Electrochemical Redox Behaviour of Temozolomide Using a Glassy Carbon Electrode

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Abstract
The electrochemical behaviour of temozolomide on a glassy carbon electrode has been investigated. The reduction of temozolomide is an irreversible process, pH dependent, and the mechanism involves the addition of one electron and one proton to C5 to form an anion radical, causing the irreversible breakdown of the tetrazinone ring. The oxidation mechanism of temozolomide is an irreversible, adsorption-controlled process, pH dependent up to value close to the pKₐ and occurs in two consecutive charge transfer reactions, with the formation of the hydroxylated product. The electroanalytical determination of TMZ led to a detection limit of 1.1 μM.

Keywords: Temozolomide, Voltammetry, Redox mechanism, Glassy carbon

1. Introduction
Triazene compounds are a group of alkylating agents that include dacarbazine (DTIC), temozolomide (TMZ) and mitozolomide (MZ) (Scheme 1). However, only the first two are presently used in clinical practice, while MZ remains an experimental antitumour drug due to its low tolerability. The active moiety of these compounds is the triazeny1 group, i.e. three adjacent nitrogen atoms responsible for the chemical, physical and antitumour properties of the molecule [1].

TMZ, a 3-methyl analog of MZ, has antitumour activity and a better safety profile in preclinical assessments [2], and was developed as a potential alternative to the more toxic DTIC. They both exert their antitumour activity through the linear triazene, 5-(3-methyltriazen-1-yl)-imidazo-4-carboximide (MTIC) (Scheme 1). DTIC is metabolically converted to MTIC in the liver (N-demethylation), whereas TMZ undergoes chemical degradation to MTIC at physiological pH [3–5]. The cytotoxicity of MTIC is thought to be primarily due to alkylation at the O6 and N7 positions of guanine, and MTIC is converted to 5(4)-aminoimidazole-4(5)-carboxamide (AIC) (Scheme 1) [6–9]. However the exact mechanism of action of TMZ has not been entirely clarified.

Detection and quantification of TMZ in biological fluids is very important. Also, to support pharmacokinetic studies with sufficient speed, suitable analytical procedures are required. High-performance liquid chromatography (HPLC) with UV detection has been developed for stability investigation or analytical determination of TMZ [10–13]. However, more recently, there has been a tendency for the use of less costly techniques and a reduction in the use of organic solvents since they result in high ecological costs.

Scheme 1. Chemical structures of mitozolomide (MZ), temozolomide (TMZ), dacarbazine (DTIC), MTIC and AIC.
Due to their high sensitivity, voltammetric methods have been successfully used for the detection and determination of various pharmaceutical compounds. Moreover, investigation of the redox behaviour of pharmaceutical compounds by means of electrochemical techniques has the potential for providing valuable insights into the redox reactions of these molecules [14–20]. Nevertheless, there are no electrochemical studies of TMZ.

The present paper describes the electrochemical oxidation and reduction mechanisms of TMZ hydrate, for a wide range of solution conditions, using cyclic, differential pulse and square-wave voltammetry, at a glassy carbon electrode. Information on the electrochemical behaviour of TMZ obtained from the results at different pHs may play a crucial role in understanding its properties as well as its metabolism in biological systems.

2. Experimental

2.1. Materials and Reagents

TMZ was obtained from Sigma and used without further purification. A stock solution of 1.00 mM TMZ was prepared in deionised water 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 Pipettes (Rainin Instrument Co. Inc., 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 in 500 μM TMZ buffered solutions at room temperature (25 ± 1 °C).

