Electrochemical Oxidation of Sanguinarine and of Its Oxidation Products at a Glassy Carbon Electrode – Relevance to Intracellular Effects

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Abstract

The electrochemical behavior of sanguinarine, a quaternary benzophenanthridine glycoside alkaloid with antimicrobial, anti-inflammatory, antioxidant and/or immune-stimulatory activities, was studied at a glassy carbon electrode using cyclic, differential pulse, and square wave voltammetry. The oxidation of sanguinarine is a quasireversible, diffusion-controlled process and occurred in a cascade mechanism with the formation of several oxidation products which adsorbed at the electrode surface. The oxidation of sanguinarine is pH dependent and involves the transfer of the same number of electrons and protons. The adsorbed sanguinarine oxidation products are reversibly oxidized at the glassy carbon electrode surface and their oxidation for a wide range of pHs was also studied by differential pulse and square wave voltammetry. A mechanism for the oxidation of sanguinarine at glassy carbon electrode is proposed.

Keywords: Sanguinarine, Alkaloid, Voltammetry, Oxidation, Adsorption

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1. Introduction

Sanguinarine, a quaternary benzophenanthridine glycoside alkaloid synthesized by plants belonging to the Papaveraceae, Fumariaceae and Rutaceae families [1], is known to exert a wide spectrum of physiological effects such as antimicrobial, anti-inflammatory, antifungal, antioxidant and/or immune-stimulatory activities [2–5]. It has been shown that sanguinarine acts against gram-positive and gram-negative bacteria and fungi [6–8] whereas other studies demonstrated that sanguinarine protects against skin cancer by up-regulating the expression of proteins involved in UV radiation-induced cell death [9]. In pharmacology, sanguinarine chloride, Scheme 1, is used principally in dental products and, due to the above mentioned properties, it reduces gingival inflammation and plaque formation [10].

From a possible therapeutic point of view, sanguinarine is important since it showed cytotoxic and apoptotic effects against various human cancer cell lines including epidermal, prostate, cervical and lymphoma [6, 11–13 and the references therein], but the mechanism through which sanguinarine initiates cell death is not fully elucidated [14, 15]. Nevertheless, several modifications in the activity of proteins involved in cell signaling have been observed [6, 16–18] but the formation of reactive oxygen species [13] as well as DNA damage [15, 19–22] were shown to be viable mechanism for triggering apoptosis of sanguinarine treated cancer cells. Recently, we have demonstrated that sanguinarine causes melanoma cell death through both mitochondrial and nuclear-mediated effects [23].

In view of the importance of sanguinarine as a potential chemotherapeutic agent or in other clinical applications,
different instrumental analytical procedures based on HPLC, capillary electrophoresis, UV spectrophotometry, dielectric relaxation and calorimetric studies were developed [24–27]. Most of these procedures were used for the quantitative determination of sanguinarine and to study its effect on specific biological structures such as DNA and proteins. However, very little is known about sanguinarine redox mechanisms although the reduction of several alkaloids has been reported at mercury electrodes [28] and the formation of redox active films from oxidation products of sanguinarine and chelerythrine was also characterized [29].

Since sanguinarine may participate in several redox reactions in the intracellular milieu, the aim of the present study is the investigation of the electron transfer properties of sanguinarine using cyclic, differential pulse and square-wave voltammetry at a glassy carbon electrode. The investigation of the electrochemical oxidation mechanisms of sanguinarine has the potential for providing valuable insights into biological redox reactions of this class of molecules, resulting in a better understanding of the data described for biological systems and increasing the overall knowledge of sanguinarine’s physiological mechanism of action.

2. Experimental

2.1. Materials and Reagents

Sanguinarine chloride was obtained from Sigma, and was used without further purification. A stock solution of 50 mM sanguinarine was prepared in DMSO and was stored at −4 °C. The desired concentration of sanguinarine was obtained by direct dilution of the appropriate quantity of stock solution into the supporting electrolyte in the electrochemical cell.

