Cyclic Lipopeptide Antibiotic Daptomycin Electrochemical Oxidation at a Glassy Carbon Electrode

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Abstract: Daptomycin was the first approved drug from a new class of antimicrobials, the cyclic lipopeptides, and presents a broad spectrum of activity against a wide range of gram-positive bacteria. The daptomycin redox behaviour, by cyclic, differential pulse and square wave voltammetry, in a wide pH range, at a glassy carbon electrode, was investigated. The daptomycin oxidation was a two-step irreversible diffusion-controlled process and the diffusion coefficient \( D_{DPT} = 2.32 \times 10^{-5} \, \text{cm}^2 \text{s}^{-1} \), was calculated. A detection limit \( \text{LOD} = 0.32 \, \mu \text{M} \), was obtained. For the first time daptomycin, in fetal bovine serum biological fluid, using DP voltammetry, was determined.

Keywords: Daptomycin · lipopeptide · antibiotic · electrochemical oxidation · glassy carbon electrode.

1 Introduction

Antimicrobial resistance (AMR) can be characterized as the ability of a microorganism (such as bacteria, fungi, viruses and some parasites) to change when it is exposed, or to stop an antimicrobial drug (such as antibiotics, antifungals, antivirals, antimalarials and anthelmintics) from working against it. As a result, standard treatments become ineffective; infections persist in the body and may increase the risk of other infections spreading [1].

The microorganism’s new resistance mechanisms have been emerging and spreading globally, which threatens the ability to treat common infectious diseases, resulting in prolonged illness, disability, and death. In this context, the development of new drugs with the ability to bypass these resistance mechanisms represents an important advance in clinical practice.

Among these new antimicrobial drugs, more closely related to the antibiotic candidate groups, cyclic lipopeptides (CLPs) are a promising class of natural products with potent antibacterial activity. CLPs are composed of a lipid tail linked to a short oligopeptide which is cyclized to form a lactone or lactam ring, either between two amino acids in the peptide chain or between an amino acid and an amino- or hydroxyl-group bearing a fatty acid moiety [2].

Daptomycin (DPT) (N-decanoil-L-tryptophyl-L-asparaginyl-L-aspartyl-L-threonylglycyl-L-ornithyl-L-aspartyl-D-alanyl-L-aspartylglycyl-D-seroyl-threo-3-methyl-L-glutamyl-3-antraniloyl-L-alanine-\( \epsilon \)-lactone), was the first macrocyclic lipopeptide antibiotic to be approved, after intensive clinical trials by the U.S. Food and Drug Administration (FDA) in 2003, and is probably among the most important antibiotics developed in the past 50 years [3–5]. It is specifically indicated for the treatment of complicated skin and skin structure infections, right-sided endocarditis and bacteremia caused by susceptible strains of Gram-positive microorganisms [6], such as Staphylococcus aureus (including methicillin-resistant strains), Streptococcus pyogenes, S. agalactiae, S. dysgalactiae subspecies equisimilis and Enterococcus faecalis (vancomycin-susceptible strains only) [7–9].

The DPT structure, Scheme 1, is composed of a peptide moiety containing 13 D- and L- amino acids, only three of these being with D-stereochemistry. The cyclic peptide core is composed of a set of 10 non-proteinogenic amino acid residues, which include three D-amino acids (D-asparagine, D-alanine and D-serine), three less common amino acids (ornithine, (2S,3R)-3-methylglutamic acid, and kynurenine), that form an ester bond with threonine and build up the macrolactone ring, and an \( n \)-decanoyl fatty acid chain linked at the N-terminus to tryptophan, the only electroactive amino acid residue [4, 10]. In addition, it has four acid residues (three aspartic acid residues and one 3-methyl glutamic acid residue) and a basic residue (ornithine) [11].

