A commercially viable electrochemical sensor for the immunosuppressant drug mycophenolate mofetil utilizing pencil graphite electrode

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Abstract: An electrochemical sensor facilitating the electro oxidation of the immunosuppressant, mycophenolate mofetil (MMF) on pencil graphite electrode (PGE) has been developed. The electrochemical characteristics of the electrode was evaluated by virtue of cyclic voltammetry (CV) and differential pulse voltammetry (DPV). The PGE was characterized by XRD analysis and FE-SEM before and after the electrooxidation of MMF at the electrode. CV of MMF exhibited two irreversible oxidation peaks at about 0.66 V and 0.84 V. In the DPV studies, two linear ranges were observed towards the determination of MMF concentration from 20 nM – 300 nM and 300 - 1000 nM in 0.1M phosphate buffer with pH 6. The limit of detection of the sensor was estimated to be 1.80 nM. The proposed sensor exhibited acceptable selectivity for quantifying the MMF in pharmaceutical dosage forms and urine samples.

1. INTRODUCTION

Mycophenolate mofetil, the pro-drug of mycophenolic acid, is a well-tolerated immunosuppressant drug advised for the preventive treatment in the immunologic damaging in transplanted tissues of the kidney [1], liver [2], heart [3] and other various organs [4-7]. MMF is also used in the treatment of scleroderma and interstitial lung disease [8]. The drug exhibits its immunosuppressive effects by preventing the actions of inosine monophosphate dehydrogenase enzyme (IMPDH), which controls the catalytic oxidation of inosine monophosphate to guanosine monophosphate. MMF performs as an IMPDH inhibitor which leads to the antiproliferation of T and B lymphocytes associated with the IMPDH catalyzed de novo synthesis of purine nucleotides. Moreover, it acts a drug which affects the working of immune system in the treatment of various autoimmune diseases [9] and anticancer therapies [10]. The determination of MMF from biological fluids has drawn substantial attention due to the fetal development defects and miscarriage associated with the use of MMF in pregnant women [11]. Hence an effective detection method for the quantification of MMF is highly expected.

Several previously reported works focused on chromatography [12-16] spectroscopy [17, 18] and electroanalytical [19-24] for the determination of MMF in recent years. Among these techniques, electrochemical methods have been made more attention by virtue of simplicity, exceptional reproducibility, high portability, stability, fast response time, low cost and lower detection limit. PGE has been emerged as a highly promising electrode material in the area of electroanalytical research [25-27]. The low cost and ease of fabrication, when compared to other carbon-based electrodes, makes PGE more viable to develop disposable electrodes. Hence the present work displays the utilization of a disposable PGE for the electro oxidation of MMF. PGEs possess good mechanical and chemical stability with alterable working surface area. Here we are reporting an electrochemical
sensor for MMF on PGE without any modification of electrode surface and a nanomolar level detection of MMF was achieved by DPV technique. The analytical results obtained were compared with few of the existing electrochemical sensors. It was found that our work is superior in terms of the detection limit compared with the other literature reports.

2. EXPERIMENTAL

2.1. Reagents and solutions
The pencil leads (Cello 0.7mm diameter and 6 cm length) were purchased from a local stationery store. MMF was received as a gift sample on the part of KVSR Siddhartha College of Pharmaceutical Sciences, India. A 10⁻²M solution of MMF was prepared in 7:3 (v/v) methanol-water mixture and stored at 4°C for further use. Phosphate buffer solutions (PBS) with pH ranging from 3.0 - 8.0 were prepared by adding suitable amounts of Na₂HPO₄ and NaH₂PO₄ in water. Na₂HPO₄ and NaH₂PO₄ were procured from Merck. The commercial pharmaceutical MMF sample (IPCA MMF-500) were purchased from a local drug store. The entire chemicals employed were of analytical grade and millipore water was utilized for the preparation of all analytical solutions.

2.2. Instrumentation
CHI610E electrochemical analyzer (CH Instruments Inc., USA) was used for the electrochemical measurements at room temperature. A three electrode system with Ag/AgCl (1M KCl) as reference electrode, platinum wire as counter electrode and pencil graphite electrode as working electrode in a single compartment cell was used for the CV and DPV studies. The pH of the various solutions was measured with pH meter at room temperature. The pencil leads were wrapped with a teflon tape by exposing a length of 0.2 cm, which acts as the working surface.

