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Short communication

Evaluation of photochemical degradation of digoxin by Na,K-ATPase assay

Vesna Vasić [a,*], Urh Černigoj [b], Katarina Krinulović [a], Gordana Joksić [a], Mladen Franko [b]

[a] *Vinča Institute of Nuclear Sciences, P.O. Box 522, 11001 Belgrade, Serbia and Montenegro*
[b] *Nova Gorica Polytechnic, Laboratory for Environmental Research, P.O. Box 301, SI-5001 Nova Gorica, Slovenia*

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Abstract

A simple Na,K-ATPase assay is described as a suitable method for testing of digoxin photodegradation. The exposure of Na,K-ATPase to the photodegraded samples exhibited reduced inhibition of the enzyme, compared to the unirradiated samples containing equal initial concentrations of drug. The degree of inhibition was dependent on the irradiation time. The concentrations of digoxin in irradiated samples were evaluated by HPLC analysis. Excellent agreement of the results obtained by both methods was observed. The investigation of the influence of irradiated samples on Na,K-ATPase inhibition revealed no side products acting as Na,K-ATPase inhibitors. The cytokinesis block micronucleus test (CBMN) was applied in order to investigate the cytotoxicity of the possible degradation products after exposure to UV irradiation. The results confirmed that the photochemical treatment did not induce the cytotoxic side products. Zero order kinetics, which was observed for digoxin photodegradation and the associated reaction mechanism are also discussed.

Keywords: Digoxin; Photochemical Degradation; Na,K-ATPase activity; HPLC; Genotoxicity

1. Introduction

Digoxin belongs to the group of ouabain like cardiac glycosides most frequently used to improve cardiac contractility in the treatment of congestive heart failure [1]. It acts through the inhibition of Na,K-ATPase, the ouabain sensitive membrane enzyme which functions to maintain gradients of sodium and potassium ions across the membranes [2,3]. The inhibition effects of digoxin related compounds on Na,K-ATPase activity are well understood [4]. Structure–activity studies on steroidal glycosides have led to conclusion that the lactones ring at C17β has been considered to be responsible for inotropic activity, bringing about conformational changes on the enzyme that would give rise to its inhibition [5,6]. Recent studies correlated the influence of digoxin on Na,K-ATPase activity to its genotoxicity measured on the harvested human lymphocytes[7].

The pharmacopoeia recommendations frequently require the protection of drugs from light. The absorption of UV–vis light by some drugs that are not stable against irradiation leads to their chemical changes followed by the formation of other compounds that may have toxic properties [8]. The influence of irradiation from different light sources to some cardiac glycosides (ouabain, digoxin and its related compounds) in crystalline state was investigated and the photoproducts were determined densitometrically [9]. Besides, the solid-state stability was tested by isothermal calorimetry [10]. The activation energy and degradation rates were determined at various temperatures. Digoxin is known as very stable when kept in dark and well-closed containers, but it undergoes acid catalysed hydrolysis in water solutions [11,12]. The degradation products of digoxin hydrolysis are digoxigenin-bisdigitoxoside, digoxigenin-monotoxoside and digoxigenin.

* Corresponding author. Tel.: +381 11 2458 222; fax: +381 11 444 7207.
E-mail address: evasic@vin.bg.ac.yu (V. Vasić).

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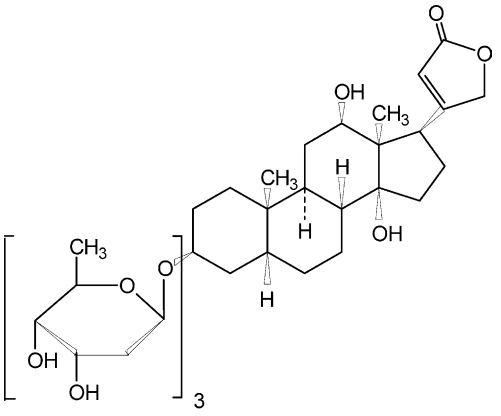
Moreover, the toxic products induced by exposure to UV irradiation may also cause cytotoxic effects on living cells [13]. Genotoxicity testing aims to detect a large range of genetic damage endpoints and evaluate such results in context of cell survival. The cytokinesis block micronucleus test (CBMN) offers the advantage of providing information of both: cell cycle progression and chromosome/genome damages simultaneously [14].



