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Electrochemical Profiling of a Promising Fused Pyrimidine-Triazole Drug Candidate

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Abstract: In this study, a novel triazolopyrimidinone derivative, 5-(Chloromethyl)-2-(2-chlorophenyl)-[1,2,4]triazolo[1,5-a]pyrimidin-7(3H)-one, abbreviated CMCP, was synthesized as a viable therapeutic candidate. Using electrochemical techniques such as Differential Pulse Voltammetry (DPV) and Cyclic Voltammetry (CV), we investigated its electrochemical characteristics and interactions with both single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA). We explored the experimental parameters, including pH, concentration and scan rate to provide best analytical results. Our methodology involves analyzing changes in electrochemical signals, specifically the peak current of guanine bases, before and after interactions with the drug. Furthermore, stability tests were performed on CMCP over several days. The results unveiled noteworthy changes in guanine bases following CMCP interactions with both ssDNA and dsDNA, underscoring the potential influence of this compound on DNA structure.

İlaç Adayı olarak Yeni Sentezlenen Pirimidin- Triazole Yapılı Bir Bileşiğin Elektrokimyasal Yapısının İncelenmesi

Anahtar Kelimeler
İlaç Adayı,
Kalem Grafit Elektrot,
İlaç-DNA Etkileşimi,
Triazolopirimidinon

Öz: Bu çalışmada, umut vadeden yeni bir ilaç adayı olarak triazolopirimidinone türevi olan 5-(klorometil)-2-(2-klorofenil)-[1,2,4]triazolo[1,5-a]pirimidin-7(*3H*)-on (kısaltılmış olarak CMCP) sentezlenmiştir. Diferansiyel Puls Voltametri (DPV) ve Döngüsel Voltametri (CV) gibi elektrokimyasal teknikler kullanılarak, elektrokimyasal özellikleri, ayrıca tek sarmal DNA (ssDNA) ve çift sarmal DNA (dsDNA) ile etkileşimleri araştırılmıştır. Optimum sonuçlar sağlamak için pH, konsantrasyon ve tarama hızı gibi çeşitli deneysel parametreler araştırılmıştır. Çalışmada, ilaçla etkileşimden önce ve sonra guanin bazlarının pik akımı gibi elektrokimyasal sinyallerdeki değişiklikleri analiz edilmiştir. Ayrıca, CMCP üzerinde birkaç gün boyunca kararlılık testleri yapılmıştır. Sonuçlar, CMCP'nin hem ssDNA hem de dsDNA ile etkileşiminden sonra guanin bazlarında dikkat çekici değişiklikler olduğunu ortaya koymuştur ve bu bileşiğin DNA yapısı üzerindeki potansiyel etkisini vurgulamaktadır. Bu deneysel bulgular, CMCP'nin DNA üzerindeki etkisi nedeniyle ilerideki ilaç geliştirme çalışmaları için umut vadeden bir aday olduğu görüşünü desteklemektedir.

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1. Introduction

Heterocyclic compounds, a versatile class of organic compounds, have attracted considerable attention due to their significant impact on the field of chemistry and their wide-ranging applications [1,2]. The most prevalent heterocycles typically consist of five- or six-membered rings and incorporate heteroatoms such as nitrogen (N), oxygen (O), or sulphur (S) [3]. Nitrogen-containing heterocyclic compounds are a key component of many natural products, drug molecules, and organic functional materials [4]. Triazolopyrimidinone, a fused pyrimidinone-triazole heterocyclic compound, has been the subject of extensive research in various fields. These compounds have exhibited a broad spectrum of activities, encompassing antibacterial [5], antifungal [6], antiviral [7], anticancer [8] and antimicrobial agents [9]. They also highlight the potential of triazolopyrimidinones to be used as starting points for the development of novel drugs for the treatment of these and other diseases [10-12].