Although DPT has been approved in more than 40 countries worldwide, few studies report the development of DPT analytical methods. Most DPT quantification and detection studies are directed towards biological fluid analysis, using mainly chromatographic methods with different detectors, such as high performance liquid chromatography with ultraviolet [12–16], mass [15, 17] and tandem mass spectrometry [18], liquid chromatography with ultraviolet [19], mass [20] and tandem mass spectrometry [21, 22], ultra and ultra-high performance liquid chromatography with ultraviolet [23–25], tandem mass spectrometry [26] and photodiode array [27]. Some other techniques have also been investigated [28, 29].
These analytical methods mainly employ techniques that require toxic solvents, or high volumes of solvents, for drug quantification, which are expensive, and unhealthy, for the laboratory staff, community, and environment. To minimize these problems and achieve good sensitivity and selectivity, the possibility of using electrochemical techniques was investigated.

The quantification and detection of the DPT oxidation mechanisms, using electrochemical techniques is important, since it will result in a better understanding and increase knowledge concerning DPT physiological mechanisms of action.

The aim of this work was to investigate the electrochemical behaviour and the electron transfer mechanism of DPT, using cyclic, differential pulse and square wave voltammetry, at a glassy carbon electrode, and to determine, for the first time, DPT in fetal bovine serum biological fluid.

2 Experimental
2.1 Materials and Reagents

Daptomycin (Dapcin (DPT)) (D2446, purity ≥ 90%), tryptophan (Trp) (T3300, purity ≥ 99%), fetal bovine serum (F0804, Research Grade), urea (U5378, for molecular biology), sodium dodecyl sulfate (SDS) (L3771, purity ≥ 98.5%) and DL-dithiothreitol (DTT) (D0632, purity 98%) were obtained from Sigma-Aldrich, Spain, and used without further purification.

The 0.3 mM DPT stock solution was prepared in 0.1 M phosphate buffer solution pH 7.0, and frozen at −20 °C, and after thawing was never used for more than one day. This procedure was in agreement with the long term storage Sigma-Aldrich Production Information suggestion for DPT storage, and ensured that the drug activity was maintained. The stock solution of 1.0 mM Trp was prepared in 0.1 M phosphate buffer pH 7.0. Solutions of different concentrations were prepared by dilution in the supporting electrolyte.

The supporting electrolyte solutions, with ionic strength I = 0.1 M, of different pH composition: pH 3.5 (HOAc + NaOAc), pH 4.2 (HOAc + NaOAc), pH 5.3 (HOAc + NaOAc), pH 6.0 (NaH₂PO₄ + Na₂HPO₄), pH 7.0 (NaH₂PO₄ + Na₂HPO₄), pH 8.2 (NaH₂PO₄ + Na₂HPO₄), pH 9.2 (NaOH + Na₂B₄O₇), pH 10.2 (NaOH + Na₂HPO₄), using analytical grade reagents, and purified water from a Millipore Milli-Q system (conductivity ≤ 0.1 µS cm⁻¹), were prepared [26].

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 Ingold combined glass electrode.

All experiments were performed at room temperature, T = 298 K (25 °C).

2.2 Voltammetric Parameters and Electrochemical Cells

Voltammetric experiments were carried out using a µAutolab, running with GPES 4.9 software, Metrohm/ Autolab, The Netherlands. The measurements were carried out using a three-electrode system in a 2 mL one compartment electrochemical cell (eDAQ, Europe). Measurements were carried out using a glassy carbon electrode (GCE) (d = 1.5 mm) as working electrode, a Pt wire as counter electrode, and an Ag/AgCl (3 M KCl) as reference electrode.

The experimental conditions for cyclic voltammetry (CV) were obtained using a potential increment of 2 mV at variable scan rates. For differential pulse (DP) voltammetry pulse amplitude 50 mV, pulse width 100 ms, step potential 2 mV, and scan rate 5 mV s⁻¹, were used. For square wave (SW) voltammetry, pulse amplitude 50 mV, frequency 13 Hz, and potential increment 2 mV, corresponding to an effective scan rate of 25 mV s⁻¹, were used.

Prior to use, the GCE was washed with ethanol and Milli-Q water, and the surface was polished using diamond spray (particle size 3 µm) (Kemet, UK). After polishing, it was thoroughly rinsed with Milli-Q water. Following this mechanical pre-treatment, the GCE was placed in the appropriate buffer supporting electrolyte and cyclic voltammograms were recorded between the potential limits of E₁ = 0.0 V and E₂ = + 1.3 V, until a stable signal was obtained (3–5 cycles at a potential scan rate of 50 mV s⁻¹). This procedure was performed before each electrochemical experiment and ensured very reproducible experimental results.