All supporting electrolyte solutions were prepared using analytical grade reagents and purified water from a Milli-pore Milli-Q system (conductivity < 0.1 μS cm⁻¹) (Table 1). Microvolumes were measured using EP-10 and EP-100 Plus Motorized Microliter Pipettes (Rainin Instrument Co., Woburn, USA). The pH measurements were carried out with a Crison micropH 2001 pH-meter with an Ingold combined glass electrode. All experiments were done at room temperature (25 ± 1 °C).

2.2. Voltammetric Parameters and Electrochemical Cells

Voltammetric experiments were carried out using a μ-Auto-lab running with GPES 4.9 software, Eco-Chemie, Utrecht, The Netherlands. Measurements were carried out using a glassy carbon (GCE) (d = 1.5 mm) working electrode, a Pt wire counter electrode, and a SCE as reference, in a 0.5 mL one-compartment electrochemical cell. The experimental conditions for differential pulse voltammetry (DPV) were pulse amplitude 50 mV, pulse width 70 ms, scan rate 5 mV s⁻¹. For square wave voltammetry (SWV) the experimental conditions were frequency 25 Hz and potential increment 2 mV, corresponding to an effective scan rate of 50 mV s⁻¹. The GCE was polished using diamond spray (particle size 1 μm) before each experiment. After polishing, the electrode was rinsed thoroughly with Milli-Q water for 30 s; then it was sonicated for 1 minute in an ultrasound bath and again rinsed with water. After this mechanical treatment, the GCE was placed in the desired buffer electrolyte and various DP voltammograms were recorded until a steady state baseline voltammogram was obtained. This procedure ensured very reproducible experimental results.

2.3. Acquisition and Presentation of Voltammetric Data

All the voltammograms were carried out in the dark. The DP voltammograms were baseline-corrected using the moving average with a step window of 5 mV included in GPES version 4.9 software. This mathematical treatment improves the visualization and identification of peaks over the baseline without introducing any artefact, although the peak height is in some cases reduced (< 10%) relative to that of the untreated curve. Nevertheless, this mathematical treatment of the original voltammograms was used in the presentation of all experimental voltammograms for a better and clearer identification of the peaks. The values for peak current presented in all graphs were determined from the original untreated voltammograms after subtraction of the baseline.

3. Results and Discussion

3.1. Cyclic Voltammetry

The oxidation behavior of sanguinarine was first studied by cyclic voltammetry (CV) in solutions containing 200 μM sanguinarine in electrolytes with different pH (Fig. 1). The oxidation occurred in a cascade mechanism with reversible steps and, depending on the pH of the supporting electrolyte, several peaks were observed.
In acid media, pH 3.8 0.1 M acetate buffer (Fig. 1A) several anodic peaks occurred on the first positive-going scan: peak 1a at $E_{pa}^1 = +0.85$ V, peak 2a at $E_{pa}^2 = +1.12$ V and peak 3a at $E_{pa}^3 = +1.36$ V. On the negative-going scan of the same voltammogram, a cathodic peak 5c occurred at $E_{pc}^5 = +0.26$ V. This peak corresponds to the reduction of the sanguinarine oxidation product formed at the GCE surface. A subsequent CV in the positive direction, in the same solution and without cleaning the GCE surface, showed a new anodic peak 5a at $E_{pa}^5 = +0.31$ V, and confirmed the reversibility of peak 5c, which is strongly adsorbed blocking the GCE surface such as on the 2nd scan sanguinarine oxidation peaks 1a, 2a and 3a practically disappeared.