DNA is the most popular pharmacological target of many drugs because it is essential for all cellular processes [13]. The interaction between drugs and DNA is important in medicine because they influence both therapy and potential side effects. Understanding these interactions is critical for creating more effective treatments and safer drugs. Investigating the mechanisms underlying drug-DNA interactions not only provides insights into pharmacological effects, but it also paves the way for novel DNA-targeted drug design [14]. DNA-drug interactions are unravelled using a variety of techniques, including optical and infrared spectroscopy, Nuclear Magnetic Resonance (NMR), circular and linear dichroism, viscosity assays, Mass Spectrometry (MS), molecular docking, and electrochemical methods. These techniques provide valuable insights into the pharmacological effects of drugs and the molecular mechanisms of disease [15-18]. Electrochemical methods, such as Cyclic Voltammetry (CV) and Differential Pulse Voltammetry (DPV), offer a unique window into the dynamic interrelationship between drugs and DNA molecules [19]. The advantages of electrochemical approaches include their portability, simplicity, and quickness. Despite the complexity of the samples, their high selectivity assures precise identification of the target analytes, and their sensitivity allows for the detection of trace quantities. Cost-effectiveness is one of their main advantages because it makes them available for routine analysis [20].

In this study, the electrochemical characteristics of 5-(Chloromethyl)-2-(2-chlorophenyl)-[1,2,4]triazolo[1,5-a]pyrimidin-7(3H)-one, CMCP, a triazolopyrimidinone derivative and its interactions with both single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) were investigated using DPV and CV. (depicted in Figure 1). The objective of this study is to contribute to the ongoing development of innovative therapeutic compounds. Specifically, attention was directed towards the novel potential drug candidate CMCP, which is recognized for its triazolopyrimidinone structure, and a comprehensive examination of its electrochemical properties was conducted. Various experimental parameters, such as pH, concentration and scan rate were systematically explored using DPV. Additionally, critical parameters such as reproducibility, precision, linearity, as well as the limits of detection (LOD) and limits of quantification (LOQ) were determined. CMCP stability and interaction with ssDNA and dsDNA were assessed under optimal storage conditions (dark room at 25°C) on specific days (day 0, day 1, day 3, day 7, and day 30). DPV was used to monitor changes in guanine bases, which are essential for DNA structure and function. The significant alterations in guanine bases observed following CMCP interaction with ssDNA and dsDNA highlight the crucial nature of these findings.

2. Material and Method

2.1. Apparatus

Analytical Thin Layer Chromatography (TLC) was conducted using Merck silica gel F-254 plates. The melting points were determined employing the Stuart SMP 30 melting point apparatus (Staffordshire, ST15 OSA, United Kingdom). These melting point measurements were conducted in open capillary tubes. The synthesis of the compounds through microwave irradiation (MW) was performed utilizing the Milestone MicroSYNTH (Milestone S.r.l., Italy) microwave apparatus. The SimpliAmp™ Thermal Cycler (Thermo Fisher Scientific, Waltham, MA) was used to apply temperature cycles in a controlled and accurate manner, allowing dsDNA denaturation and the separation of the two DNA strands in the sample. The Heating Thermoshaker (Thermo Fisher Scientific, Waltham, MA) was employed for various tasks, including incubating samples at specific temperatures and gently mixing samples while maintaining precise temperature control. A PalmSens4 potentiostat/galvanostat/impedance analyser was used for all DPV and CV measurements. The PalmSens4 was connected to a computer via a USB cable and controlled using the PSTrace 5.8 program. A three-electrode system was used in the experiment, with a pencil graphite electrode (PGE) as the working electrode, an Ag/AgCl reference electrode, and a platinum wire counter electrode. The graphite lead was held in place with a Rotring T 0.5 mm pencil (Rotring, Germany). A local bookstore provided HB pencil leads (Tombo, Japan) measuring 60 mm in length and 0.5 mm in diameter. A conductive cable was attached to the metallic part of the pencil to provide electrical connection between the graphite lead and the holder.

2.2. Chemicals

Ethanol absolute (99.9%) and glacial acetic acid were acquired from Isolab Chemicals. Double-stranded salmon sperm DNA (dsDNA) was procured from Sigma Chemical Co. (St. Louis, USA), while all other chemicals were sourced from reputable suppliers, including Merck (Darmstadt, Germany), Tokyo Chemical Industry C. LTD. (Tokyo, Japan), Alfa Aesar-Acros Organics (Thermo Fisher Scientific), and Carlo Erba Reagents (Milano, Italy), ensuring their high purity. Solvents used were of analytical grade and were used as received without additional purification.

