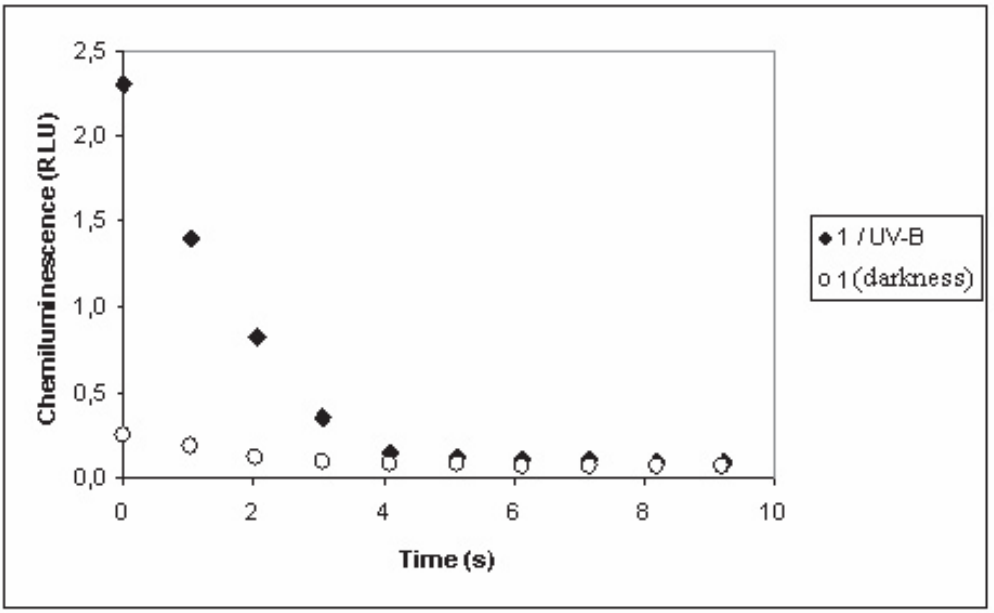


Figure 3. Production of ·OH and H_2O_2 after photolysis (UV-B) of allopurinol (**1**) in presence of NADH, detected by chemiluminescence of luminol. RLU = relative light units.

Allopurinol (**1**) was able to induce photohemolysis of human erythrocytes (red blood cells, RBC) only under UV-B irradiation (not under UV-A light). The photohemolysis assay, as an *in vitro* phototoxicity test, has evidenced the involvement of radical-mediated cellular membrane damage in the skin photosensitization by allopurinol.

In vitro experiments of photohemolysis under UV-A irradiation in the presence of **1** and cinoxacin, a phototoxic antibacterial quinolone (Vargas *et al*., 1994; Vargas and Rivas, 1997), showed that the photohemolytic effect of cinoxacin was diminished (Figure 4). Allopurinol alone did not produce any phototoxic effect. These results showed that the anti-oxidant activity of allopurinol is specifically to reactive oxygen species and not at all to toxic photoproducts or free radical intermediates.

When allopurinol was irradiated with UV-B light in the presence of linoleic acid, significant amounts of dienic hydroperoxides were evidenced by the appearance of the new UV-absorption band at 233 nm (Recknagel and Glende, 1984). The photoinduced lipid peroxidation by allopurinol is illustrated in Figure 5.

