Redox mechanism of lumazine at a glassy carbon electrode

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\textbf{A B S T R A C T}

The electrochemical behaviour of lumazine, a pterin compound which acts as substrate for the lumazine synthase/riboflavin synthase complex, has been studied at a glassy carbon electrode using cyclic, differential pulse and square wave voltammetry. The oxidation of lumazine was possible only in electrolytes with pH > 7.0 since it occurred at very high potential values. It is an irreversible, pH-dependent process that occurred with the transfer of two electrons and two protons and did not involve the formation of any electroactive oxidation product. The reduction of lumazine was studied over a wide pH range and occurred with the transfer of two electrons and two protons. In acid electrolytes the reduction was quasi-reversible and occurred with the formation of several reduction products that were oxidized at the glassy carbon surface and for higher pH the reduction became reversible. The diffusion coefficient of lumazine was calculated in pH 7.0 0.1 M phosphate buffer to be $D_{LMZ} \approx 9.1 \times 10^{-6}$ cm$^2$ s$^{-1}$. The electroanalytical determination of lumazine was carried out in pH 7.0 0.1 M phosphate buffer using differential pulse voltammetry with a detection limit LOD = 0.76 $\mu$M. A mechanism for both oxidation and reduction of lumazine is proposed.

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1. Introduction

Pteridines are heterocyclic compounds widespread in biological systems, participating in relevant biological functions [1–4]. Pteridines contain the bicyclic ring system formed by a pyrimidine and a pyrazine, and include pterins and lumazines, Scheme 1. The chemical difference between these two groups of compounds lies in the pyrimidine ring. The participation of pterins in different biological processes has been demonstrated, and so there is interest in clarifying the chemistry of this group of compounds.

Lumazine (LMZ), Scheme 1C, is a pterin compound which acts as substrate for the lumazine/riboflavin synthase complex, an enzyme complex that catalyzes the conversion of two molecules of the LMZ into riboflavin (vitamin B2) [1–3] which, in turn, is the precursor of flavin mononucleotide and flavin adenine dinucleotide, an essential cofactor for a wide variety of redox enzymes [5,6]. From this point of view, the most promising targets for new antimicrobial agents in riboflavin biosynthesis are lumazine synthase and/or riboflavin synthase, both enzymes being involved in the last two steps of the riboflavin pathway [7]. These enzymes have been studied in detail [2] and crystal structures of lumazine synthases have been determined [2,8] providing an excellent basis for antibacterial drug design [9–12].

The coordination chemistry of pteridine derivatives has been a subject of interest for many years, and has been studied from different perspectives [8,10,11,13–17]. A large number of LMZ-metal complexes [14,17,18] has been studied to mimic the environment and reactivity of the metal site of the enzymes. Some of these complexes have been characterized by X-ray crystallography [18] and their biological activity evaluated [7]. Although only a few of these compounds have been the subject of more detailed studies, generally it has been shown that the target for these inhibitors has to be carefully selected in order to avoid unwanted effects. Nevertheless, pterin analogues certainly have the potential to serve as basic structures for the development of novel anti-infectives [7].

On the other hand, in recent years the DNA-probe and antisense approach technology has stimulated the synthesis of various modified oligonucleotides [19–21]. In this context, different LMZ derivatives have been regarded as structural analogues of pyrimidine nucleosides and incorporated into DNA strands [19]. It has been shown that the interaction between LMZ and a complementary base such as adenine, can lead to the formation of stable double strands. The variation of some substituents in the LMZ moiety allowed a broad alteration in its physical properties. The incorporation of 6,7-dimethyl-lumazine on a complementary DNA strand enhanced the binding affinity to adenine and the
selectivity of adenine over thymine, compared to the parent molecule, LMZ [20].

In view of the importance of LMZ and its derivatives in biological systems, several studies have investigated the photochemistry of LMZ under UV-A irradiation in both acidic and alkaline aqueous solutions [4].

The electrochemical behaviour of pterin compounds was studied and the electroactive group is in the pteridine ring. The reduction of pteridine derivatives was studied using a HMDE for its trace determination [24]. However, as the use of HMDE is limited to the negative potential range, the information obtained allowed conclusions only about the reduction of LMZ.