Structural formula of digoxin

Some previous results [15,16] showed that Na,K-ATPase can be used for determination of its inhibitors. The objective of this work was to investigate the suitability of Na,K-ATPase for determination of digoxin, and to follow the effect of irradiation using Na,K-ATPase activity as the measure of digoxin concentration. The aim was to develop a simple method for testing the photodegradation of digoxin based on the measurement of inorganic orthophosphate (P_i) liberated by hydrolysis of adenosinetriphosphate (ATP), since P_i is strongly concentration dependent on Na,K-ATPase inhibitors. In addition, the formation of degradation products, eventual Na,K-ATPase inhibitors, could be recognised by the proposed method.

In the present work photochemical degradation of digoxin in aqueous media was carried out. Since several HPLC methods for evaluation of digoxin concentration in various samples are known [17,18] the performance of Na,K-ATPase assay was verified with HPLC analysis of photodegraded samples.

Moreover, cytotoxic effects of degradation products in phytohemaglutinin-stimulated human lymphocytes were estimated using CBMN test.

2. Experimental

2.1. Chemicals

All chemicals were of analytical grade. Na,K-ATPase, acetate and 1,10-phenanthroline were purchased from Sigma Co. Digoxin [19] was obtained from "Zdravlje", Leskovac. Ethanol was a product of Riedel de Haen, ammonium acetate was from Merck, potassium oxalate from Fluka, ferric sulphate from Aldrich, sulphuric(VI) acid was provided by Carlo Erba and acetonitrile was HPLC grade and was a J.T. Baker product. High purity water (<18 MΩ/cm) was obtained by deionisation using the NANOpure system (Barnstead). 30% ethanol stock solution containing 1×10^{-3} M of digoxin was prepared by dissolution of the weighted amount in a small volume of hot ethanol (96%) and immediately diluted by high purity water.

2.2. Photodegradation studies

A 125 W Cermax xenon parabolic lamp emiting light with wavelengths above 200 nm was used as a light source. Experiments were carried out in a quartz sample cell with the optical path length of 1.0 cm placed 65 cm from the light source. Since no significant photodegradation of digoxin was achieved in reasonable time using only wavelengths above 320 nm, the entire emission of the Xe-lamp (including wavelengths between 200 and 320 nm) was exploited. The photon flux for the range of wavelengths below 410 nm, was evaluated by potassium ferrioxalate actinometry [20], and detemined to be 2.6×10^{16} cm^{-2} s^{-1}. In a typical run the 30% ethanol solutions containing different molarities of digoxin at pH 7.5 were prepared and 2.5 mL of the solution was placed into the sample cell. Solutions were stirred with magnetic stirrer during the irradiation. The temperature of irradiated solutions was constant at 45 °C. Samples were exposed for various irradiation times, when the aliquots of each sample were analysed using the Na,K-ATPase assay and by HPLC.

2.3. HPLC analysis

HPLC measurements were carried out according to the slightly modified procedure Jedlička et al. [17]. The HPLC equipment consisted of a HP 1100 Series chromatograph (G1322A degasser, G1311A quaternary pump, G1315A diode array detector, G1313A auto injector, G1316A column thermostat). Irradiated solutions were analysed on a reversed-phase C8 column (Du Pont Zorbax, 4.6 mm × 25 cm, 5 μm particle size) using a 70:30 mixture of 10 mM aqueous NH_4NO_3 and acetonitrile as the initial eluent, which was changed into a pure acetonitrile by applying a linear gradient between 8 and 12 min of each chromatographic run. The flow rate was 2 mL/min and the injection volume was 10 μL. The digoxin elution was monitored at 218 nm, while simultaneously recording absorption spectra of eluting compounds in the wavelength range from 190 to 400 nm.

2.4. ATPase assay

Na,K-ATPase activity was determined in a standard incubation medium (200 μL), containing 50 mM Tris–HCl (pH 7.4), 100 mM NaCl, 20 mM KCl, 5 mM ATP and protein (2 mg/mL) in the presence of 20 μL of the digoxin solution. Incubation mixtures were preincubated for 10 min at

37 °C in the presence of digoxin or distilled water (control). The reaction was started by the addition of ATP, allowed to proceed for 10 min, and interrupt by the addition of the ice cold $HClO_4$. The concentration of inorganic orthophosphate (P_i) liberated from the hydrolysis of ATP as the measure of Na,K-ATPase activity in the presence and absence of the inhibitor was determined using modified spectrophotometric procedure [15]. The spectrophotometric measurements were performed on a Beckman 5260 UV–vis spectrophotometer.