2.2. Voltammetric Parameters and Electrochemical Cells

Voltammetric experiments were carried out using a μAutolab running with GPES 4.9 software, Metrohm/Autolab, Utrecht, The Netherlands. Measurements were carried out using a glassy carbon electrode (GCE) (d=1.5 mm) working electrode, a Pt wire counter electrode, and an Ag/AgCl (3 M KCl) as reference, in a 1 mL one-compartment electrochemical cell. The experimental conditions for differential pulse (DP) voltammetry were: pulse amplitude 50 mV, pulse width 70 ms, scan rate 5 mV s⁻¹. For square-wave (SW) voltammetry the experimental conditions were frequency 25 Hz and potential increment 2 mV, corresponding to an effective scan rate of 100 mV s⁻¹. The GCE was polished using diamond spray (particle size 1 μm) before every electrochemical assay. After polishing, the electrode was rinsed thoroughly with Milli-Q water. Following this mechanical treatment, the GCE was placed in buffer 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

All DP voltammograms presented were background-subtracted and baseline-corrected using the moving average application with a step window of 2 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 current 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 base line.

3. Results and Discussion

3.1. Cyclic Voltammetry

The electrochemical behaviour of TMZ was investigated by CV, at 100 mV s⁻¹, in N₂-saturated and different pH buffer electrolytes (Figure 1).

In acid media, pH 2.2 0.1 M HCl/KCl, TMZ oxidation occurs in a single step, peak 1a, at Epa = +0.8 V, and on the reverse negative-going scan of the first cycle a well-defined cathodic peak 2 was observed at Epc = −0.65 V (Figure 1A). In the subsequent CV scan the height of both peaks 1a and 2a decreased, and in the positive scan a new anodic peak 2a, at Epa = +0.6 V, appeared.

Due to the complexity of the redox processes occurring at the CGE surface and to obtain more information about the origin of the peak 2a, an experiment was performed using a clean CGE surface scanning only in the positive potential region (not shown) and in successive scans only peak 1a was observed. Thus it was verified that the anodic peak 2a corresponds to the oxidation of the TMZ reduction product formed at the CGE surface after TMZ reduction during the negative scan of the first cycle.

A CV in a solution of TMZ, in pH 7.1 0.1 M phosphate buffer, showed, in the first scan, an anodic peak 1a, at

Table 1. Supporting electrolytes, 0.1 M ionic strength.

<table>
<thead>
<tr>
<th>pH</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2</td>
<td>HCl + KCl</td>
</tr>
<tr>
<td>3.1</td>
<td>HAcO + NaAcO</td>
</tr>
<tr>
<td>4.0</td>
<td>HAcO + NaAcO</td>
</tr>
<tr>
<td>5.2</td>
<td>HAcO + NaAcO</td>
</tr>
<tr>
<td>6.0</td>
<td>NaH₂PO₄ + Na₂HPO₄</td>
</tr>
<tr>
<td>7.1</td>
<td>NaH₂PO₄ + Na₂HPO₄</td>
</tr>
<tr>
<td>8.0</td>
<td>NaH₂PO₄ + Na₂HPO₄</td>
</tr>
<tr>
<td>9.1</td>
<td>NaOH + Na₂B₄O₇</td>
</tr>
<tr>
<td>11.6</td>
<td>NaOH + KCl</td>
</tr>
</tbody>
</table>

In alkaline medium, pH 9.1 0.1 M NaOH/Na₂B₂O₇ buffer, the CV showed that scanning in the negative direction on the first scan no peak was observed in the cathodic region but on the reverse scan the peak 1a at $E_{pc1} = +0.42$ V, and the peak $3_a$ at $E_{pc3} = +0.78$ V, were observed. (Figure 1C). However, on the second scan in the negative direction, a new reduction peak 4, appeared at $E_{pc4} = -0.19$ V. This peak 4 corresponds to the reduction of the TMZ oxidation product formed at the GCE surface. The reverse scan showed a new oxidation peak $4_a$ at $E_{pa4} = -0.10$ V, thus confirming the reversibility of peak $4_c$.

Nevertheless, the CV results showed that the oxidation and reduction mechanisms of TMZ occur independently of each other so they were investigated separately.