Since LMZ may participate in vivo in several redox reactions, the clarification of its redox mechanisms using electrochemical techniques, namely voltammetric methods, which have the potential for providing valuable insights into the understanding of the role of LMZ in exercising biological activity, seems timely. The present study is concerned with the investigation of the complex LMZ redox processes, using cyclic, square wave and differential pulse voltammetry at a glassy carbon electrode. The oxidation and reduction of LMZ are studied separately.

2. Experimental

2.1. Materials and reagents

Lumazine (LMZ) from Fluka was used without further purification. A stock solution of 2.0 mM LMZ was prepared in purified water from a Millipore Milli-Q system and was stored at 4 °C. All supporting electrolyte solutions, Table 1, were prepared using analytical grade reagents and purified water from a Millipore Milli-Q system (conductivity < 0.1 μs cm⁻¹).

Nitrogen saturated solutions were obtained by bubbling high purity N₂ for a minimum of 10 min in the solution and continuing with a flow of pure gas over the solution during the voltammetric experiments.

Microvolumes were measured using EP-10 and EP-100 Plus Motorized Microliter Pippettes (Rainin Instrument Co. Inc., Woburn, USA). The pH measurements were carried out with a Crison microPH 2001 pH-meter with an indgold 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 µAutolab running with GEPES 4.9 software, Eco-Chemie, The Netherlands. Measurements were carried out using a glassy carbon (GCE) (d = 1.5 mm) working electrode, a Pt wire counter electrode, and a Ag/AgCl as reference, in a 0.5 ml 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 and placed in supporting 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

The DP voltammograms were baseline-corrected using the moving average with a step window of 5 mV included in GEPES 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

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**Table 1**

Supporting electrolytes, 0.1 M ionic strength.

<table>
<thead>
<tr>
<th>pH</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.3</td>
<td>HCl + KCl</td>
</tr>
<tr>
<td>3.1</td>
<td>HAcO + NaAcO</td>
</tr>
<tr>
<td>4.5</td>
<td>HAcO + NaAcO</td>
</tr>
<tr>
<td>5.4</td>
<td>HAcO + NaAcO</td>
</tr>
<tr>
<td>6.1</td>
<td>NaH₂PO₄ + Na₂HPO₄</td>
</tr>
<tr>
<td>7.0</td>
<td>NaH₂PO₄ + Na₂HPO₄</td>
</tr>
<tr>
<td>8.0</td>
<td>NaH₂PO₄ + Na₂HPO₄</td>
</tr>
<tr>
<td>10.3</td>
<td>NH₃ + NH₄Cl</td>
</tr>
<tr>
<td>12.0</td>
<td>NaOH + KCl</td>
</tr>
</tbody>
</table>

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**Scheme 1.** Chemical structures: (A) pteridine, (B) pterine and (C) lumazine.
peak current presented in all graphs were determined from the original untreated voltammograms after subtraction of the baseline.

3. Results and discussion

The voltammetric behaviour of LMZ at a clean GCE was investigated in pH 7.0 0.1 M phosphate buffer between +1.6 V and −1.0 V. The cyclic voltammograms were obtained in a N2-saturated solution of 500 μM LMZ, Fig. 1. During the voltammetric measurements a constant flux of N2 was kept over the solution in order to avoid the diffusion of atmospheric O2 into the LMZ solution.

The scans were started from 0 V going in the positive direction and one main anodic peak 1a was obtained. On the negative-going scan of the first voltammogram, a main reduction peak 2c occurred and after changing the scan direction, three more anodic peaks were observed. Due to the complexity of LMZ redox processes, the oxidation and reduction of LMZ were studied separately.

3.1. Oxidation

3.1.1. Cyclic voltammetry

The oxidation of LMZ was initially studied by CV in a 500 μM LMZ solution in pH 7.0 0.2 M phosphate buffer. On the first voltammogram, scanned from 0.0 V to the positive potential limit of +1.60 V, one main oxidation peak 1a, at \( E_{pa} = +1.49 \) V, was observed, Fig. 2. After changing the scan direction, no reduction peak occurred showing that the oxidation process of LMZ is an irreversible process. The small decrease of LMZ oxidation peak current observed on the second and third voltammograms obtained in the same conditions was due to the adsorption of LMZ oxidation product at the GCE surface. However, the appearance of no additional anodic peaks on recording successive scans showed that the oxidation product of LMZ is not electroactive.

