IMPEDANCE STUDY OF HYPOXIC CELLS RADIOSENSITIZER ETANIDAZOLE RADICAL ANION IN WATER

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This contribution presents a study made as a part of commemoration of Professor Jaroslav Heyrovský to whom the Nobel Prize was awarded fifty years ago.

Electrochemical transfer of the first electron to cytotoxic radiosensitizer etanidazole (ETN) and ETN radical anion formation in the alkaline buffered aqueous solution were studied by means of electrochemical impedance spectroscopy (EIS). The heterogeneous electron transfer rate constant for the first reduction of ETN (radical anion production), k^0, was calculated. The value of k^0 from EIS measurements and the previously obtained value of so-called E_7^1 potential indicate that ETN, compared with other possible chemical radiosensitizers, requires lower energy to accept the first electron during the metabolic pathway. However, the necessity of the alkaline pH may decrease the ability of ETN to act as hypoxic radiosensitizer in the human body.

Keywords: Etanidazole; Impedance spectroscopy; Radical ions; Radiochemistry; Electrochemistry; Hypoxic cell; Electrochemistry.

Radiation therapy is a form of cancer treatment that prevents malignant cells from growing and dividing; ionizing radiation implies free radical production. Radiosensitizers are drugs that make cancer cells more sensitive to radiation therapy. They change the way how cells respond to the radiation.

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The cytotoxic properties of such compounds are due to the fact that, in the metabolic pathway, for example nitro compounds undergo one-electron reduction to generate nitro radical anions exhibiting cytotoxicity towards cellular systems[1–3]. As DNA is the critical target in the living cells, it is possible that these nitro radical anions interact with cellular DNA and DNA components, causing DNA damage within the cell. Moreover, it is evident that nitro radical anions from heterocyclic drugs abstract electrons from the bases[4], forming corresponding base cations that undergo secondary reactions to give different compounds. As a result, the base is degraded.

Some electrochemical results indicate that several drugs would act in a two-step redox mechanism. The first step involves oxidation of one moiety of a drug, and the second one then further reduction of the second moiety of the already oxidized molecule at a considerably lower reduction potential.

The reactivity of radical anions from most of the nitrogen-containing compounds was studied mainly by the pulse radiolysis technique[5–8]. Recently, electrochemical techniques have been used to study the behavior of the nitro radical anions[9–21].

Pharmacological and toxic effects are mediated by cytotoxic reactions and depend on the in vivo reduction of the reactive group producing the radical anion or cation species. It is probably possible to modulate both the reduction ability and radical stability and, consequently, capability of being enzymically reduced.

Drugs with higher electron affinity (EA; with smaller reduction potential) are generally the most toxic and mutagenic species, being also more quickly metabolized. Furthermore, there are a number of other studies[22–24] dealing with the relationship between reduction potentials and pharmacological activity, showing that this parameter is of interest from both electrochemical and pharmacological point of view.

Etanidazole (ETN; Fig. 1) belongs to the important class of electron-affinic radiosensitizers[25–27]. An important property of the electron-affinic compounds is that they appear to radiosensitize hypoxic cells, but have no measurable effect in well-oxygenated cells, at least in vitro[28]. This is probably

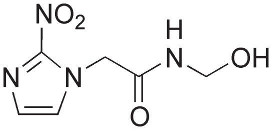

FIG. 1
Molecular structure of etanidazole

not because of some remarkable inherent property, but because of simple kinetic competition between oxygen and drugs (chemicals) for reaction with key DNA base radicals. This agent may also be useful as an imaging agent for identifying hypoxic, drug-resistant regions of primary tumors or metastases. ETN, like other nitroaromatic radiosensitizers, displays its biological activity probably not only through the main active nitro group but also through the side-chain attached to the nitrogen in the imidazole ring in position 1.

The electrochemical behavior of substituted nitroaromatic compounds has been studied at the different types of electrodes. The experiments usually focused on the study of nitro radical anion formed during the reduction step in various, mostly aprotic and mixed solvents. However, according to our best knowledge there is no evidence in the literature for an impedance study of ETN in pure water solution.

EXPERIMENTAL

Etanidazole (98%) was obtained from Sigma–Aldrich and was used without any purification. All other chemicals were of p.a. or higher quality. Buffers were prepared using Millipore MilliQ water (resistance >18.2 MΩ). Oxygen was removed from the solution by purging with argon (Messer, min. 99.998 vol.%) prior to the each measurement.