2.3. Analytical procedures
The electrochemical behavior of MMF on PGE was explored by CV in a potential range 0.4V to 1.0 V with a scan rate of 100 mV/s. The electrochemical response of the proposed sensor were further analyzed and optimized using DPV, being it more sensitive than CV [28]. For DPV, the potential step, pulse amplitude and pulse width used were 0.004 V, 0.5 V and 0.025 s respectively in a potential window of 0.5 V to 0.8 V. The electrochemical measurements were carried in the electrochemical cell containing PBS (0.1M pH 6) by the addition of appropriate amounts of standard MMF solution.

For the quantification of MMF in the urine samples, urine samples were freshly collected from a healthy volunteer who abstained any medication during the week preceding the analysis. The urine samples were analyzed as such without any pre-treatment. For this, known volumes of standard MMF solution were spiked into the electrochemical cell containing 10 µL of urine sample which was further diluted to a final volume of 10 mL with PBS (0.1M pH 6). The concentration of MMF in the resulting sample solutions was then found out by DPV using calibration graph. Analysis of MMF were carried out on commercial pharmaceutical formulations such as tablet. To prepare tablet solutions, 5 tablets of the MMF were finely powdered into a homogeneous mixture in a mortar. A 10 mL solution of the tablet was prepared by adding adequate amount of the sample with 7:3 methanol -water solution. The solution was then filtered using an ordinary filter paper to remove the extra non-dissolved solids. Different volumes of the same sample solutions were transferred directly to an electrochemical cell containing 10 mL PBS6, and then DPV analysis of MMF were done. The concentration of the analyte was then obtained from the previously obtained calibration curve.
3. RESULTS AND DISCUSSIONS

3.1. FE-SEM and XRD analysis of PGE
The surface morphology of PGE was depicted by Field emission scanning electron microscopy (FE-SEM) before and after oxidation of MMF at the electrode (figure 1A and 1B). From the figure, it is evident that a refined individual flake like structure of graphite is seen on the bare PGE, whereas after oxidation the flakes on the surface are removed due to oxidation.

![Figure 1. (A) FE-SEM image of PGE before electro-oxidation of the MMF on PGE (B) FE-SEM image of PGE after electro-oxidation of MMF](image)

3.2. X-Ray Diffraction (XRD) pattern of the PGE
The XRD pattern of the bare PGE is shown in figure 2. Two peaks at 26.596° and 54.691° were obtained for bare PGE corresponding to (002) and (004) plane of graphite respectively. The layered structure of graphite is the basis of the above two peaks. The peak at 2θ = 26.596° is the distinctive peak of graphite with d-spacing of 0.3489 nm. The intensity of the peak at the plane (002) suggests a higher crystallinity for the PGE.

![Figure 2. XRD image of PGE](image)

3.3. Electrochemical response of MMF at PGE
The electrochemical response of MMF on PGE in PBS (0.1M pH 6) was studied by CV and the result obtained in the case of 500 nM MMF solution is exemplified in figure 3A. The response curve revealed two irreversible anodic peaks at around 0.69 V (peak1) and 0.89 V (peak2). The peak 1 was well-defined at higher concentrations compared to the peak 2 and was considered for further studies. Peak 1 and peak 2 were absent when CVs were recorded without MMF in 0.1 M PBS, which clearly depicts that PGE is electro inactive in the studied potential range.

3.4. Effect of supporting electrolyte and pH of the solution
The electro oxidation of MMF (400nM) in 0.1M PBS with varying pH from 3 – 8 were investigated by the DPV technique. Also, analytical response of MMF in different supporting electrolytes (0.1M)
such as acetate buffer, PBS, H$_2$SO$_4$, HNO$_3$, NaOH and NaCl were analysed. A better oxidation peak with augmented peak current was observed for the supporting electrolyte of 0.1 M PBS with pH 6 in comparison to other electrolytes studied. The impact of pH on the DPV response of MMF in the pH range 3 – 8 are displayed in figure 3B. As it is seen, the anodic peak current reaches a maximum value at pH 6 and thereafter it decreased with increase in pH. Moreover, a slope of -58.56 mV/pH has obtained for the plot of pH versus anodic peak potential (E$_{pa}$), with the linear regression equation, E$_{pa}$(V) = 0.96085 + 0.05856pH (R$^2$ = 0.99206). This pointed out the transfer of same number of protons and electrons in the electro oxidation of MMF as previously reported [22]. Based on the above observations PBS with pH 6 was selected as the suitable supporting electrolyte for subsequent analysis.