2.5. Toxicity tests

For evaluation of geno and cytotoxicity of digoksin cytokinesis block micronucleus test (CBMN) according to the method of Fenech and Morley [14] was used to analyze induction of micronuclei and proliferation of the harvested human lymphocytes. Aliqouts of 0.5 mL of whole blood from a healthy volunteer were set up in cultures containing 4.5 mL of medium RPMI-1640 supplemented with 15% of calf serum and 2.5 μg/mL of phytohemaglutinin (Gibco, BRL). Cultures were exposed to 100 μL of photochemicaly treated digoxin or physiological solution (control value). Micronuclei were analyzed in binucleated cells. For each sample 1000 binucleated cells (BN) were analyzed. Incidence of micronuclei was expressed as the number of micronuclei per 1000 BN cells. Cell proliferation potential was evaluated by classifying 1000 cells (from the same slides) according to the number of nuclei. Cytotoxicity index (CBPI) [21] was calculated according the formula: CBPI = 1MI + 2MII + 3(MIII + MIV)/(total number of cells), where MI to MIV represent the number of cells with one to four nuclei.

3. Results

3.1. Hydrolysis and thermal stability of digoxin at pH 7

To exclude the effect of hydrolysis and temperature on the spontaneous degradation of digoxin, the stability of 1×10^{-3} M 30% ethanol solution at 45 °C was investigated for 12 h by following the absorption spectra as a function of time in absence of irradation. Besides, the samples were analyzed by HPLC. The results revealed that the contributions of hydrolysis and thermal degradation under the investigated experimental conditions were insignificant (<1%), since the absorption spectra and HPLC analysis did not indicate the change of digoxin concentration nor the formation of new products.

3.2. Detection of digoxin by Na,K-ATPase assay after irradiation

Photochemical degradation of digoxin was investigated using 1.00×10^{-3} and 0.33×10^{-3} M standard solutions. The samples were irradiated for 1 to 40 min. Each irradiated sample exposed to UV–vis light for a particular time was diluted to concentrations corresponding to 1×10^{-9} to 1×10^{-4} M digoxin in an untreated solution. The inhibition of Na,K-ATPase activity by irradiated digoxin was followed. In all experiments the enzymatic activity in the presence of digoxin was calculated as the percentage of the control value of the standard incubation mixture, without the inhibitor. The concentration dependent inhibition curves for various irradiation times in the case of 1.00×10^{-3} M irradiated standard digoxin solutions are presented in Fig. 1. It is obvious from Fig. 1 that the degree of the inhibition decreased with the increase of irradiation time. Similar results were also obtained for 0.33×10^{-3} M irradiated solution.

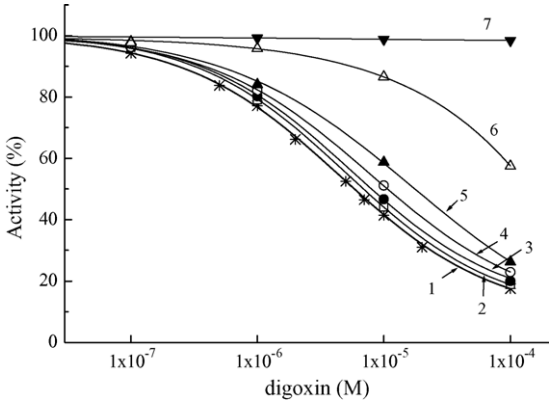


Fig. 1. Concentration-dependent curves of digoxin induced inhibition of Na,K-ATPase before (1) and after irradiation (2–7) of 1×10^{-3} M standard solution. Exposure times: (2) 2.5 min; (3) 5.0 min; (4) 7.5 min; (5) 10.0 min; (6) 20.0 min; (7) 40 min.

Besides, the concentration dependent inhibition curve (Fig. 1, curve 1) representing Na,K-ATPase activity versus digoxin concentration (unirratiated) was constructed. It served as the calibration graph for determination of digoxin concentration in irradiated sample. Twenty microlitres of irradiated sample was added to the Na,K-ATPase assay and the activity of the enzyme was measured as described in Section 2. The concentration of digoxin in the irradiated samples was determined from the calibration graph, and the results are given in Table 1. The results show, that the increasing of the irradiation time strongly influenced the digoxin concentration. Moreover, after 40 min irradiation time, the inhibition of the enzyme was not noticeable, indicating the absence of enzyme inhibitor.

3.3. HPLC analysis of the irradiated samples

The irradiated samples were analyzed using HPLC. No degradation products were detected by DAD detector. The results of the determination of digoxin concentration in the irradiated samples obtained by HPLC analysis are also given in Table 1, together with the results obtained by means of Na,K-ATPase assay. Good agreement using two independent methods for determination of digoxin concentration in the irradiated samples was observed.