### 3.2. Reduction

#### 3.2.1. Cyclic Voltmetry

Since the adsorption of TMZ and/or its reduction product is greater in acid electrolytes, further studies of the reduction of TMZ were carried out in pH 7.1 0.1 M phosphate buffer N₂-saturated solutions. CVs showed only one reduction peak $2_c$ at $E_{pc2} = -0.91$ V (Figure 1B). Scanning in the positive direction, no oxidation peak was observed, showing that the reduction of TMZ in pH 7.1 is an irreversible process.

CVs were also obtained for different scan rates, and with increasing scan rate peak $2_c$ potential was slightly shifted to more negative values (Figure 2), in agreement with the irreversible nature of the electrochemical process for TMZ. The difference between peak potential $E_{pc}$ and the potential at half height of peak $E_{p/2c}$ was $j = 47.7/\alpha c n'$ where $\alpha c$ is the charge transfer coefficient and $n'$ the number of electrons in the rate-determining step [21], it can be calculated that $\alpha c n' = 0.72$.

Increasing the scan rate, the current of peak $2_c$ increased linearly with the square root of scan rate (not shown), consistent with the diffusion-limited reduction of a solution species. The peak current in amperes for a diffusion-controlled irreversible system is given by $I_{pc} (A) = -2.99 \times 10^5 n (\alpha c n')^{1/2} A C O v^{1/2}$ where $n$ is the number of electrons transferred during the reduction of TMZ ($n = 1$ as shown in Section 3.2.2), $A$ is the electrode area in cm², $D_O$ is the diffusion coefficient in cm² s⁻¹, $C_O$ is the concentration in mol cm⁻³ and $v$ is in V s⁻¹ [21]. By plotting $I_{pc}$ vs. $v^{1/2}$, the value of $D_O$ is obtained.

For the measured slope of $6.0 \times 10^{-6}$ A/(V s⁻¹)² the diffusion coefficient of TMZ in pH 7.1 0.1 M phosphate buffer is $D_O = 1.8 \times 10^{-5}$ cm² s⁻¹. For this calculation, the GCE electroactive area was determined from a plot of $I_{pc}$ vs. $v^{1/2}$ using a solution of 0.5 mM hexacyanoferrate(III) in phosphate buffer and the value of the diffusion coefficient of hexacyanoferrate(III) of $D_{HCF} = 7.35 \times 10^{-6}$ cm² s⁻¹.

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**Fig. 1.** CVs of 500 µM TMZ in N₂-saturated solutions, (—) first and (-----) second scans: (A) pH 2.2, (B) pH 7.1 and (C) pH 9.1, $v = 100$ mV s⁻¹.
An electroactive area of 0.0111 cm² for the glassy carbon electrode was determined.

3.2.2. Differential Pulse Voltammetry

The effect of pH on the electrochemical reduction of TMZ was studied over a wide pH range between 2 and 12 using DP voltammetry. The DP voltammograms were recorded in different electrolytes with 0.1 M ionic strength (Figure 3A and Table 1) with a constant flux of N₂ in order to avoid the diffusion of atmospheric oxygen into the solution of TMZ.

The peak 2 c occurs only in electrolytes with pH < 8 and the potential of peak 2 c is shifted to more negative values with increasing pH. The dependence is linear over the whole pH range between 2 and 8 (Figure 3A). The slope of the dotted line, 59 mV per pH unit, shows that the same number of electrons and protons is involved in the reduction mechanism of TMZ (Figure 3B). The width at half-height of peak 2 c is \( W_{1/2} \approx 100 \text{ mV} \), showing, as does CV, that the reduction of TMZ occurs with one electron and one proton transfer. The variation of peak 2 c current versus pH shows that the highest current is obtained at pH 2.2 (Figure 3B). The cathodic peak 2 c, involves the addition of one electron and one proton to C5 to form an anion-radical, causing the irreversible breakdown of the tetrazinone ring. A mechanism for the reduction of TMZ in acid media is proposed (Scheme 2).