In a new experiment carried out in a solution of 200 µM sanguinarine in pH 7.0 0.1 M phosphate buffer (Fig. 1B) the three oxidation peaks also occurred, but at lower potentials showing that the oxidation process of sanguinarine is pH dependent. On the other hand, peak 1a occurred with a higher current relative to the voltammogram obtained in pH 3.8, and this allowed clarifying which peak, 1a, 2a or 3a, leads to the oxidation product corresponding to peak 5c. Therefore, CVs were recorded under the same conditions and with a clean GCE surface, but the scan direction was inverted immediately after peak 1a, but before peak 2a, and the reduction peak 5c did not appear, Fig. 1C. In a new experiment, increasing the positive potential limit, the scan direction was inverted immediately after peak 2a, but before peak 3a, and peak 5c occurred at $E_{pc}^5 = +0.21$ V (Fig. 1C).

Thus, peak 5c corresponds to the reduction at the GCE surface of the product of sanguinarine oxidation formed at peak 2a at $E_{pa}^2 = +0.95$ V. Moreover, on a subsequent scan in the positive direction (not shown) peak 5a occurred at $E_{pa}^5 = +0.25$ V, and $\Delta E^5 = E_{pa}^5 - E_{pc}^5 = 60$ mV, i.e. a reversible couple, corresponding to the redox reactions of sanguinarine oxidation product formed at the electrode surface. The decrease of the peak heights observed on the second scan (not shown) was due to the formation of sanguinarine oxidation product on the GCE surface which reduces the available electrode surface area.

### 3.2. Differential Pulse Voltammetry

The electrochemical oxidation of sanguinarine was studied over a wide pH range between 2.0 and 13.0. The DP voltammograms (Fig. 2A) were all recorded in solutions of 100 µM sanguinarine in different electrolytes of 0.1 M ionic strength (Table 1). For pH < 2.0 and pH > 8.0 no oxidation peak was found even for higher concentration showing that sanguinarine is not oxidizable in these conditions, and for pH 2.0 and pH 8.0 only the two peaks 2a and 3a were observed.

For 3.3 < pH ≤ 7.0, the four peaks 1a, 2a, 3a and 4a occurred and their potential decreased with increasing pH of the supporting electrolyte. The dependence was always linear as shown in Figure 2B, the slope of the lines, $-60$ mV per pH unit showing that the oxidation of sanguinarine occurred with the same number of electrons and protons transferred. Taking into consideration the peak width at half height of ca. 50 mV, the oxidation of sanguinarine occurred with the transfer of 2 electrons and 2 protons [30, 31]. However, for pH < 3.3 and pH > 7.0, for peak 2a, and for pH < 3.3, for peak 3a, the peak potentials were pH independent. These changes can be attributed to sanguinarine chemical protonation/deprotonation processes.

Successive DP voltammograms were recorded in a solution of 100 µM sanguinarine in pH 4.2 0.1 M acetate buffer (Fig. 3A). As already mentioned, in these conditions the oxidation of sanguinarine occurred in four steps and in the first DP voltammogram peak 1a was observed at

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**Fig. 1.** CV baseline subtracted obtained at $v = 20$ mV s$^{-1}$, in 200 µM sanguinarine: (A) pH 3.8 0.1 M acetate buffer (—) first and (⋯⋯) second scan; and in pH 7.0 0.1 M phosphate buffer from: (B) 0.0 to 1.3 V (—) first and (⋯⋯) second scan and (C) first scans from (—) 0.0 to 1.0 V and 0.0 to (⋯⋯) 0.8 V.
On the second DP scan, two new anodic peaks, 5 a at $E_{pa5} = +0.25$ V and 6 a at $E_{pa6} = +0.39$ V, appeared. These peaks correspond to the oxidation of sanguinarine oxidation products. The current of peaks 1 a, 2 a, and 4 a decreased gradually with the number of potential scans due to a decrease of the available electrode surface area after adsorption of sanguinarine oxidation products. Due to the strong adsorption of sanguinarine oxidation products, a DP voltammogram was obtained showing only peak 5 a after transferring the electrode to buffer electrolyte.