2.3 Acquisition and Presentation of Data

All the voltammograms presented were background-subtracted and baseline-corrected using the moving average with a step window of 5 mV included in GPES version 4.9 software. This mathematical treatment improved 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.

2.4 Sample Preparation for DPT Determination in Biological Fluid

The DPT determination was investigated in fetal bovine serum biological fluid by addition/recovery experiments. The samples were deliberately contaminated with 2.00 × 10⁻³ mol L⁻¹ DPT. The measurements were made in 0.1 M phosphate buffer solution pH 7.0, using DP voltammetry, in the potential range from 0.4 to 0.8 V. After recording DP voltammograms in the absence of DPT, an aliquot of the spiked sample was added to the electrochemical cell to give a final concentration of 2.00 × 10⁻⁶ mol L⁻¹ DPT and then was directly analysed. This measurement were performed in triplicate, and the GCE was always cleaned.

3 Results and Discussion

3.1 Cyclic Voltammetry

The electrochemical behaviour of DPT, at GCE, was first studied using CV, in 0.1 M acetate buffer solution pH 4.0, and 0.1 M phosphate buffer solution pH 7.0, using a scan rate ν = 25 mV s⁻¹. The CVs showed two oxidation peaks, the first a well-defined, oxidation peak P₁, at $E_{pa1} = +0.85$ V for pH 4.0, Figure 1, and at $E_{pa1} = +0.74$ V for pH 7.0, and the second a smaller oxidation peak P₂, at $E_{pa2} = +0.85$ V for pH 4.0, and at $E_{pa2} = +1.05$ V for pH 7.0. Reversing the scan direction, no reduction peak appeared for either pH, indicating that the oxidation process is irreversible [29]. Recording successive scans in the same solution, without cleaning the electrode surface, both peak currents decreased, due to the adsorption of DPT and/or its oxidation products at the GCE surface, Figure 1.

The scan rate effect on the oxidation process of DPT oxidation peak P₁ was investigated recording CVs, in 25 µM DPT, in 0.1 M phosphate buffer pH 7.0, at scan rates between 5–50 mVs⁻¹, always using a clean GCE. The oxidation peak P₁ current, $I_{pa} (A) = 2.69 \times 10^{-5} (a_n')^{1/2} A [R] D_{O}^{1/2} v^{1/2}$, increased linearly with $v^{1/2}$, indicating that the oxidation process was diffusion-limited [29] and the value of the DPT diffusion coefficient in 0.1 M phosphate buffer pH 7.0, was calculated as $D_{DPT} = 2.32 \times 10^{-5}$ cm² s⁻¹. The GCE electroactive area, $A = 4.17 \times 10^{-3}$ cm², was determined using 1.0 mM hexacyanoferrate(II) in phosphate buffer solution ($D_O = 7.35 \times 10^{-6}$ cm² s⁻¹).

3.2 Differential Pulse Voltammetry

The effect of pH on the electrochemical oxidation of DPT at GCE was investigated, over a wide pH range. The DP voltammograms, in 25 µM DPT solutions, always using a clean GCE, were recorded, Figure 2.

The DP voltammograms presented two oxidation peaks, P₁ and P₂, for all values of pH investigated. For 3.2 < pH < 10.2, DPT oxidation peaks P₁ and P₂ are pH-dependent, and for pH > 10.2 both oxidation peaks become pH-independent, in agreement with a one electron and one proton transfer mechanism in the tryptophan...
(Trp) amino acid residue [35], in the DPT structure, see Section 3.5.

3.3 Square Wave Voltammetry

In SW voltammetry the current is sampled in both positive and negative-going pulses, the electroactive compound oxidation and reduction peaks, at the electrode surface, are obtained in the same experiment. SW voltammetry permits investigation of the reversibility of the electron transfer reactions by plotting the forward and backward components of the total current [30].

The SW voltammograms, in 25 μM DPT, in 0.1 M phosphate buffer pH 7.0, at a clean GCE surface, were obtained, Figure 3.