Impedance measurements were carried out in the range of 1 Hz to 25 kHz, using an AUTOLAB PGSTAT 30 equipped with FRA2 module (Eco Chemie, The Netherlands). AC amplitude of 10 mV was derived from an internal oscillator. Each impedance curve consisted of 100 measured points. Electrochemical data from electrochemical impedance spectroscopy (EIS) were analyzed using the AUTOLAB software. A three-electrode electrochemical cell was used. The reference electrode (RE), Ag|AgCl|1 M LiCl, was separated from the test solution by a salt bridge. The working electrode (WE) was a valve-operated static mercury electrode (SMDE2, Laboratorní Přístroje, Prague) with an area of 5.15×10^{-3} cm^2. The counter electrode (CE) was a cylindrical platinum wire with an area approximately 100 times higher than the area of WE. All pH measurements were carried out with a WTW pH Meter (Model 340) equipped with a WTW Sentix 50 glass electrode.

RESULTS AND DISCUSSION

The first part of this paper describes the basic electrochemical behavior of ETN in the buffered water solution. Similarly to the other substituted nitrobenzenes, the electrochemical reactions of ETN in aqueous media at mercury electrode are affected by the buffer pH. Consequently, the cyclic voltammograms of ETN are also pH dependent.

Figure 2 shows cyclic voltammograms (CV) at various concentrations of ETN in the buffer solution. The first two redox peaks I and II are due to the four-electron four-proton reduction (*1*) and subsequent two-electron two-proton reduction of the nitro group (*2*) to produce corresponding hydroxylamine and amine derivatives of ETN, respectively.

$$\text{ETN–NO}_2 + 4\ \text{e} + 4\ \text{H}^+ \rightarrow \text{ETN–NHOH} + \text{H}_2\text{O (peak I)} \tag{1}$$

$$\text{ETN–NHOH} + 2\ \text{e} + 2\ \text{H}^+ \rightarrow \text{ETN–NH}_2 + \text{H}_2\text{O (peak II)} \tag{2}$$

ETN–hydroxylamine is on the anodic sweep oxidized to nitroso–ETN (peak III in Fig. 2) (*3*). On the second cathodic sweep, two-electron reduction of the nitroso– ETN is observed[29].

$$\text{ETN–NHOH} \rightarrow \text{ETN–NO} + 2\ \text{e} + 2\ \text{H}^+ \text{ (peak III)} \tag{3}$$

A detailed investigation of the reduction of nitrosobenzene indicates that, similarly to the reduction of nitrobenzene, a rapid surface reaction of the adsorbed compound took place simultaneously with the diffusion controlled reduction[30].

The cytotoxic properties of nitro radiosensitizers are based on the fact that, in the cell, they undergo the one-electron reduction to generate the nitro radical that is extremely toxic towards cellular systems (probably to

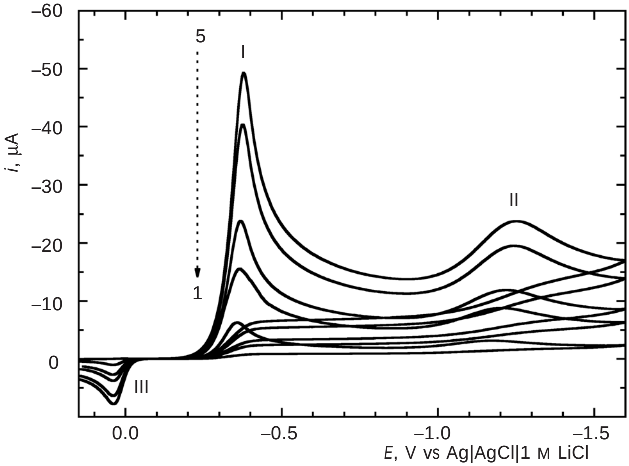

FIG. 2
CV of ETN in 0.1 M BR buffer at various ETN concentrations, pH 5.95; [ETN] (in mmol l^{-1}): 1 0.27, 2 0.51, 3 0.69, 4 1.11, 5 1.32