Various buffers, such as 0.5 M Acetate (ACB) at pH levels of 3.8, 4.8, and 5.6, 0.05 M Phosphate (PBS) at pH 7.4, 0.1 M sodium borate (BBS) at pH levels of 8.2 and 9.8, as well as 0.02 M NaCl and 0.05 M Tris-EDTA (TE) buffer at pH 8.0, were employed in the experiments. CMCP was synthesized at Izmir Katip Celebi University, Faculty of Pharmacy, Department of Pharmaceutical Chemistry, and generously provided. A stock solution of CMCP was prepared in Dimethylformamide (DMF) and stored in a dark room at 4°C to prevent degradation.

2.3. Experimental

2.3.1. Synthesis of CMCP

Triazolopyrimidinones are attainable through a range of chemical processes, such as cycloaddition, condensation, and cyclization reactions. These reactions generally involve the combination of appropriate precursor compounds to create the triazole and pyrimidinone rings. 5-(2-chlorophenyl)-3-amino-1,2,4-triazole (10 mmol) and ethyl 4-chloroacetoacetate (20 mmol) in 12-18 ml acetic acid (MW, 20 min, 180°C) was refluxed. The formed solid was filtered off, washed with acetic acid then dried to yield **CMCP**. Compounds **CMCP** (5-(Chloromethyl)-2-(4-methoxyphenyl)-[1,2,4]triazolo[1,5-a]pyrimidin-7(3H)-one) used in the next step without purification.

5-(Chloromethyl)-2-(2-chlorophenyl)-[1,2,4]triazolo[1,5-a]pyrimidin-7(3H)-one (CMCP).

Yellow solid; yield, 52 %; m.p., 113 °C; 1H-NMR (DMSO-d6, 400 MHz) δ 8.02 (d, J = 8.7 Hz, 2H), 7.07 (d, J = 8.8 Hz, 2H), 6.16 (s, 1H), 4.66 (s, 2H), 3.81 (s, 3H) ppm; 13C-NMR (DMSO-d6, 100 MHz) δ 161.6, 156.1, 151.8, 128.7, 114.8, 100.4, 55.8ppm; HRMS m/z: 290.0555 (Calcd for ($C_{13}H_{11}ClN_4O_2$), 290.0571). We reported HRMS data to within 5 µg/mL to support molecular formula and purity assignment.

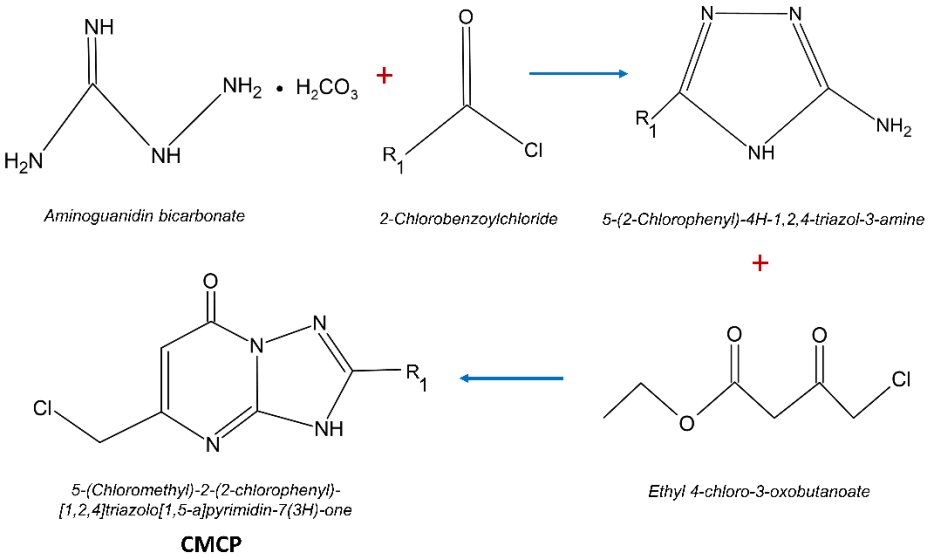

Figure 1. General synthesis scheme of the pyrimidinone-triazole derivative: CMCP.

2.3.2. Pre-treatment of PGE

In preparation for our experiments, the pencil graphite electrodes (PGEs) were activated by applying a potential of +1.4 V for a duration of 30 seconds within ACB at pH 4.8. The primary objective of this activation was to minimize background current, thereby enhancing the precision of subsequent electrochemical measurements.