Because the oxidation of LMZ occurs at very high potential values, near the glassy carbon positive potential limit in aqueous solutions [25], it was only possible to study the oxidation of LMZ in pH > 7.0, and a similar behaviour was observed.

3.1.2. Differential pulse voltammetry: pH effect

The pH dependence of LMZ oxidation was investigated in 500 μM LMZ solutions in a pH range from 7.0 to 12.0 using DP voltammetry, Fig. 3. The DP voltammograms showed a single oxidation peak 1a. With the increase in pH, the potential of peak 1a was displaced to less positive values. The dependence was linear over the whole pH range according to the equation: \( E_{pa} = 1.836 - 0.060 \) pH. The slope of 60 mV per pH unit, suggested that the oxidation process of LMZ involved the same number of electrons and protons. Additionally, the width at half-height of peak 1a, \( W_{1/2} = 55 \) mV, close to the theoretical value for the transfer of two electrons enabled the conclusion that the oxidation of LMZ occurs with the transfer of two electrons and two protons. The variation of the LMZ redox peak current with pH is a measure of the variation of the electron transfer rate constant with varying electrolyte solution pH, and always occurs when there is electron and proton transfer.

3.2. Reduction

3.2.1. Cyclic voltammetry

The reduction of LMZ was first studied in N2-saturated in a 200 μM LMZ solution in pH 7.0 0.1 M phosphate buffer. The CVs were recorded from 0.0 V to the negative limit of −1.00 V and the positive potential limit of +0.80 V. On the negative-going scan of the first voltammogram, one main cathodic peak 2c occurred at \( E_{pc} = −0.69 \) V, Fig. 4A.

Changing the scan direction the anodic peak 2a occurred at \( E_{pa} = −0.63 \) V. The potential difference between peaks 2a and 2c is \( \Delta E_{p} = 60 \) mV, and the difference in currents corresponding to a quasi-reversible reaction. However, continuing the scan towards the positive potential limit of +0.80 V, two new oxidation peaks 3a, at \( E_{pa} = +0.01 \) V, and peak 4a, \( E_{pa} = +0.47 \) V, were observed. After recording successive scans in the same solution without cleaning the electrode surface, the currents of all peaks were not changed.

CVs were also obtained at different scan rates in a 200 μM LMZ solution in N2-saturated pH 7.0 0.1 M phosphate buffer, Fig. 4A. Between measurements, the electrode surface was always polished in order to ensure a clean surface to avoid possible problems due to adsorption. Increasing the scan rate, the potential of peak 2a was slightly shifted to more negative values, and \( |E_{pc} - E_{p2c}| = 38 \) mV. Since for a diffusion-controlled irreversible system \( |E_{pc} - E_{p2c}| = 47.7/(n'zF) \) where \( zF \) is the cathodic charge transfer coefficient and \( n' \) the number of electrons in the rate-determining step [25], it can be calculated that \( zF/n' = 1.28 \).
Increasing the scan rate, the current of peak 2c increased linearly with square root of \(v\), consistent with the diffusion-limited reduction of solution species, Fig. 4A. The peak current in amperes for a diffusion-controlled irreversible system is given by

\[ I_{pc}(A) = -2.99 \times 10^6 n^2 (\alpha n)^{1/2} A [R]_{\infty} D_R^{1/2} v^{1/2} \]

where \(n\) is the number of electrons transferred during the reduction, \(A\) is the electrode area in \(\text{cm}^2\), \(D_R\) is the diffusion coefficient in \(\text{s}^{-1}\), \([R]_{\infty}\) is the concentration in \(\text{mol cm}^{-3}\) and \(v\) is in \(\text{V s}^{-1}\) [25]. By plotting \(I_{pc}\) vs. \(v^{1/2}\), considering \(n = 2\) for LMZ in pH 7.0 (see Section 3.2.2), the value of \(D_{LMZ}\) was obtained. The GCE electroactive area of \(A = 0.012 \text{ cm}^2\) was determined as described elsewhere [26]. From the measured slope of 5.06 \(\times 10^{-6} \text{ A/ V s} \text{s}^{1/2}\), the diffusion coefficient of LMZ in pH 7.0 0.1 M phosphate buffer was determined to be \(D_{LMZ} = 9.1 \times 10^{-6} \text{ cm}^2 \text{s}^{-1}\).