DNA). In the previous study we found that, in the case of ETN, in the strong alkaline solutions (pH > 10) the peak I (reduction of nitro moiety to hydroxylamine) is divided into two peaks. The first peak corresponds to the nitro radical anion production ($ETN{-}NO_2 + e \rightarrow ETN{-}NO_2^{\bullet -}$) and the second one to the hydroxylamine production ($ETN{-}NO_2^{\bullet -} + 3\ e + 4\ H^+ \rightarrow ETN{-}NHOH + H_2O$). If the vertex potential E_λ is set closely behind the potential of the first peak, the nitro radical production becomes reversible. However, this electron transfer is coupled by the weak adsorption of ETN on the electrode surface[29]. In order to see the split of the peak I into peaks Ia and Ib, it was necessary to accumulate ETN at the electrode surface. The optimum accumulation potential was found approximately $E_{accum} = -0.3$ V vs RE, and the best separation time was $t_{accum} = 600$ s (ref.[29]).

EIS Measurement

Drugs with higher electron affinity are generally more toxic and mutagenic. This mutagenic potential can be characterized by two "electrochemical" parameters.

The first one, E_7^1 values, is a parameter that accounts for the energy necessary to transfer the first electron to an electroactive group at pH 7 in an aqueous medium to form a radical anion. Therefore, in the case of nitro compounds, the E_7^1 values represent the ability to form the nitro radical anion. This parameter was discussed for ETN in our previous paper. For ETN we found the value $E_7^1 = -0.374 \pm 0.006$ V, which is in a good agreement with pulse radiolysis measurements[29].

The second parameter is the heterogeneous electron transfer rate constant, k^0, which also characterize (quantify) the ability of the compound to accept the electron. In this study we focused on the k^0 determination by EIS measurements.

The electrochemical behavior of the ETN radical anion production has been represented by EIS as an equivalent circuit. For the evaluation of the equivalent circuit parameters from the experimentally recorded complex impedances the AUTOLAB software was used (see Experimental). The heterogeneous rate constant of the reaction $ETN{-}NO_2 + e \rightarrow ETN{-}NO_2^{\bullet -}$ was determined using graphical complex plain representation of the cell impedance[31].

Figure 3 shows plots of the typical impedance diagrams at different potentials. The Randless and modified Randless circuits are the simplest equivalent circuits that fit our experimental data with acceptably low χ^2. In the modified Randless circuit (Fig. 4) the Warburg element W was replaced by the Gerischer element *G* and pure capacitance element *C* by the constant phase element *Q* defined by Eqs (*4*) and (*5*), respectively:

$$G = \frac{1}{G_0\sqrt{K_a + i\omega}} \tag{4}$$

$$Q = \frac{1}{Y_0(i\omega)^n} = \frac{1}{Y_0\omega^n}e^{[ni(\pi/2)]} \tag{5}$$

where ω is an angular velocity, i imaginary unit, K_a is "rate constant" parameter, Y_0 and G_0 are parameters that characterize the Gerischer and constant phase elements, respectively.

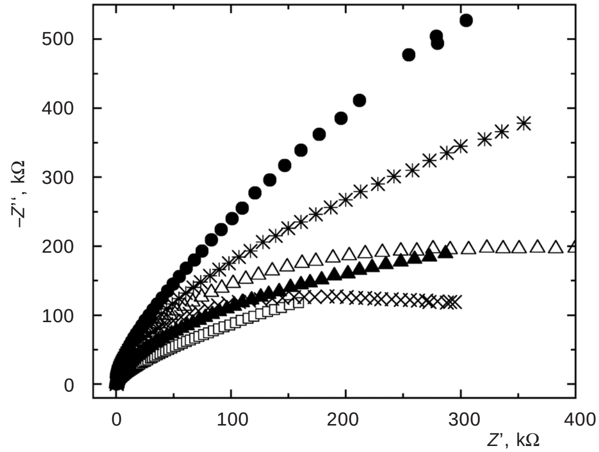

FIG. 3
Argand's diagram of ETN in 0.1 M BR buffer at various potentials vs RE; pH 11; ● –0.41 V, □ –0.585 V, ▲ –0.61 V, △ –0.64 V, × –0.76 V, ✳ –0.90 V