The PGEs, having undergone this electrochemical pre-treatment, were subsequently employed as the working electrodes in all ensuing experiments. Electrochemical surface pre-treatment serves to increase the surface area of electrodes, introduce functional groups on the electrode surface for stronger interactions with DNA molecules, and eliminate impurities from the electrode surface that might interfere with DNA analysis [21]. These attributes are pivotal in expediting electron transfer at the electrode interface [22], a fundamental factor contributing to the effectiveness of our experimental investigations.

2.3.3. Interaction

The electrochemical behaviour of CMCP was investigated through the application of DPV, considering scenarios with and without the presence of both ssDNA and dsDNA. The investigation began by examining the interaction of CMCP with ssDNA, employing a denaturation method as outlined in a previously referenced study [23]. To commence this particular phase of the experiment, we initiated a precise thermal treatment. Specifically, we subjected the sample to a brief heating step lasting 1 minute, with the temperature set at 95°C. Subsequently, we rapidly cooled the sample by placing it on ice. Subsequently, CMCP was introduced, and the ssDNA was immobilized onto the PGEs. Following this immobilization step, DPV measurements were conducted. In the subsequent phase, the interaction of CMCP with dsDNA was scrutinised. Solutions containing dsDNA and CMCP were gently agitated at 45°C and 600 rpm for a duration of 30 minutes. Following the interaction period, PGEs were immersed in these solutions, and DPV was employed for analysis.

2.3.4. Measurement

DPV and CV measurements were carried out across a voltage range spanning from +0.4 to +1.4 V, with a scan rate set at 50 mV/s. These tests were consistently executed under typical room temperature conditions (25°C). The visual representation of the experimental process was illustrated in **Figure 2.**

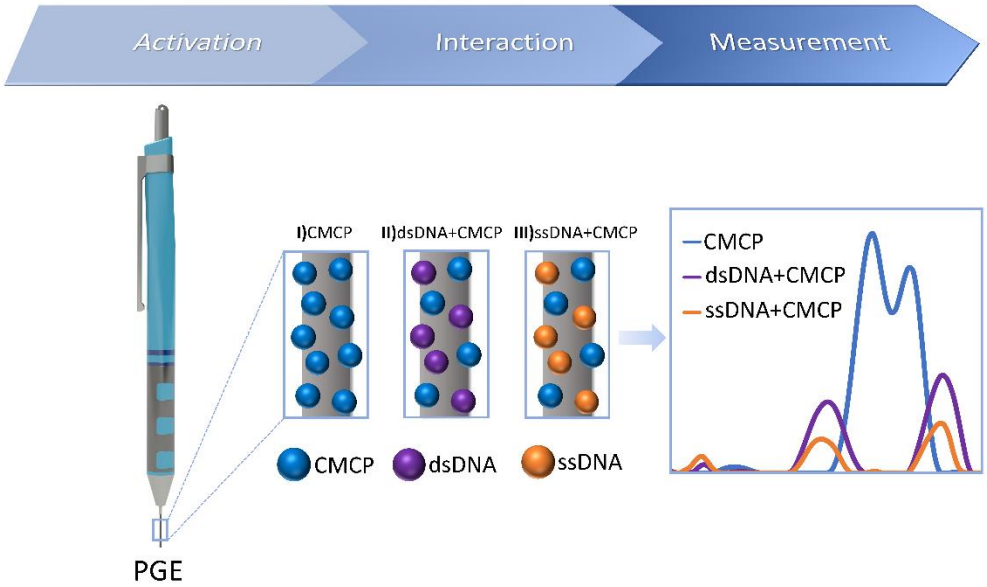

Figure 2. The experimental steps were as follows: activation of PGE with ACB, CMCP interaction with both dsDNA and ssDNA, and DPV measurements.

3. Results

3.1. Electrochemical Properties of CMCP

In this section, an investigation into the electrochemical behaviours of the drug candidate molecule was conducted using DPV. Initially, the impact of pH on the electrochemical oxidation signals of the drug candidate was assessed, and the results are illustrated in **Figure 3**. pH plays a pivotal role in drug metabolism and therapeutic efficacy, making it essential to identify the optimal pH conditions for the drug candidate. In the pH study, a range from 3.8 to 9.8 was examined using DPV. The objective was to identify the pH conditions that induce significant changes in peak current and peak potential.