Similarly to CV and DP voltammetry, two consecutive electrode reactions were observed. Plotting the forward (I_f) and backward (I_b) components of the total (I_t) current, the irreversibility of the two redox peaks was confirmed.

3.4 Effect of Denaturing Agents on DPT Oxidation

The denaturing process of peptides causes morphological changes to the 3D structure, which unfold on the electrode surface, and the electroactive amino acid residues' oxidation becomes easier.

As denaturing agent, urea affects the secondary and tertiary structure without affecting the primary structure, the surfactant SDS dissolves hydrophobic proteins, and the reducing agent DTT disrupts disulphide bonds, which are essential for qualitative and quantitative analysis in proteomic mechanism investigation [31]. Urea and SDS are not electroactive, unlike DTT, which is electroactive [32].

The effect of the denaturing agents on the DPT electrochemical oxidation was followed by DP voltammetry comparing the changes in the DPT electrochemical behaviour before and after the interaction with each denaturing agent. For each experiment, 25 μM DPT was separately incubated with 25 μM urea, SDS or DTT, for 3 hours, and DP voltammograms, always at a clean GCE, in 0.1 M phosphate buffer solution pH 7.0, were recorded. In the DPT solution incubated with urea, no oxidation peak was observed. The oxidation of DPT incubated with SDS or DTT, showed the DPT oxidation peak.

3.5 Tryptophan Redox Behaviour

The tryptophan (Trp) amino acid residue in the DPT structure is the only amino acid residue that can be oxidized in aqueous solution [35].

Therefore, in order to clarify the DPT oxidation mechanism, for the same experimental conditions, the Trp redox behaviour was also investigated. DP voltammograms, in 25 μM DPT and Trp, at a clean GCE, in 0.1 M phosphate buffer solution pH 7.0, were recorded, Figure 4.

In the DP voltammograms, two oxidation peaks for Trp, at E_{pa1} = +0.61 V and E_{pa2} = +1.00 V, and similarly for DPT, at E_{pa1} = +0.68 V and E_{pa2} = +0.91 V, were observed, Figure 4. The shift of the DPT oxidation peak to higher potentials, compared with Trp, is explained taking...
into consideration the size of the molecule, the 3D configuration, as well as the DPT spatial orientation on the electrode surface, making the access of the Trp amino acid residue for oxidation more difficult.

### 3.6 Daptomycin Standards

The electroanalytical determination of DPT standards, by DP voltammetry, measuring the DPT oxidation peak P1 current, after standard additions of DPT, corresponding to bulk concentrations between 0.5 and 25 μM, was carried out, Figure 5. Each standard measurement was always performed using a clean GCE surface, after mechanical and electrochemical pre-treatment, a process that gave rise to small changes in the GCE surface area, which can in turn cause small variations in the peak currents measured.

![Fig. 5. DP voltammograms baseline corrected, at GCE, in 0.1 M phosphate buffer pH 7.0, for 0.5, 1.0, 2.5, 5.0, 7.5, 10.0, 15.0 and 25.0 μM [DPT].](image)

From the data of three calibration curves, the values of $R^2 = 0.993$, in the linear range (0.5–7.5 μM DPT, $n = 5$), following the equation $I_{pa} (\text{nA}) = 2.63 \times (\text{nM}) + 1.15 \times (\text{nA})$, and a relative standard deviation (R.S.D.) less than 7 nA, were calculated. The results are summarized in Table 1.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear Range</td>
<td>0.5–7.5 μM</td>
</tr>
<tr>
<td>Sensibility</td>
<td>2.63 nA/μM</td>
</tr>
<tr>
<td>Intercept</td>
<td>1.15 nA</td>
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<tr>
<td>Limit of Detection</td>
<td>0.32 μM</td>
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<tr>
<td>Limit of Quantification</td>
<td>1.06 μM</td>
</tr>
<tr>
<td>SD</td>
<td>0.28 nA</td>
</tr>
<tr>
<td>$R^2$</td>
<td>0.993</td>
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</table>

The DPT detection limit (LOD) was determined using the equation $\text{LOD} = 3 \times \text{SD} \times (\text{sensitivity})^{-1}$, where SD is the standard deviation of the peak current, for DP voltammetry, and the sensitivity is the slope of the calibration plot. The quantification limit (LOQ), the lowest concentration that can be quantified with acceptable precision and accuracy, is given by $\text{LOQ} = 10 \times \text{SD} \times (\text{sensitivity})^{-1}$. A LOD = 0.32 μM and LOQ = 1.06 μM, were determined.