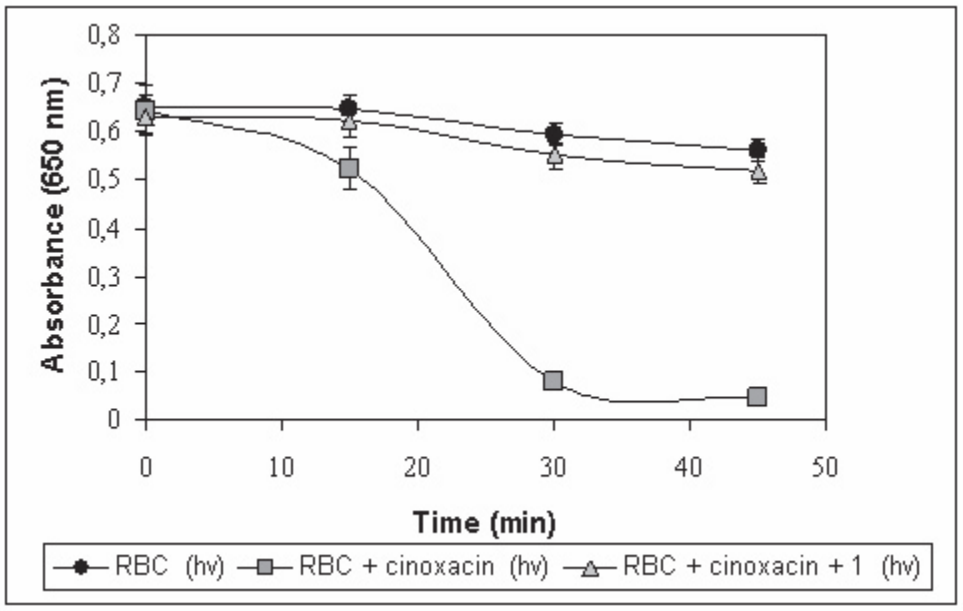


Figure 4. Effects of allopurinol (**1**) on the photohemolysis of RBC induced by cinoxacin under UV-A light. Controls in the darkness of RBC, cinoxacin and **1**, didn't show any photohemolytic effect.

When the photohemolysis and lipid peroxidation tests were carried out in the presence of SOD at 10%, the phototoxic effect was reduced. Furthermore, when the photohemolysis and lipid peroxidation tests were carried out under argon atmosphere, a decrease of the photoactivity induced by **1** was observed. The values obtained were approximately 10% and 15% lower for the photohemolysis and for the lipid peroxidation, respectively, than those obtained in aerobic conditions. Neither photohemolysis nor lipid peroxidation were observed when allopurinol was irradiated with UV-A light in the corresponding assays.

Radical-mediated damage to protein may be initiated by electron leakage and photoinduced oxidation of lipids and amino acids. This fact was demonstrated in the *in vitro* experiments where allopurinol irradiated with UV-B light photoinduced lipid peroxidation and oxidation of glutathione. Lipid photoperoxidation certainly correlates with the damage produced in cell membranes and thus with the observed photohemolysis. The phototoxicity mechanism for allopurinol most probably involves reaction of free radical species, hydroxyl radical and superoxide anion with cellular components. This may be illustrated by the decrease in GSH and cysteine, two highly important water-soluble antioxidants, which were converted into the corresponding disulfides (Adam *et al*., 1988), immediately after irradiation (UV-B) in the presence of allopurinol.

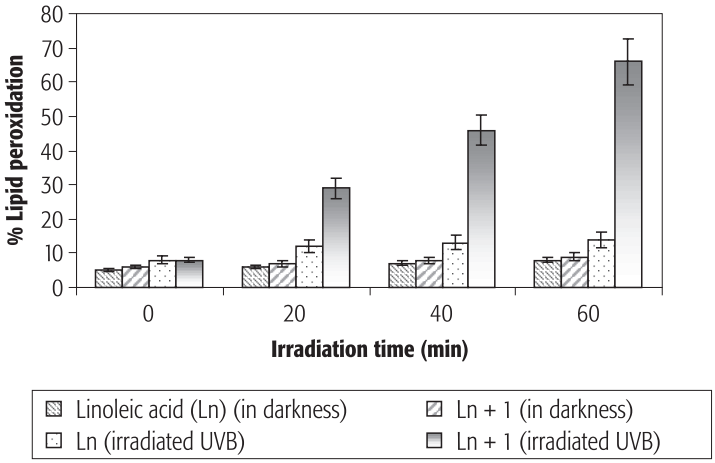


Figure 5. Photoperoxidation of linoleic acid (10^{-3} M) photosensitized by allopurinol (**1**) with UV-B light.

Antioxidant effects of allopurinol

In a sense, contrary to the phototoxic character of **1** under UV-B light, this drug in darkness is able to produce anti-oxidant effects (Moorhouse *et al*., 1987). The chemiluminescence (CL) observed both in the processes induced by H_2O_2 as well as in those by ferrous ion in luminol or else by HRP in isoluminol was used to evaluate the scavenging capacity of allopurinol (**1**) on reactive oxygen species (ROS). In the presence of **1** a dose-dependent inhibition period was observed in this type of system CL assayed. Figure 6 shows the inhibitory effect of the luminescence (measured as intensity or relative light units (rlu) vs time) in presence of **1** on the peroxide-luminol system.

The CL activity generated by isoluminol and HRP reflects the release of reactive oxygen species (ROS) especially ·OH (Lundqvist and Dahlgren, 1996). Hydroxyl radical was also generated by the addition of a freshly prepared $FeSO_4$ solution to the mixture containing luminol as measured by chemiluminescence. These results are in agreement with the previous observations with other drugs (Green *et al*., 1994; Yildiz and Demiryürek, 1998) where the addition of a ferrous ion salt to buffered solutions generates the hydroxyl radical-mediated oxidative reactions. The scavenging activity of allopurinol on ROS is shown in Figure 6. In this assay system allopurinol was compared with standard antioxidants such as a-tocopherol (vitamin E) and ascorbic acid (vitamin C).