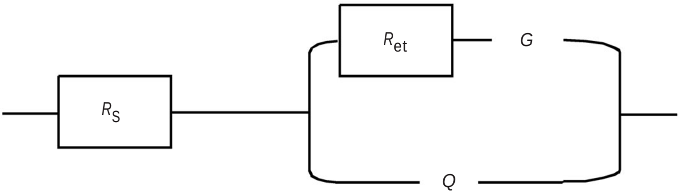

FIG. 4
Equivalent circuit representing the impedance of the cell containing ETN in 0.1 M BR buffer; R_S, electrolyte resistance; R_{ct}, charge transfer resistance; *Q*, constant phase element; *G*, Gerischer element

The Gerischer (*G*) circuit element was first derived for a preceding chemical reaction. This is a so-called CE mechanism. Therefore, the replacement of the Warburg element by the Gerischer element is in a good agreement with our previous observations[29].

The accuracy of the proposed circuit is demonstrated using the Bode plot (Fig. 5).

TABLE I

Simulated values of the electrical elements used in the equivalent circuit shown in Fig. 4

E, V	R_S, Ω	$Q/Y_0 \times 10^{-7}$ Ω^{-1} s^n	Q/n	G/G_0 Ω^{-1} $s^{1/2}$	$G/K_a \times 10^{-5}$ s^{-1}	R_{ct}, kΩ
–0.410	603	0.4023	0.9754	0.2782	2.096	170.5
–0.460	585	0.3527	0.9581	0.1976	1.997	36.10
–0.525	566	0.2526	0.9210	0.6108	2.076	7.690
–0.585	537	0.2177	0.8912	0.1890	2.925	35.10
–0.610	540	0.3039	0.9254	0.1193	4.372	97.50
–0.640	558	0.3689	0.9530	0.1207	7.733	245.4
–0.700	561	0.4710	0.9735	0.3643	7.006	231.8
–0.760	573	0.4654	0.9725	0.2827	2.141	220.9

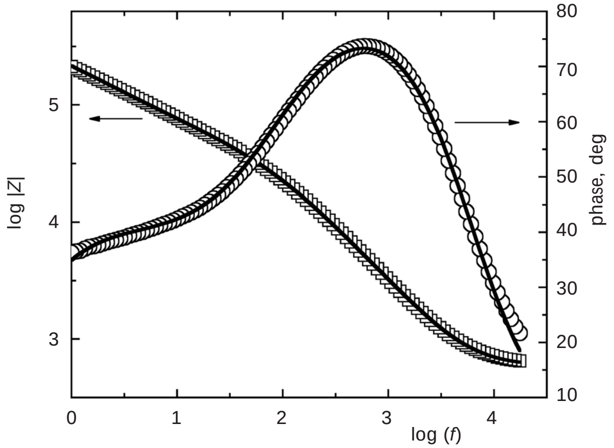

FIG. 5

Bode plot. ETN in 0.1 M BR buffer, pH 11; E = –0.585 V vs RE. Points represent experimental data, curves nonlinear regression analysis according to the equivalent circuits (Fig. 4)

Experimental data connected to ETN radical anion production are summarized in Table I. From the table we can see that almost all elements reach the maxima or minima for $E = -0.585$ V vs RE. Moreover, from the logarithmic dependence of the charge transfer resistance R_{ct} on the potential applied, E, one can estimate the electron transfer coefficient, α (as well as $1 - \alpha$). This dependence yields two asymptotic lines (slopes) corresponding to two limiting cases: (i) the reduction rate is much larger than the oxidation one; (ii) the oxidation prevails over the reduction[31]. The ratio of the slopes is very close to the unity. Therefore, one can conclude that the transfer coefficient $\alpha \approx 0.5$ [31]. This value is very close to our previous calculations ($\alpha \approx 0.46$) based on the voltammetric measurements[29]. These calculations indirectly confirm the suitability of the proposed equivalent circuit (Fig. 4). Moreover, these results can be used to evaluate kinetic parameters of the system.

Under suitable experimental conditions it is possible to study the kinetics of the electrode reaction, and the heterogeneous electron transfer rate constant, k^0, can be evaluated from the impedance measurements.