Increasing the scan rate, the anodic peak 2a current increased relative to the cathodic peak 2c current, Fig. 4A, as the potential was scanned faster than the rate of oxidation of LMZ reduction product, peak 2a. This experiment showed that LMZ redox reaction will approach reversibility increasing the scan rate.

CVs were also obtained in \(N_2\)-saturated solutions of 200 \(\mu\text{M}\) LMZ at \(v = 100 \text{ mV s}^{-1}\) in supporting electrolytes with different pH values, Fig. 4B. On the CV obtained with the clean GCE in the LMZ solution in pH 4.5 acetate buffer, the pair of peaks 2c–2a occurred at low potentials but the difference between the cathodic and the anodic currents showed the quasi-reversibility of the LMZ reduction. On the positive – going scan of the same CV only one large peak 3a appeared.

In a new experiment carried out in pH 7.0 after cleaning the GCE surface, the pair of peaks 2c–2a was displaced to more negative potentials. Although an increase in the anodic current of peak 2a relative to the cathodic current of peak 2c was observed, the current of peak 2a was still smaller than peak 2c, and on the positive – going scan of the CVs, a new peak 4a occurred at a more positive value then peak 3a.

In another experiment carried out in pH 10.0 after cleaning the GCE surface, the cathodic and anodic currents of peak 2a reached the same value, indicating the reversibility of the LMZ reduction in these conditions, but peaks 3a and 4a did not occur any more.

3.2.2 Differential pulse voltammetry; pH effect

The pH study of LMZ reduction was performed in \(N_2\) saturated in a 10 \(\mu\text{M}\) LMZ solution in a wide pH range from 2 to 12 using DP voltammetry, Fig. 5A.

The potential of peak 2c was shifted to more negative values with increasing the pH, and a linear dependence, according to the equation 

\[ E_{pc}^0 = -0.220 - 0.060 \log_{10} \text{pH} \]

was obtained over the whole pH range, Fig. 5B. The slope of 60 mV per pH unit, suggests that LMZ reduction process involve the same number of electrons and protons. The width at half-height of the peak, \(W_{1/2}\), 50 mV, close to the theoretical value for the transfer of two electrons and allowed to conclude that the reduction of LMZ occurs with the transfer of two electrons and two protons. The peak 2c current was higher in alkaline electrolyte solutions, Fig. 5A and B.
3.2.3. Square wave voltammetry

SW voltammograms were initially obtained in a N2-saturated solution of 10 µM LMZ in pH 4.5 and 5.4 0.1 M acetate buffer, Fig. 6A and B, showing similar features to CV and DP voltammetry. LMZ reduction peak 2c occurred on the first scan at $E_{pc} = 0.52$ V.

Since in SWV the current is sampled in both positive and negative-going pulses, peaks corresponding to the oxidation and reduction of the electroactive species at the electrode surface can be obtained in the same experiment. Thus, by plotting the forward and backward components of the total current, the quasi-reversibility of peak 2c was observed since the backward current showed a smaller value than the corresponding forward one. However, in this situation both the forward and the backward currents occurred at the same potential.

A similar experiment was carried out in the same condition but in different electrolytes. For pH 7.0 were observed two peaks in the total current due to the displacement of the peak potentials obtained for peak 2c in the forward and for peak 2a the backward currents, demonstrating the quasi-reversibility of LMZ reduction.

For higher pH values, pH > 10.3, the peak 2c forward current and peak 2a backward current are equal so the total current doubled, and peaks 2c and 2a occur at the same potential showing that in these conditions the system become completely reversible.