The electrochemical oxidation of sanguinarine oxidation products adsorbed at the GCE surface was also studied for different pH values. After two consecutive DP voltammograms in 100 $\mu$M sanguinarine solution in different buffer electrolytes, the second DP voltammograms were plotted vs. pH (Fig. 4A). Peaks 5 a and 6 a were pH dependent, their potential decreasing linearly with the pH of the supporting electrolyte (Fig. 4B). In both cases, the slope of the lines was found to be $+60$ mV per pH unit, meaning that the number of protons transferred during the oxidation of sanguinarine oxidation product is equal to the number of electrons. However, for pH $>6.2$, peak 5 a is pH independent, indicating a mechanism above pH 6.2 involving only two electrons and no protons, corresponding to the sanguinarine oxidation product undergoing chemical deprotonation.
On the other hand, the peak 6a current is practically constant over the whole pH range whereas peak 5a showed a maximum on the pH interval between 2.0 and 4.2. Above this value, the anodic current of peak 5a decreased rapidly and after pH 5.0 remained constant (Fig. 4B).

3.3. Square Wave Voltammetry

SW voltammograms were recorded in 200 µM sanguinarine solution in pH 7.0 0.1 M phosphate buffer (Fig. 5). Although in the first voltammetric scan three peaks 1a, 2a and 4a were easily observed (Fig. 5A) peak 1a was broader indicating two overlapping consecutive charge transfer reactions, peak 1a and 1a". Such an effect can be explained taking into consideration the hindering of the oxidation reaction of sanguinarine as a consequence of GCE surface blockage due to the adsorption of sanguinarine oxidation product. Thus, sanguinarine molecules in close proximity to the electrode surface are oxidized giving rise to peak 1a and the sanguinarine oxidation product adsorbed on the GCE surface forms an incomplete noncompact monolayer. Further oxidation of sanguinarine molecules diffusing from bulk solution towards the electrode, peak 1a" is more difficult because it occurs through the incomplete layer of adsorbed sanguinarine oxidation product. However, after the redox process corresponding to peak 1a" the layer of sanguinarine oxidation product becomes more compact, covering the electrode surface and impeding further oxidation of sanguinarine molecules that diffuse from the solution.

Plotting the forward and the backward components of peak 1a total current, two new cathodic peaks 1c and 1c" were observed. The potentials of these cathodic peaks are similar to those of the anodic peaks, but the currents are smaller showing that the oxidation of sanguinarine is a quasireversible process.

![Fig. 4.](image1.png)

Fig. 4. (A) 3D plot of the second consecutive DP voltammograms in 100 µM sanguinarine vs. pH. (B) Plot of (*) $E_{pa}$ and (○) $I_{pa}$ of peaks 5a and of (▲) $E_{pa}$ and (△) $I_{pa}$ of peaks 6a vs. pH. The slope of the dotted lines is 60 mV per pH unit.

![Fig. 5.](image2.png)

Fig. 5. SW voltammograms base line corrected in 100 µM sanguinarine in pH 7.0 0.1 M phosphate buffer: (A) first scan and (B) second scan; $f = 10$ Hz, $\Delta E_s = 2$ mV, pulse amplitude 50 mV, $v_{eff} = 20$ mV s⁻¹, $I_t = $ total current, $I_f = $ forward current, $I_b = $ backward current.

In a second consecutive SW voltammogram, the formation of sanguinarine reversible oxidation product peak 5a (Fig. 5B) was confirmed by plotting the forward and backward components of the total current, the oxidation and the reduction currents being seen to be equal. Moreover, the identical value of the potential of peak 5 on the forward and backward current components is an indication of the adsorption of sanguinarine oxidation products on the GCE surface.

3.4. Oxidation Mechanism

To understand the redox mechanism of sanguinarine, its electrochemical behavior was compared with the electrochemical oxidation of compounds with similar moieties to those present in the sanguinarine structure such as 3,4-methylenedioxyamphetamine (MDA) and 3,4-methylene-dioxymetamphetamine (MDMA).