As it is seen from the Figure 3, the drug candidate has an oxidation signal at around +1V. The results revealed that CMCP consistently exhibited stable responses, with the most prominent peak observed at pH 3.8. Consequently, pH 3.8 was selected as the dilution buffer. This thorough exploration of pH conditions provides crucial insights into the electrochemical behaviour of the drug candidates and their potential therapeutic applications.

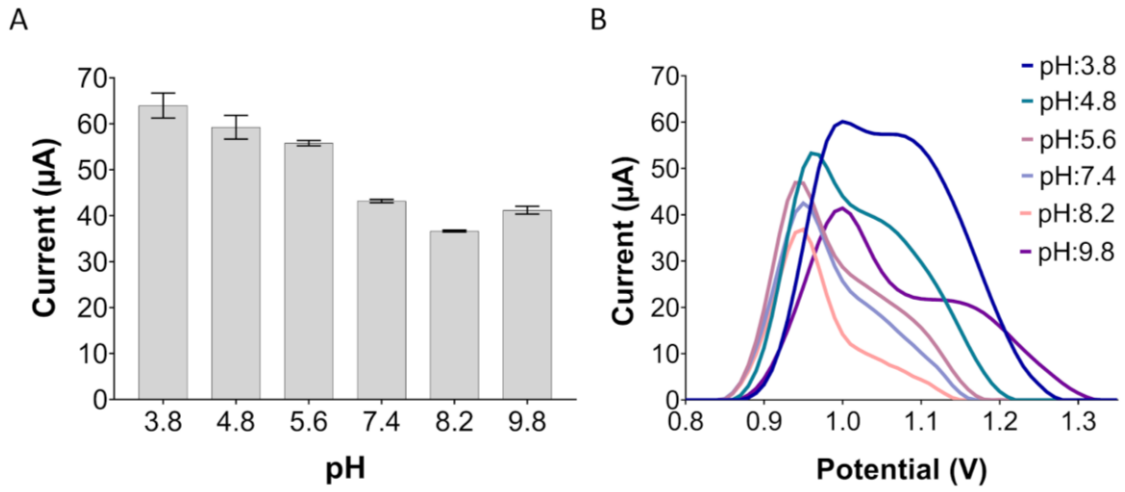

Figure 3. The effect of pH on peak currents and peak potentials. The bar graph showcases the behaviour of the CMCP across various pH values spanning from 3.8 to 9.8. Notably, the highest peak currents for CMCP were observed at pH 3.8.

The subsequent phase of the study focused on establishing the analytical concentration ranges of the drug candidate through DPV measurements conducted at a scan rate of 50 mV/s. Calibration plots, depicted in **Figure 4**, were constructed across varying concentrations of CMCP. Notably, the resulting calibration graph exhibited a linear relationship between current response and concentrations. Specifically, the calibration plot for CMCP exhibited a correlation coefficient of 0.9975 (as depicted in Figure 4). The precise and reliable quantification of CMCP concentrations achieved using the developed methodology is clearly evident from these results.

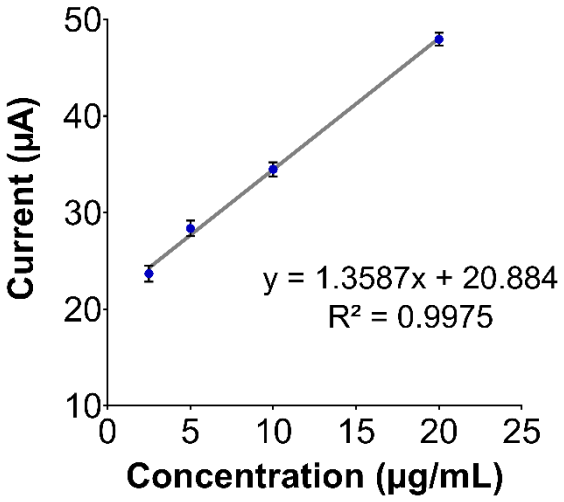

Figure 4. Calibration graph of CMCP was obtained by plotting drug concentrations against the current peaks. DPV measurements were performed with different concentrations from 2.5 µg/mL to 20 µg/mL.