### 3.7 Daptomycin Determination in a Biological Fluid

For the quantification of DPT in fetal bovine serum biological fluid, by DP voltammetry, the addition/recovery method was used. The fetal bovine serum biological fluid was spiked with $2.00 \times 10^{-4} \text{ mol L}^{-1}$ DPT, and was diluted directly into the electrochemical cell, to a final concentration of $2.00 \times 10^{-4} \text{ mol L}^{-1}$ DPT. The DP voltammogram, in 0.1 M phosphate buffer pH 7.0, measuring DPT peak P1 oxidation current, was recorded.

The matrix effect was evaluated, and showed no significant interferences, since a concentration of $1.99 \pm 0.09 \times 10^{-6} \text{ mol L}^{-1}$ DPT and an average recovery value of 99.5%, were determined.

### 3.8 Discussion

The electrochemical behaviour of DPT at GCE, in a wide electrolyte pH range, using CV, DP and SW voltammetry, was investigated. The DPT oxidation occurred in a two-step mechanism. For $3.2 < \text{pH} < 10.2$ both DPT anodic reactions, oxidation peaks P1 and P2, were pH-dependent.

Among the 13 amino acids present in the DPT composition, only Trp amino acid is electroactive, in aqueous solution. The Trp oxidation occurred in two oxidation steps, similarly to DPT, but for less positive potentials. The higher oxidation peak potentials, of Trp amino acid residue in the DPT, are explained by the macrocyclic lipopeptide 3D structure, Scheme 1, making the access of the Trp amino acid residue to the GCE surface more difficult, thence the higher DPT oxidation peak potentials.

The Trp amino acid is an indole derivative substituted at the C3 position, and the DPT electrochemical oxidation, in aqueous solution, occurs at the Trp amino acid residue. Considering the fact that the pyrrole ring of indoles is more reactive than the benzene ring, the first oxidation reaction, oxidation peak P1, corresponds to oxidation at the C2 position on the pyrrole ring [33–35], since all the indole derivatives, such as DPT-1 are substituted at the C3 position. The second oxidation reaction, oxidation peak P2, of the indole derivatives with a substituent at the C3 position, corresponds to one electron being removed from DPT-2, followed by deprotonation, and the direct nucleophilic attack by water, with the hydroxylation of C7 of benzene moiety, DPT-3. The proposed DPT oxidation mechanism is presented in Scheme 2.
The electroanalytical determination of standards carried out by DP voltammetry, measuring the first DPT oxidation peak P1 current, gave a LOD = 0.32 μM and a LOQ = 1.06 μM. This electrochemical technique shows lower detection and quantification limits than other methods [17, 24, 25].

The electroanalytical quantification of DPT, for the first time, in fetal bovine serum biological fluid, by the addition/recovery method, with high efficiency and sensitivity, was performed. The final concentration of 1.99 ± 0.09 × 10⁻⁶ mol L⁻¹ DPT, by DP voltammetry, was found in fetal bovine serum biological fluid, in agreement with the DPT concentration added, showing that DPT electroanalysis has great potential, with high efficiency and sensitivity, to be applied in the quantification of DPT in real biological samples.

4 Conclusions

The electrochemical oxidation of daptomycin, the first approved drug from a new class of macrocyclic lipopeptide antibiotics, at GCE, by CV, DP and SW voltammetry, in a wide pH range, was studied. The DPT oxidation is a diffusion-controlled process, occurring in two irreversible pH-dependent steps, and a mechanism was proposed.

The electroanalytical quantification of DPT, in fetal bovine serum biological fluids, by the addition-recovery method, was performed. The novel DP voltammetric method, at GCE, is simple, fast, inexpensive, highly sensitive, and an excellent option for DPT cyclic lipopeptide antibiotic quantification in biological fluids.

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