Table 1
Comparison of concentrations of digoxin determined by HPLC and ATPase assay in the irradiated samples

Time of irradiation (min)	$10^3 \times$ digoxin concentration (mol/L)		Efficiency of irradiation (%)
	Determined by HPLC	Determined by ATPase assay	
0	1.00		–
2.5	8.83 ± 0.04	8.70 ± 0.04	11.7
5.0	7.23 ± 0.04	7.32 ± 0.04	27.7
7.5	5.41 ± 0.03	5.44 ± 0.03	45.9
10.0	3.26 ± 0.02	3.48 ± 0.02	67.4
20.0	0.38 ± 0.02	0.38 ± 0.02	96.2
40.0	0.00 ± 0.00	0.00 ± 0.00	100.0
0	0.33		–
1	2.78 ± 0.02	2.75 ± 0.02	18.2
3	1.94 ± 0.01	1.95 ± 0.01	42.9
5	1.16 ± 0.01	1.20 ± 0.01	65.9
10	0.20 ± 0.01	0.20 ± 0.01	94.1
15	0.00 ± 0.00	0.00 ± 0.00	100.0

3.4. Toxicity studies of digoxin degradation products

The proliferation index (CBPI) and incidence of micronuclei were studied as two possible toxic effects of irradiated digoxin solution on the human lymphocytes. The cells were exposed to 100 μL of digoxin solutions which were photochemically treated from 2.5 to 40 min. The concentration of digoxin in the cultures was calculated from the results obtained by Na,K-ATPase assay. It was in the range from 1.66×10^{-5} M (2.5 min irradiated sample) to 7.52×10^{-6} M (20 min irradiated sample).

In the first experiment the cell proliferation was followed using the unirradiated digoxin in the concentration range from 1×10^{-9} to 1×10^{-4} M. The same experiment was performed using the irradiated samples. The results presented in Fig. 2 show the cell proliferation versus digoxin concentration curves in the presence of the treated and untreated samples. It is obvious, that the results obtained for the irradiated samples fit very well to the control curve obtained for the unirradiated samples. These findings suggest, that the photochemical treatment did not induce toxic degradation products, capable to induce additional damages of DNA or distrurb proliferation ability of lymphocytes.

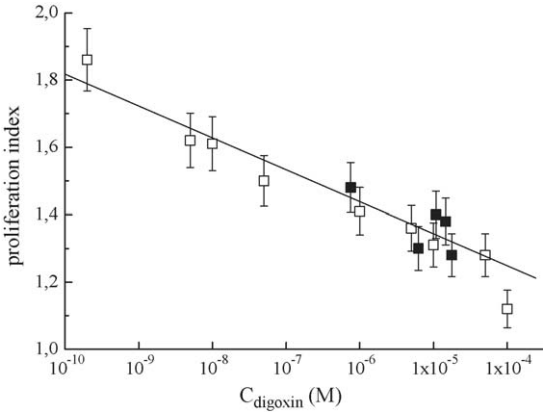


Fig. 2. Dependence of proliferation index on digoxin concentration in untreated (open symbols) and irradiated (solid symbols) samples.

In the second experiment the incidence of micronuclei in treated samples was measured. The mean value of 5 ± 2 micronuclei per 1000 BN was obtained. The result was independent on digoxin concentration in the cultures. It is worthy to note that it was in the range of background values described in the literature [13] and did not differ significantly from the control value.

3.5. Kinetics of digoxin degradation

The efficiency of degradation was calculated from the results obtained by Na,K-ATPase assay. However, it depends on initial digoxin concentration. As an example from Table 1, after 5 min irradiation 27.7 and 65.9% degradation efficiency of digoxin was achieved using 1.00×10^{-3} and 0.33×10^{-3} M digoxin solution, respectively. This indicates that the reaction does not proceed according to first-order kinetics, as it would be expected for a reaction where digoxin would be the rate determining species. For this reason the photodegradation kinetics was studied in more detail using data from HPLC analysis to avoid sporious results due to small but possible contribution of other ATPase inhibiting species.

The concentration of digoxin versus irradiation time after exposure of 0.33×10^{-3} and 1.00×10^{-3} M digoxin solutions at pH 7.4 is shown in Fig. 3. The experimental points represent the mean values of concentrations of digoxin determined by HPLC in duplicates. The parallel straight lines in [digoxin] versus *t*-plots indicate zero-order kinetics, rather than the pseudo-first order reaction. The zero-order rate constant obtained as a mean value of the plots for two initial concentrations of digoxin is $k = (4.6 \pm 0.3) \times 10^{-5}$ min^{-1}.