Calibration graph played a pivotal role in calculating the LOD and LOQ for the CMCP. Specifically, the concentrations 2.5, 5, 10 and 20 µg/mL were employed for the determination of LOD and LOQ for CMCP. The LOD and LOQ for CMCP were calculated to be 1.2 µg/mL and 4.1 µg/mL, respectively using the equation LOD: 3 s/m, and LOQ: 10 s/m (s is the standard deviation for the blank solution and m is the slope of the related calibration curve)[28].

By varying the scan rate, valuable insights can be gained into the kinetics and mechanism of electrochemical reactions. Adjusting the scan rate enables the exploration of different reaction pathways, assessment of the reversibility of redox processes, and analysis of the comprehensive electrochemical characteristics of compounds. Therefore, the study of scan rate provides a comprehensive understanding of the underlying processes and aids in the design and optimization of electrochemical detection methods and sensor devices. In the last part of the study the influence of scan rate (V) on peak current (Ip) values was explored utilizing CV, covering a scan rate range from 25 mV/s to 150 mV/s.

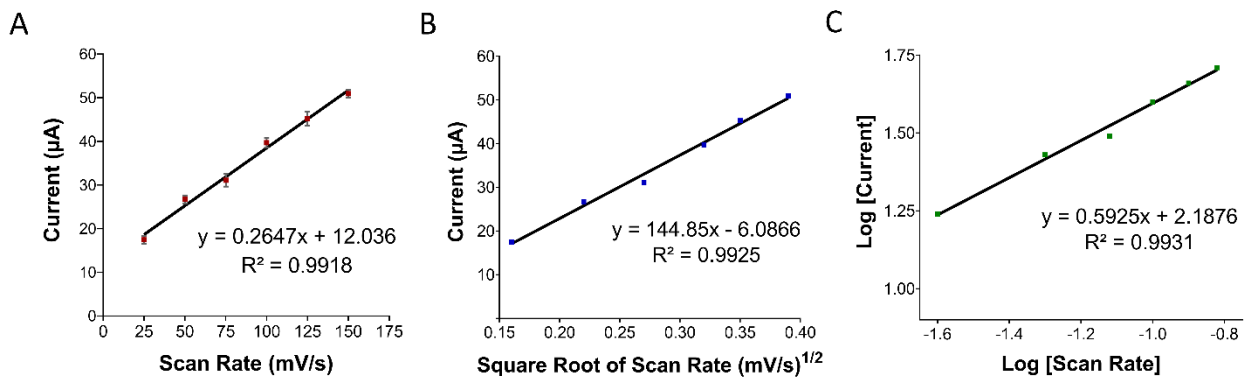

Figure 5. Effect of scan rate on peak currents (A), peak current vs. the square root of the scan rate (B), effect of scan rate on the log of peak currents of CMCP (C).

The anodic peak currents (Ip_a) exhibit a strong linear relationship with scan rates (ν) (**Figure 5A**). This correlation can be mathematically expressed by the following linear equation:

$$Ip_a(\mu A) = 0.02647\nu + 12.036\ (R^2 = 0.9918) \quad (1)$$

The relationship between Ip_a and the root of the scan rate ($\nu^{1/2}$) also possesses a linear behaviour (**Figure 5B**) the linear equation is as follows:

$$Ip_a(\mu A) = 144.85\nu^{(1/2)} - 6.0866\ (R^2 = 0.9925) \quad (2)$$

Linear behaviour was also determined between log(Ip_a) and log(ν) within the scan rate range between 25 mV/s and 150 mV/s (**Figure 5C**):

$$log\ Ip_a = 0.5925 log\nu + 2.1876\ (R^2 = 0.9931) \quad (3)$$

Considering the insights from the literature, these obtained slope values closely align with theoretical expectations, particularly in the case of a value close to 0.5. This alignment suggests the prevalence of diffusion-controlled processes. In contrast, a theoretical value of 1 indicates processes influenced by adsorption [24,25]. For *Equation 3*, the determined slopes were calculated as 0.5925. This result distinctly implies that the electrochemical oxidation of CMCP diffusion-controlled processes.