3.5. Effect of scan rate
The nature of electro oxidation that occurs at the working electrode surface can be best understood from the scan rate study. The impact of scan rate on the electro oxidation of MMF (500 nM) on PGE in PBS (pH 6) was analyzed by CV technique with a scan rate ranging from 20-200 mV/s as depicted in the figure 3C. A linear increase in the oxidation peak current (I$_{pa}$) of MMF was noticed with the increase in the scan rate and is given in figure 3D. The linearity in the peak current versus scan rate follows the regression equation, I$_{pa}$(µA) = 0.13863 + 0.00381 ʋ (mV/s) (R$^2$ = 0.99482). This implies that the electro oxidation of MMF on PGE is an adsorption-controlled process. Besides, the oxidation peak potential moved to more positive values as the scan rate increases, which is attributed to irreversible electrochemical reactions. According to Laviron equation, the number of electrons (n) transferred during the electro-oxidation of MMF was calculated from the slope of the E$_{pa}$-ln ʋ plot with the linear regression equation, E$_{pa}$(V) = 0.56321 + 0.02093 ʋ (mV/s) (R$^2$ = 0.99593). The value of n was found to be 2.45, if the value of charge transfer co-efficient $\alpha$ = 0.5 for a totally irreversible electrode process [29]. Based on the obtained results, the electro-oxidation mechanism of MMF on PGE could be considered as a two proton two-electron transfer process as reported in the previous literature [22].

3.6 Optimization of DPV parameters for PGE
To maximize the analytical signal for DPV, the experimental parameters like potential step, pulse width and pulse amplitude were studied at PGE in 0.1M PBS at pH 6 containing 100 nM MMF. Initially, the potential step was varied from 2 mV to 6 mV by keeping pulse width as a constant of 25 ms and pulse amplitude of 50 mV. In such conditions, the peak current was improved with increasing potential step up to 4 mV and then decreased. Hence 4 mV was chosen as the best DPV potential step. Fixing the potential step at 4 mV, the effect of pulse width was investigated by varying from 5 ms to 25 ms and a maximum peak current was obtained for 25 ms. Thus 25 ms was selected as the optimum pulse width. Then pulse amplitude was varied from 30 mV to 70 mV by keeping the optimum potential step and pulse width, a maximum anodic peak current is observed for pulse amplitude 50 mV. Thus, the DPV parameters, potential step, pulse amplitude and pulse width selected for the analytical detection were 4 mV, 50 mV and 25 ms respectively.
3.7. Impact of accumulation potential and accumulation time on the oxidation of MMF

The figure 4A shows the variation of accumulation potential in a potential window, 0.1 V to 0.4 V with a constant accumulation time of 10 s for 100 nM MMF. The maximum peak current was obtained an accumulation potential of 0.2 V and was selected for further analysis. Furthermore, the variation in the peak current regarding accumulation time was then investigated from 5 s to 20 s with the accumulation potential of 0.2V (figure 4B). The best suitable result was obtained for accumulation time of 10 s. As accumulation time increases above 10s, the peak current decreases may be due to adsorption of the analyte on the electrode surface.

3.8. Calibration plot

The electrochemical response of MMF from 20 nM to 1000 nM using DPV under optimum conditions were given in Figure 5A. As the concentration of MMF increased from 20 nM to 1000 nM, the anodic current was found to be increased linearly. This anodic current response was attributed to the electro oxidation of MMF in PBS at pH 6. The anodic peak current versus concentration of MMF was plotted and is showed in the Fig. 5B. The calibration curve consists of two linear segments in the range of 20 nM - 300 nM and 300 nM- 1000 nM with the linear equation of $I_{pa} (\mu A) = 0.00137 \times C \ (nM) + 0.06591 (R^2 = 0.99045)$ and $I_{pa} (\mu A) = 8.50115 \times 10^{-4} \times C \ (nM) + 0.20131 (R^2 = 0.99828)$.