3.3. Analytical determination

Although a DP voltammetry procedure for the analytical determination of LMZ following the reduction peak 2c is more time-consuming, requiring the deaeration of the solutions, it is preferable because the oxidation of LMZ occurs at very high potential values, near the glassy carbon potential window limit in aqueous solutions and with small current peaks. Although in pH 7.0 the reaction is quasi-reversible, DP voltammetry is preferred for the determination of LMZ to SW voltammetry because of the appearance of two consecutive peaks on the SW voltammograms, Fig. 6C.

DP voltammograms, Fig. 7, were recorded for standard additions of LMZ corresponding to bulk concentrations between 0.49 and 10.71 µM LMZ in N2-saturated pH 7.0 0.1 M phosphate buffer.
The electrode surface was polished and conditioned between measurements in order to ensure a clean GCE surface. The dependence of LMZ reduction peak 2c current with concentration was linear for the whole concentration interval studied and expressed by the equation:

$$I_{pc}(A) = \frac{8.50 \times 10^{-3.24}}{C_{LMZ}(M)}$$

($R^2 = 0.995$, $N = 11$, S.D. = $8.28 \times 10^{-5}$).

The sensitivity of the technique for the determination of LMZ was evaluated by calculating the detection limit (LOD) which was determined as the concentration that led to a peak with a height three times the baseline noise level. Using the line parameters of $I_{pc} = I_{pc}(C_{LMZ})$, the detection limit was $LOD_{LMZ} = 0.76 \mu M$. The quantification limit (LOQ) is the lowest concentration of a substance that can be quantified with acceptable precision and accuracy. A typical signal/noise ratio of 10 is generally considered to be acceptable; therefore: $LOQ_{LMZ} = 2.55 \mu M$.

The reproducibility of the method was evaluated by plotting different calibration curves. The relative standard deviations (R.S.D.) calculated from the sensitivities of three calibration curves was around 8%. It must be mentioned that each measurement was done using a freshly polished GCE, a process that cause small modifications of the electrode surface which can in turn cause variations in the current, and this should be the main source of error in the procedures described.

### 3.4. Redox mechanism

The results obtained indicated that LMZ undergoes oxidation in one step as indicated by the appearance of a single oxidation peak in the positive going direction, and reduction in two steps as revealed by the occurrence of two cathodic peaks in the negative going direction, Scheme 2.

The oxidation peak 1a is irreversible, Fig. 2, and corresponds to the electro-oxidation of LMZ (1) to the hydroxy derivative (2) by a mechanism involving two electrons and two protons in the presence of water, Scheme 2A. It has been found that the hydroxy derivative (2) is formed in the enzymatic oxidation of lumazine [27]. The electrochemical oxidation of lumazine is reported here...
for the first time, the mechanism being proposed by comparison with the oxidation mechanism of similar compounds [28].

The reduction of LMZ, reduction peak 2c, involves two electrons and two protons and is attributed to the reduction of the pteridine ring to yield the dihydro derivative (3). The formation of such a product has also been reported in the literature [22–24]. The appearance of the anodic peaks 3a and 4a in the reverse scan corresponds to the two step oxidation of LMZ derivative (3), the product of LMZ reduction formed at the GCE surface, to generate compounds (4) and (5), Scheme 2B. However, as observed by cyclic voltammetry, Fig. 4B, in alkaline solution LMZ reduction became reversible, meaning that the product of LMZ reduction (3) may undergo hydrolysis in solution.

4. Conclusions

The present study has shown that LMZ can be oxidized and reduced at a glassy carbon electrode. The oxidation of LMZ is pH-dependent irreversible process and involves the transfer of two electrons and two protons. The reduction of LMZ is a reversible pH-dependent process involving the transfer of two electrons and two protons. For acid and neutral electrolytes, it was a quasi-reversible process that led to the formation of two reduction products at the electrode surface. Increasing the pH, the reduction of LMZ became a reversible process. Based on the results presented, a redox mechanism of LMZ was proposed.

The diffusion coefficient in pH 7.0 0.1 M phosphate buffer was calculated to be $D_{LMZ} \approx 9.1 \times 10^{-6}$ cm$^2$ s$^{-1}$. The electroanalytical determination of LMZ was carried out in pH 7.0 0.1 M phosphate buffer, using DPV. The investigation of the electrochemical behaviour of LMZ 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 LMZs’ physiological mechanism of action.

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