3.2. Interaction

The electrochemical behaviours of CMCP were examined using DPV in the presence and absence of dsDNA. We begin by conducting a test to assess the interaction of CMCP with ssDNA. To facilitate this, a common method involving physical denaturation of dsDNA fragments was employed, as detailed in a referenced study [23]. In this process, a stock solution of dsDNA was initially prepared, and subsequently, 100 µL of dsDNA was aliquoted and sealed in centrifuge tubes. Following this, all samples were subjected to a 1-minute heating step at 95°C in a thermal cycler, followed by cooling on ice for 10 minutes. After adding 10 µg/mL of CMCP, the resulting ssDNA was immobilized onto pre-treated PGEs for 30 minutes through adsorption. After this immersion, DPV measurements were carried out.

In the second stage, the interaction of CMCP with ssDNA was examined. Solutions were prepared by mixing 50 µg/mL of dsDNA and 10 µg/mL of the CMCP in ACB. Following this, these solutions underwent thermal agitation at 45°C and 600 rpm using a thermal shaker for 30 minutes. Subsequently, 100 µL of the resultant interaction solution was carefully transferred into tubes. Then, PGEs were submerged in the interaction solutions for a duration of 30 minutes. After this immersion, DPV measurements were carried out to evaluate the outcomes of the interaction.

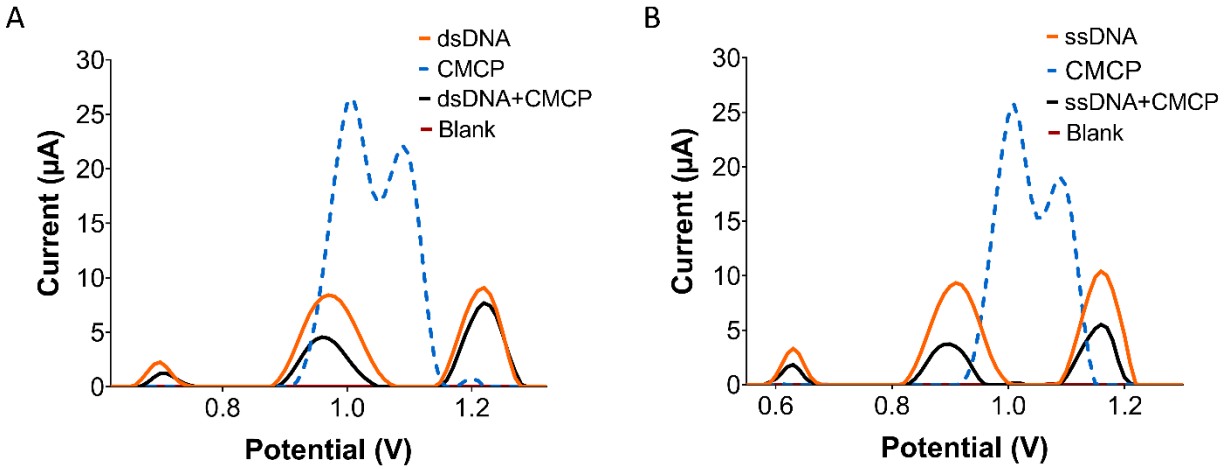

Figure 6. Impact of CMCP Interaction on dsDNA and ssDNA. DPV results present guanine oxidation currents for dsDNA (A) and ssDNA (B) following interaction with CMCP.

In **Figure 6A**, the CMCP peaks were recorded as 26.45 µA at 1.01 V and 22.10 µA at 1.09 V. The two significant peaks of dsDNA in ACB (pH: 3.8) were measured at 8.32 µA at +0.91 V and 9.13 µA at 1.16 V. The addition of the CMCP drug decreased the two significant peak values of dsDNA to 5.41 µA and 7.66 µA, respectively. Following the interaction with CMCP, the peak potential of dsDNA shifted to +0.90 V, while the other peak potential remained unchanged. This shows that CMCP specifically affects the electrochemical properties of dsDNA.