3.3. Oxidation

3.3.1. Cyclic Voltammetry

The oxidation of TMZ at a GCE was studied in pH 7.1 0.1 M phosphate buffer. The CVs, obtained at a scan rate \( v = 100 \text{ mV s}^{-1} \), show two well-separated consecutive oxidation peaks (Figure 1B). Both peak 1 a, at \( E_{pa} = +0.54 \text{ V} \), and peak 3 a, at \( E_{pa} = +0.90 \text{ V} \), are irreversible. The second scan shows a decrease of peak 1 a current and the absence of peak 3 a due to the adsorption of TMZ and oxidation products on the GCE surface.

CVs were also obtained for different scan rates (not shown). Increasing the scan rate, the peak 1 a current also increases, but there is not a linear relationship between \( I_{pa} \) of peak 1 a and the square root of the scan rate, as expected for an irreversible diffusion-controlled oxidation process.
process. This is explained taking into consideration the adsorption of TMZ molecules and/or their oxidation products on the GCE surface.

3.3.2. Square-Wave Voltammetry

The SW voltammograms showed similar features to CV, i.e., oxidation peaks 1_a and 3_a in the first scan and the appearance of new peak 4_a corresponding to the oxidation products absorbed on the electrode surface in the second scan (Figure 4A). The irreversibility of both peaks 1_a and 3_a was confirmed by plotting the forward and backward components of the total current obtained on the first scan (Figure 4B). Whereas the forward component showed both peaks at similar potentials as found by DP voltammetry and the same total current was obtained, the backward component showed no cathodic peak. On the second consecutive SW voltammogram, the formation of TMZ reversible oxidation product, peak 4_a, was confirmed by plotting the forward and backward components of the total current (Figure 4B), showing equal oxidation and the reduction currents. Moreover, the identical value for the potential of peak 4_a, on the forward and backward current components, confirms the adsorption of TMZ oxidation products on the GCE surface.

3.3.3. Differential Pulse Voltammetry

The DP voltammograms showed that only one oxidation reaction, peak 1_a, occurs in all supporting electrolytes, and the effect of pH on the electrochemical oxidation of TMZ was studied over a wide pH range between 2 and 12 (Figure 5A).

For buffer solutions with $2 < \text{pH} < 12$, the potential of peak 1_a was displaced to more negative values with increasing pH (Figure 5B). The slope of the dotted line, $59 \text{ mV per pH unit}$, shows that the mechanism of this oxidation process in aqueous media involves the same number of electrons and protons. The number of electrons transferred, $n$, was determined by the peak width at half height $W_{1/2}$, which is close to the theoretical value of $90 \text{ mV}$, corresponding to an electrochemical reaction involving the transfer of one electron. Consequently, it can be concluded that the oxidation process occurs with the transfer of one electron and one proton.

However, for $\text{pH} > 5$ a new peak 3_a appeared (Figure 5A). The oxidation potential of the peak 3_a is also pH dependent between 6 and 10 and its potential decreased with increasing pH (Figure 5B). The slope of the dotted line, $59 \text{ mV per pH unit}$, shows that the mechanism of this second oxidation process in aqueous media also involves the same number of electrons and protons. Taking into consideration that the width at half height of TMZ oxidation peak 3_a was $W_{1/2} \approx 90 \text{ mV}$, meaning that the oxidation process involves the transfer of one electron and one proton. The variation of peaks 1_a and 3_a current versus pH shows that the current increases with the pH with a maximum for $7.0 < \text{pH} < 10.0$ (Figure 5B).

For $\text{pH} > 9$, the oxidation peak 1_a does not depend on pH indicating a mechanism involving only one electron, the TMZ oxidation product can undergo chemical deprotonation in alkaline electrolytes [23], and the TMZ $pK_a \approx 9$ determined (Figure 5B).