3.9. Limit of detection and repeatability

The lower detection limit (LOD) was calculated using the equation $3S_b/m$, where $S_b$ is the standard deviation of the blank, $m$ is the slope of the calibration plot and the LOD was found to be 1.80 nM. The repetitiveness of the proposed sensor was assessed by monitoring the anodic peak current of 100 nM solution of MMF. The relative standard deviation (RSD) for five successive measurements was found to be 5.40 % signifying the good recurrence of the proposed sensor.
3.10. Interference study
The influence of various biomolecules such as urea (6mM), glucose (6mM), uric acid (100µM), ascorbic acid (50µM) and ions like Na⁺ (50mM) and K⁺ (50mM) on the electrochemical response of 100 nM MMF were studied to analyze the specificity of the proposed sensor. It is observed that only a 4.9 % change in the oxidation peak current of MMF was noticed in the presence of the above added species. Since no profound interference was observed on the anodic peak current of MMF, the developed sensor could be employed effectively for the detection of MMF from real samples.

3.11. Analytical application of MMF from real samples
The proposed sensor was utilized practically by monitoring the concentration of MMF from the spiked urine samples and pharmaceutical formulation such as tablet using DPV technique. Without any pretreatment, the urine samples were diluted with phosphate buffer solution before the electrochemical measurement. The tablet solutions were directly spiked into the PBS with pH 7 and electrochemical analysis was done. The recoveries of MMF from the spiked urine samples and tablet solutions were calculated from calibration curve and the results obtained were given in Table 1. The recoveries obtained for MMF in urine samples and tablet samples were found to be in the range of 97.7 to 102 %.

| Sample | Spiked (nM) | Detected (nM) | Recovery (%) | RSD % (N=3) |
|--------|-------------|---------------|--------------|-------------|
| Urine  | 40          | 39.11         | 98.5         | 5.9         |
|        | 200         | 204.8         | 102          | 1.40        |
| Tablet | 40          | 39.11         | 97.7         | 6.1         |
|        | 100         | 98.09         | 98           | 3.7         |

Figure. 5 (A) Differential pulse voltammograms recorded for 20 nM – 1000 nM MMF on PGE (B) Calibration plot for the electro oxidation of MMF at PGE.
Table 2. Comparison of the previously reported electrochemical methods for MMF determination with the present work

| Sl.No. | Electrodes | Concentration range (µM) | LOD (M) | References |
|--------|------------|--------------------------|---------|------------|
| 1      | GCE        | 5-750                    | 1.48 × 10⁻⁷ | [19]       |
| 2      | CPE modified with ionic liquid and MgO/SWCNTs | 0.1-450 | 7 × 10⁻⁸ | [23]       |
| 3      | GCE modified with Fe₃O₄ nanoparticles and MWCNT | 0.05-200 | 9 × 10⁻⁹ | [22]       |
| 4      | GCE modified with graphene Oxide | 0.04-15 | 11.3 × 10⁻⁹ | [30]       |
| 5      | Modified CPE with MIP and MWCNT | 0.0099-87 | 7 × 10⁻⁹ | [21]       |
| 6      | NiO/SWCNTs/MBBr/CPE | 0.08-900 | – | [24]       |
| 7      | Modified MWCNT/GCE | 5-160 | 9 × 10⁻⁷ | [20]       |
| 8      | PGE        | 0.02-1                   | 1.8 × 10⁻⁹ | Present work |

3.12. Comparison of the analytical aspects of the fabricated sensor with reported literature

Analytical characteristics of the developed sensor were compared with some of the previously reported works and is summarized in table 2. From the comparison table, the sensor is superior to reported literature in terms of LOD. Accordingly, the bare PGE can be considered as a simple and cost-effective single use electrode for the evaluation of MMF.

4. CONCLUSION

A commercially viable disposable electrochemical sensor was developed for the determination of mycophenolate mofetil utilizing pencil graphite. DPV was used to investigate the analytical performance of the electrode. The developed sensor exhibited two linear concentration ranges from 20nM–300 nM and 300 nM -1000 nM a with a lower detection limit of 1.80nM. The electrode possesses good repeatability and selectivity for the estimation of the drug from real samples. Nano molar level detection of mycophenolate mofetil was achieved in the real samples with recoveries in the range of 97.7 % - 102%. The obtained LOD was better when compared with reported literature.

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