In **Figure 6B,** the peaks of CMCP were measured at 25.73 µA at 1.01 V and 19.03 µA at 1.09 V. The two notable peaks for ssDNA were initially measured at 9.35 µA at 0.91 V and 10.39 µA at 1.16 V. Adding the CMCP drug significantly reduced the significant peak values for ssDNA to 4.27 µA and 6.16 µA, respectively. The presence of CMCP shows a distinct impact on the oxidation potentials of ssDNA. One peak potential for ssDNA shifted negatively from +0.91 V to +0.89 V, but the other peak potential remained unchanged. In this context, variations in peak potential-whether they are positive or negative-offer insights into the interaction mechanism between the drug candidate CMCP and DNA. Positive shifts in peak potential commonly signify intercalative binding, whereas negative shifts are indicative of electrostatic binding [26]. In the scope of our investigation, the observed negative shift in peak potential could be attributed to an irreversible electrode process. This phenomenon might be explained by electrostatic binding occurring between CMCP and DNA.

In order to investigate the toxicity effects of CMCP on dsDNA and ssDNA, guanine peak changes were calculated before and after interaction. Here is a simplified example of an equation that could be used to calculate the percentage change in peak current as an indicator of toxicity:

$$Toxicity\ (\%) = \frac{Peak\ current\ (before\ interaction) - Peak\ Current\ (after\ interaction)}{Peak\ Current\ (before\ interaction)} x100 \qquad (4)$$

This equation yields a percentage value reflecting the extent of alteration in the electrochemical response attributable to the drug-DNA interaction. A greater percentage change may imply a more pronounced impact, potentially indicating heightened toxicity. In our investigation, this metric was determined as 53% for ssDNA and 41% for dsDNA. Broadly, an S% value exceeding 85 typically signifies non-toxicity, while a value within the range of 50 to 85 suggests a level of moderate toxicity. Conversely, a value below 50 is indicative of significant toxicity [27].

3.3. Stability

In our study, we investigated the stability of CMCP by subjecting freshly prepared solutions to controlled storage conditions within a light-protected environment at a constant temperature of 25°C. The tests were carried out over distinct time intervals, specifically on day 0, day 1, day 3, day 7, and day 30. Temporal changes in the peak currents of the drug were observed.

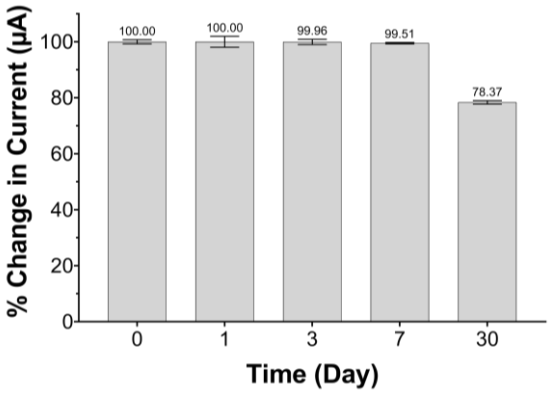

Figure 7. Change in current for CMCP examined at 25 °C for different days, e.g., 0, 1, 3, 7, and 30. The current value of the CMCP was about 78 % by the end of 30 days.

Here, CMCP exhibited good stability for 30 days of storage without significant percentage changes in current values, e.g. At the end of day 30, the percentage of the current value for CMCP was determined as 78%. The findings provide strong evidence that the stock solution of CMCP was stable for 30 days. This observation highlights the potential benefits of long-term use, as most of the drug candidates retained their essential pharmaceutical properties over an extended period.

4. Discussion and Conclusion

In conclusion, our study investigated the electrochemical properties of triazolopyrimidinone derivative, CMCP and its interaction with ssDNA and dsDNA, employing DPV and CV. Our investigation centred on the newly identified drug candidate, CMCP characterized by its triazolopyrimidinone structure, and systematically probed its electrochemical behaviour. Through DPV experimentation, we systematically assessed the parameters including pH, concentration and scan rate . The ensuing determination of key parameters such as detection limits, reproducibility, precision, linearity, and LOD and LOQ further enriched our understanding. In the pursuit of stability, we conducted comprehensive assessments under optimal storage conditions, illuminating the durability of this compound on specific occasions. Employing DPV before and after interaction revealed substantial modifications in guanine bases upon the interaction of CMCP with dsDNA, thereby highlighting the pivotal role of our research findings. Our endeavour underscores the intricate interplay between electrochemistry, drug candidate, and DNA, underscoring the potential for innovative drug development and therapeutic advancement in the field of biomedicine.

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