Allopurinol is a scavenger of the highly reactive hydroxyl radical but not in the least of singlet oxygen. No degradation of allopurinol was observed when irradiation was carried out in the presence of PP or Rose Bengal as photosensitizer, using a potassium

chromate solution (100 mg/l) as a filter (which allows I> 400 nm) and maintaining all other experimental conditions the same. Therefore, all of the observed results showed that an interaction with or quenching of singlet oxygen with allopurinol was negligible.

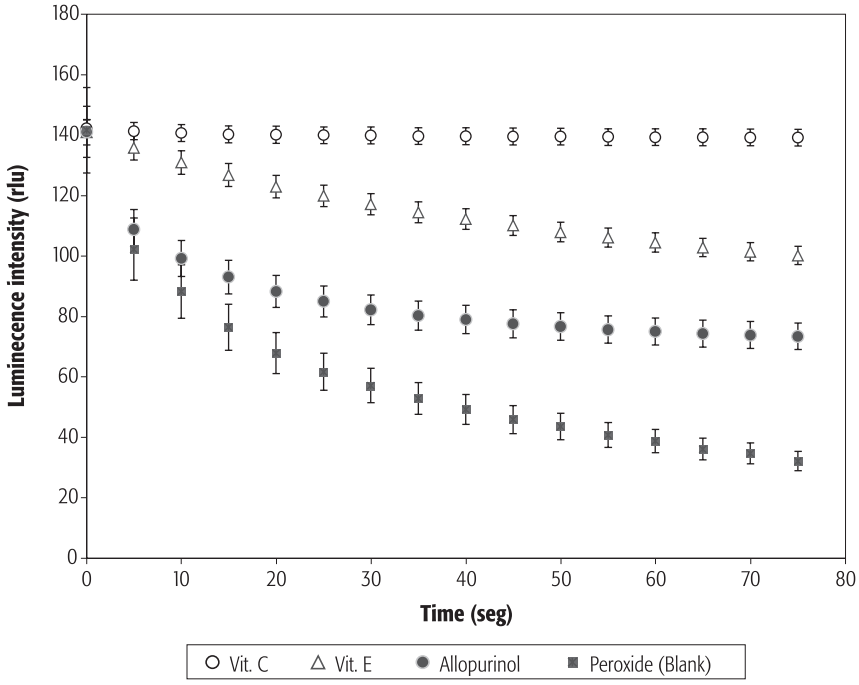


Figure 6. Quenching effect of the luminol and isoluminol chemiluminescence (H_2O_2, HRP and $FeSO_4$) taken place for allopurinol (**1**), vitamins E and C. Data are the mean ± SD (n = 4, $p < 0.05$ vs. control).

On the other hand, allopurinol showed an efficient scavenging activity of galvanoxyl radical in ethanolic solutions (Figure 7). In the next graph a comparison is also shown with the antioxidant activity of vitamin C (Figure 8). The latter showed to be more efficient than allopurinol.

The singlet oxygen and hydroxyl radical scavenging processes can be related directly with the lipid peroxidation anti-oxidative mechanism. Lipid peroxidation is widely known and it has thought to be responsible for numerous effects observed in biological systems, in that it concurrently proceeds after the initiation step by a free-radical reaction mechanism (Kappus, 1985; Vargas and Rivas, 1997). Therefore, it is considered that this process plays an important role in anti-oxidative skin toxicity, for example, due to the damaging effects of induced peroxidation to cell membranes. On the other hand, oxygen radicals and singlet oxygen are responsible for lipid peroxidation and therefore, lipid peroxidation is a very attractive reaction that explaining the

features of many diseases, drug-induced toxicity and antioxidant activity. As a model, we studied the linoleic acid peroxidation using this compound as radicals target in presence of several antioxidants including allopurinol.