4. Discussion

The presented study demonstrates the suitability of the Na,K-ATPase assay for evaluation of digoxin degradation. The method is based on well known spectrophotometric

procedure for evaluation of inorganic P_i, that is concentration dependent of Na,K-ATPase inhibitor [4,15]. Substantial number of simple and reliable methods based on HPLC chromatography for the evaluation of digoxin concentration in human plasma and in aqueous media were developed [17,18], but these methods do not give any information about the influence of degradation products of this drug after photochemical treatment on its target enzyme, Na,K-ATPase. The proposed method is very simple, easy, and enables determination of at least 96 probes on microtiter plates, using ELISA reader during no more that 10 min [15]. The excellent agreement of the results obtained using Na,K-ATPase assay with HPLC method was observed.

The results obtained by HPLC and Na,K-ATPase assay excluded the presence of toxic degradation products that could inhibit the enzyme activity. The toxicity of irradiated samples was further investigated by the CBMN test. As the results presented in Fig. 2 show, the proliferation index decreased with the increasing of digoxin concentration in unirradited samples. The proliferation index obtained for the irradiated samples containing digoxin (the concentration of which was determined by HPLC or Na,K-ATPase assay) fitted very well to this function. This study confirmed that toxic products, which could damage the living cell, did not form upon digoxin degradation.

Literature data indicate that the irradiation of some cardiac glycosides in solid state, such as ouabain, digoxin or α-acetyldigoxin, led to the decomposition or the cleavage of the lactone ring [9] with various efficiencies, forming photoproducts of isomerisation and dimerisation [22]. Digoxin usually yielded a lower percentage of photoproducts, showing two main products of irradiation [9]. The lactonic carbonyl absorption disappeared in the IR spectra. These results show that the α,β-unsaturated lactone ring was decomposed or cleaved [23]. Replacement of the lactone ring by open chains of some semi synthetic digitoxigenin derivatives induced the decrease of the relative potency to inhibit Na,K-ATPase [24]. Although no comprehensive mechanistic study of digoxin photodegradation was done in the present study, some obvious conclusions about the mechanism of photodegradation of the digoxin could be mentioned. The absorption maximum of digoxin solutions was at 218 nm. This band belongs to α,β-unsaturated lactone chromophore, because it is the only possible chromophore in the molecule, which could absorb light above 200 nm. During the photodegradation there are no degradation products detected by HPLC with DAD detector above 200 nm. The only noticeable effect is reduction of the 218 nm absorption peak of parent digoxin molecule. This result leads to the conclusion that the unsaturated chromophore and consequently the lactone ring is degraded during the photochemical treatment.

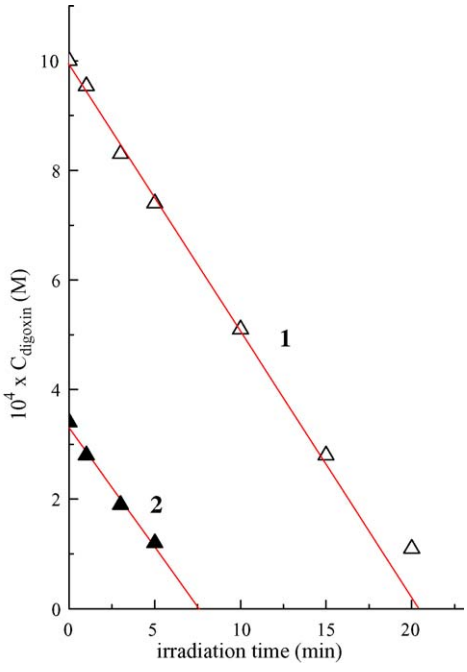


Fig. 3. Dependence of digoxin concentration on irradiation time. Concentration of standard solution: 1–1.0×10^{-3} M, 2–0.33×10^{-3} M.

The results of the kinetic experiments using two digoxin concentrations in this work suggest that digoxin did not participate in the rate-determining step. However, it must be emphasized that in principle homogenous reactions of total zero order do not exist, and in most cases the complicated kinetic behavior of the catalyzed reactions reduces to zero-order [25]. In the case of digoxin solutions, it seems that the species being monitored did not participate in the rate-determining step.

In conclusion, the determination of digoxin in solution by Na,K-ATPase assay is a simple alternative method suitable for routine evaluation of digoxin and presence of other Na,K-ATPase inhibiting compounds. However, the main advantage of this method in comparison to HPLC is the possibility of analyzing large number of samples in a short time.

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