The frequency dependence of the faradaic phase angle ϕ_F can be calculated according to Eq. (*6*):

$$\cot\phi_F = \frac{Z'_F}{Z''_F} = 1 + \frac{1}{\zeta} = 1 + \frac{(2\omega D^0)^{1/2}}{k^0} \qquad (6)$$

where D^0 is a diffusion coefficient, ζ is the rate parameter defined as $\zeta = k_f/(2\omega D_R)^{1/2} + k_b/(2\omega D_O)^{1/2}$; k_f, k_b are the potential dependent rate constants of reduction and oxidation, respectively, D_R, D_O are the respective diffusion coefficients, Z' and Z'' are the components of the faradaic impedance. Assuming that $D_R = D_O = D^0$ and $k^0 = k_f + k_b$, the heterogeneous electron transfer rate constant, k^0, for redox couple $\text{ETN–NO}_2 + e \rightarrow \text{ETN–NO}_2^{\bullet-}$ can be estimated according to Eq. (*6*).

The plot of cot ϕ_F against $\omega^{1/2}$ should be a straight line and the rate constant can be evaluated from the slope[31]. For ETN the diffusion coefficient was calculated to be $D^0 = (2.8 \pm 0.1) \times 10^{-5}$ cm^2 s^{-1} (ref.[32]).

The heterogeneous electron transfer rate constant for the first reduction of ETN (radical anion production) in 0.1 M BR buffer at pH 11 and at $E = -0.585$ V vs RE was calculated to be $k^0 = (12.8 \pm 2.3) \times 10^{-2}$ cm s^{-1} (Fig. 6). This value is comparable with those obtained for other nitro compounds in mixed water/ethanol media[21]. Therefore, one can say, that our approach to determine k^0 of the $\text{ETN–NO}_2/\text{ETN–NO}_2^{\bullet-}$ couple in the purely aqueous solution, i.e., without any organic and/or aprotic co-solvent, using electrochemical impedance spectroscopy measurements, is correct. Moreover, similar results were obtained using cyclic voltammetry[29].

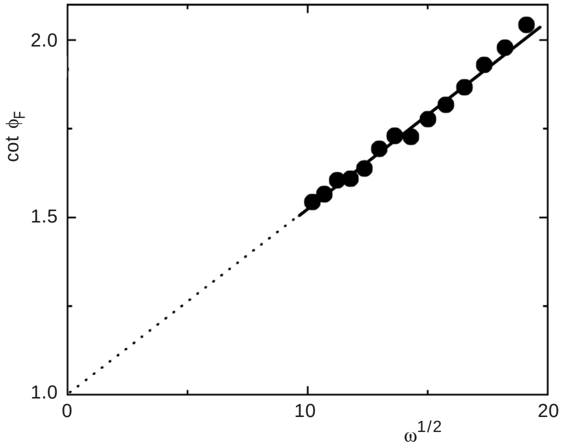

FIG. 6

The dependence of cotangent of the faradaic phase angle cot ϕ_F on square root of the angular frequency $\omega^{1/2}$ ($\omega = 2\pi f$). ETN in 0.1 M BR buffer, pH 11; $E = -0.585$ V vs RE

CONCLUSION

Etanidazole can be easily reduced in buffered aqueous solution without any organic co-solvent at the mercury electrode. At high pH of the solutions after applying suitable deposition potential, the first reduction peak I of ETN becomes divided into two peaks. The first reversible one corresponds to the process where the nitro radical anion of ETN is produced: $ETN{-}NO_2 + e \rightarrow ETN{-}NO_2^{\bullet-}$. It was proved that the $ETN{-}NO_2/ETN{-}NO_2^{\bullet-}$ couple is sufficiently stabilized in the aqueous medium at strong alkaline pH without any co-solvent added. It is a very rare case studied by electrochemical impedance spectroscopy at relatively low frequencies.

Under such conditions the heterogeneous electron transfer rate constant $k^0 = (12.8 \pm 2.3) \times 10^{-2}$ cm s^{-1} was determined. This value is in a good agreement with those obtained for similar nitro drugs. This also confirms that EIS is a good technique for study and characterization of drug radical production. If our previous results[29] of E_7^1 determination are combined with the heterogeneous electron transfer rate constant evaluation by the EIS measurements in this work, one can conclude that ETN can obviously act as a good chemical radiosensitizer. Both results indicate that a lower energy, compared with other possible chemical radiosensitizers, is necessary for the system to transfer the first electron to ETN. However, the necessity of the alkaline pH may decrease the ability of ETN to act as a hypoxic radiosensitizer in the human body.

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