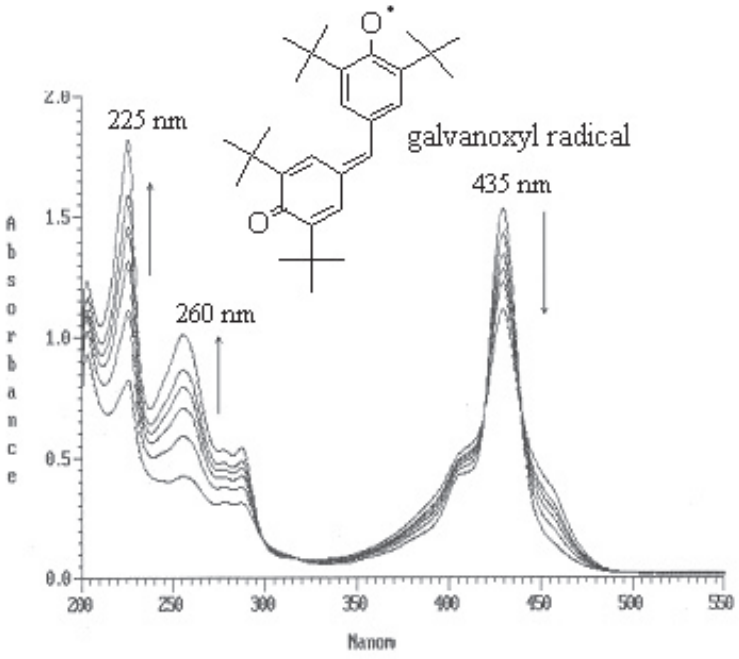


Figure 7. UV monitoring of the galvanoxyl radical quenching by addition of allopurinol. The arrows indicate the sequence of disappearance of the initial compound (galvanoxyl radical, absorbance at 435 nm) and the formation of their alcoholic product (bands at 225 and 260 nm) registered every 5 min after the addition of the allopurinol.

The process of lipid peroxidation in the presence of allopurinol (**1**) was circa 30% delayed; while in the presence of the same concentration of vitamins C and E the induced lipid peroxidation was inhibited about 65 and 70% respectively (more efficient that the allopurinol). According to the *in vitro* experiments, the anti-oxidative defense observed may be able to protect biomacromolecules from free radicals.

By means of studies on the association of **1** to HSA, it was possible to determine no-binding of **1** to HSA. These studies were evidenced by no increase of the emission intensity of allopurinol at 496 nm upon addition of HSA. This could be an effect of allopurinol binding to HSA with the resulting energy transfer between the protein and **1**. Contrarily to the binding, a quenching effect could be suggested because of the decrease in the fluorescence intensity of HSA (1.0 mM) upon addition of **1**. In a control experiment the presumably unbound drug was separated from the protein using a Sephadex G-25 column equilibrated with PBS. After this, albumin was obtained

without sample of binding of the allopurinol. This is an important discovery, since this method shows the potential of allopurinol to be used therapeutically without producing an allergic effect in human.

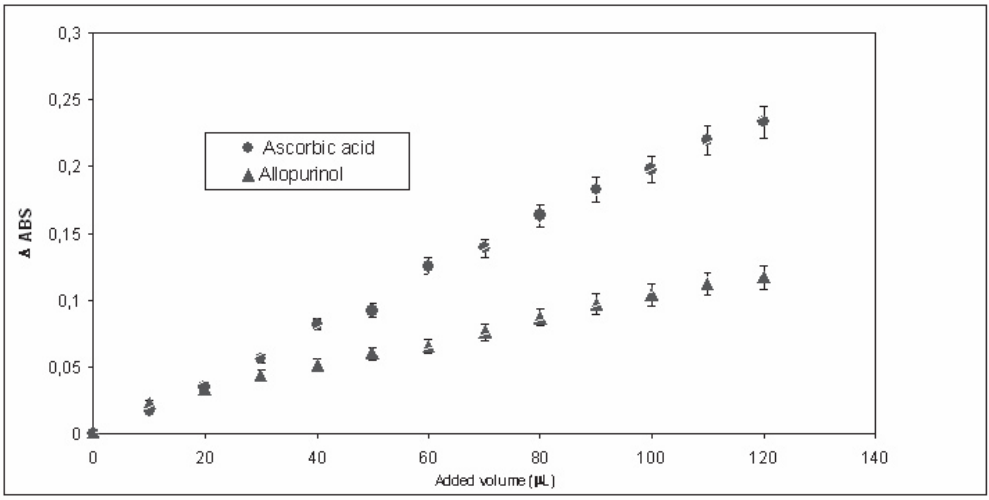


Figure 8. Scavenging effect by monitoring decreasing of the absorbance of galvanoxyl radical at their wavelength maxima vs concentration of allopurinol and ascorbic acid. Data are shown as mean ± SD (n = 3).

The study of the dual characteristics of the allopurinol, as photo and anti-oxidant, leads us to the following reflections: Although UV-B has been believed to be responsible for solar carcinogenesis and UV-A-induced DNA damage in the presence of endogenous photosensitizers may play an important role in solar carcinogenesis. In addition, certain drugs, such as allopurinol, could be capable of causing similar damage in a comparable manner, and such drugs can act as exogenous or endogenous photosensitizers. On the other hand, most of the new antioxidant compounds have not been adequately tested for their effectiveness and safety for humans in clinical trial. Therefore, there is a need to continue trials for selected antioxidants for cancer chemoprevention and oxidative stress disease.

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