Successful DP voltammograms were recorded in different pH buffer electrolytes (Figure 6). In the first scan in pH 7.1 only the peaks 1_a and 3_a occurred, but in the second scan a new peak 4_a appeared and the peak 1_a potential was positively shifted. Also the peaks 1_a and 3_a current decreased significantly with the number of scans, in the first scan in alkaline media, pH 9.1 (Figure 6B) the two-oxidation peaks 1_a and 3_a appeared, but in the second scan a new peak 4_a appeared and the peak 1_a potential was positively shifted. Also the peak 1_a and 3_a current decreased significantly with the number of scans,
which shows the strong adsorption of the oxidation product of TMZ on the surface of the electrode at this pH.

Successive DP voltammograms showed that the adsorption of TMZ oxidation product in alkaline electrolyte is stronger than in acid media. This was observed when after recording several successive scans in the TMZ solution, the electrode was washed with a jet of deionized water and then transferred to the supporting electrolyte where only oxidation peak 4 was observed.

Based on the results in the first step, peak 1, one electron and one proton are removed from the tetrazine ring following the direct nucleophilic attack by water with the production of the hydroxylated product, causing the irreversible breakdown of the tetrazine ring. In the second step, peak 3, one electron and one proton are removed from the nitrogen in the already opened tetrazine ring. A mechanism for the oxidation of TMZ in acid media is proposed (Scheme 3).

### 3.3.4. Analytical Determination

For the analytical determination of TMZ, oxidation peak 1 was chosen, since it occurs at a low positive potential. A plot of peak current of peak 1 vs. TMZ concentration, in the range 0 to 13 μM TMZ, in pH 7.1 0.1 M phosphate buffer is shown in Figure 6C. For each concentration three measurements were performed and after each measurement the electrode surface was thoroughly rinsed with deionized water. Good linearity was found between peak current and concentration described by the equation:

\[ I_{pa} (\text{nA}) = 10.2 \left[ \frac{\text{TMZ}}{\mu \text{M}} \right] + 2.9 \]

where \( r = 0.997, n = 7, P < 0.0001 \). Others parameters to define the sensitivity were calculated and the value obtained for the limit of detection, \( LOD = 1.1 \mu \text{M} \), was based on three times the noise level, and the limit of quantification was \( LOQ = 3.7 \mu \text{M} \), based on ten times the noise level. The electroanalytical determination of TMZ in biological fluids is foreseen and will provide very important and useful data for clinicians.

### 4. Conclusions

This study shows that TMZ, a chemotherapeutic agent for the treatment of brain tumors, undergoes oxidation and reduction at a CGE. The electrochemical behavior of TMZ was investigated by cyclic, differential pulse and square wave voltammetry over a wide pH range. The reduction of TMZ is an irreversible, diffusion-controlled, pH dependent process and occurs in a single step, in one electron and one proton transfer, leading to the formation of a non-electroactive reduction product. The oxidation of TMZ is also an irreversible process, pH dependent and occurs in one step for electrolytes with pH < 5.2, whereas in solutions with higher pH two oxidation peaks were observed corresponding to consecutive charge transfer reactions with the formation of a main oxidation product which adsorbs strongly at the GCE surface in alkaline solutions and undergoes reversible oxidation, blocking the electrode surface. The electroanalytical determination of TMZ led to a detection limit of 1.1 μM.

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Fig. 6. DP voltammograms recorded in 500 μM TMZ: (A) pH 7.1 0.1 M phosphate buffer and (B) pH 9.1 0.1 M NaOH/Na₂B₂O₇ buffer; (—) first and (----) second and (·····) third scans. (C) 1.96, 2.91, 4.76, 6.54, 8.26, 9.90 and 13.0 μM TMZ in pH 7.1 0.1 M phosphate buffer.

Scheme 3. Proposed oxidation mechanism of TMZ in acidic medium.

References


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