Previous studies carried out at physiological pH have showed the occurrence of two anodic peaks at +1.05 V and +1.26 V for the oxidation of MDMA, whereas for MDA only one oxidation peak has been observed at a potential similar to the first peak of MDMA [32]. Considering the structural differences between MDMA and MDA (MDMA has a secondary and MDA a primary amine group) it was proposed that the first oxidation peak is due to the removal of electrons from the aromatic electrophore whereas the second peak was attributed to oxidation of the secondary amine group.

Enzymatic studies [33, 34] have shown that both MDA and MDMA can be first oxidized to a catecholamine derivative in a process involving the transfer of 2 electrons [33] in several steps, the most important being hydrolysis and demethylation. However, these studies have also shown that the demethylation process is not limited to the action of the enzymes but can also be mediated by hydroxyl radicals [35]. Furthermore, the catecholamine derivatives of MDA and MDMA could be reversibly oxidized to form ortho-quinone specie.

It is proposed that the oxidation peak 1 corresponds to the removal of electrons from one of the methylenedioxy rings of the sanguinarine moiety. From the experimental results presented it is not possible to determine which of the methylenedioxy rings of sanguinarine undergoes oxidation. Taking into consideration previous results concerning the oxidation of sanguinarine and chelerythrine (a similar structure without a methylenedioxy ring) [29] it can be considered that peak 1 corresponds to the oxidation of the methylenedioxy ring found in the vicinity of ring D, Scheme 1A and 2. Increasing the potential applied to the electrode, peak 2 occurs due to the removal of electrons from the double bond of ring D leading to demethylation and formation of the catecholamine derivative of sanguinarine which is reversibly oxidized, at peak 5, to an ortho-quinone species (Scheme 2, Fig. 1C). Peak 3, could be due to the loss of electrons from the tertiary amine group in ring B in a process that can lead to the formation of oxosanguinarine [36]. However, for pH > 7.0 peak 3 does not occur and this can be explained taking into consideration that in alkaline electrolytes sanguinarine, similarly to other alkaloids [37, 38], can undergo chemical hydrolysis, Scheme 1B. Finally, it is proposed that peak 4 is due to the removal of electrons from the double bond of ring A leading to demethylation of the secondary methylenedioxy ring of sanguinarine and the formation of a quinine which is reversibly oxidized, at peak 6.
4. Conclusions

The present study showed that sanguinarine chloride, a quaternary benzophenanthridine glycoside alkaloid with a wide spectrum of biological effects, undergoes oxidation at a glassy carbon electrode. The electrochemical oxidation of sanguinarine chloride was investigated by cyclic, differential pulse and square wave voltammetry over a wide pH range. Cyclic voltammetry experiments have shown that sanguinarine undergoes a quasireversible, diffusion-controlled oxidation process. It has been shown that the oxidation occurs in complex, pH dependent mechanism. In acid electrolytes, it occurs in four steps whereas in alkaline media three oxidation peaks were observed.

In all electrolytes a main sanguinarine electroactive oxidation product that undergoes reversible oxidation was always formed, blocking the electrode surface. The electroactive centers of sanguinarine were also determined by comparing the present results with data obtained for molecules with similar structure. Since sanguinarine is mostly accumulated in the nuclei of tumor cells [39], also presenting mitochondrial-mediated effects [23], the present study is pertinent since it clearly identifies several distinct fates of the molecule upon oxidation. One particular aspect of sanguinarine intra-cellular interactions came from the work by Holy et al [39] where sanguinarine nuclear self-fluorescence was noticeably diminished six hours after drug addition, with cells being essentially nonfluorescent after 24 hours of sanguinarine treatment. The data from the present work may imply that sanguinarine undergoes redox reactions in specific cell compartments (including the nuclei), which resulted into conversion into different non-fluorescent products, whose biological effects are still unknown.

The present research is a clear indication that further studies of this type are required to understand the redox reactions of molecules with potential clinical use in order to identify different reactions and product formation which would vary according to the intracellular accumulation site.

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6. References