A Novel Inexpensive Electrochemical Sensor for Pyrazinoic Acid as a Potential Tool for the Identification of Pyrazinamide-Resistant *Mycobacterium tuberculosis*

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**Abstract**

**Introduction:** Tuberculosis (TB) is a significant cause of morbidity and mortality worldwide. The patient compliance with the long treatment regimens is essential for successful eradication. Pyrazinamide (PZA) shortens these regimens from 9 to 6 months, and therefore, improves treatment completion rates. Although PZA is a first-line medication for the treatment of TB, no simple or reliable assay to determine PZA resistance is yet available. In the presence of PZA, only susceptible *Mycobacterium tuberculosis* strains release pyrazinoic acid (POA). Therefore, the measurement and quantification of released POA is an indicator of PZA resistance. **Methods:** Two electrochemical sensors were constructed and tested with alternative working electrodes in conjunction with a portable potentiostat to measure the current produced when a potential difference of 2 V is applied to varying concentrations of POA in controlled solutions. **Results:** The large (13.2 mm) electrochemical sensor was able to detect POA at a minimum concentration of 40 μM to a statistically significant level (P = 0.0190). Similar graphical trends were obtained when testing the electrochemical sensor in the supernatant of a negative microscopic observation drug susceptibility assay culture, irrespective of the presence of PZA. **Conclusion:** Inexpensive and reusable electrochemical sensors with a portable potentiostat are a promising tool for the detection of POA, a biomarker of PZA susceptible *M. Tuberculosis*.

**Keywords:** Electrochemical sensor, pyrazinamide, pyrazinoic acid, resistance, tuberculosis

**INTRODUCTION**

*Mycobacterium Tuberculosis* (*MTB*) is the leading cause of death worldwide from a single microorganism.¹ Infections with *MTB* are highly prevalent affecting a third of the world’s population, predominantly in a latent form.² The patient compliance with tuberculosis (TB) chemotherapy is vital for successful *MTB* eradication, avoidance of *MTB* resistance, and for reduced *MTB* transmission. The long treatment regimens of TB are a barrier to patient compliance, and hence shortening the duration of these is important. For this reason, pyrazinamide (PZA) is now established as an essential first-line drug in TB chemotherapy regimens due to its shortening of treatment courses from 9 to 6 months.³⁻⁶ This property of PZA is attributed to its unique action on semi-dormant *MTB* under stressed conditions.⁷ However, the emergence of PZA-resistant *MTB* is threatening this and therefore is of significant public health concern.

The lack of accurate phenotypic and genotypic tests for PZA resistance⁸,⁹ as well as their expense means that testing is generally not performed in resource-poor settings. Therefore, it is probable that there are more cases of inappropriate PZA use in the countries where TB is most prevalent.

Access this article online

Quick Response Code:  
Website: www.ijmyco.org

DOI: 10.4103/ijmyco.ijmy_63_18

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How to cite this article: Rueda D, Furukawa R, Fuentes P, Comina G, Rey de Castro NG, Requena D, et al. A novel inexpensive electrochemical sensor for pyrazinoic acid as a potential tool for the identification of pyrazinamide-resistant *Mycobacterium tuberculosis*. Int J Mycobacteriol 2018;7:275-81.
PZA is a prodrug that enters MTB by passive diffusion where it is converted into pyrazinoic acid (POA) by bacterial pyrazinamidase [Figure 1].\textsuperscript{3,10,11} POA is expelled from MTB by an efflux pump system into the acidic extracellular space where it is protonated. This acidic environment is believed to occur as a result of active inflammatory cells within the tissue of the infected host. The protonated form then reenters MTB where on the acquired proton is released. This cycle is repeated multiple times resulting in intracellular POA accumulation, reduced pH, and a lethal disruption of membrane permeability resulting in cellular damage.\textsuperscript{11-13}

The methods currently used for determining PZA resistance include lengthy cultures such as MGIT 960,\textsuperscript{14} BACTEC 460,\textsuperscript{15} and culture proportion methods as well as the relatively fast but subjective Wayne assay. The Wayne assay is a qualitative test based on the detection of POA in the extracellular environment through a colorimetric biochemical reaction.\textsuperscript{16} This method uses the presence and absence of POA as evidence of MTB susceptibility and resistance to PZA, respectively. Due to the Wayne assay’s simplicity, it is extensively used in developing countries. However, it has two main limitations. First, it is qualitative and, therefore, subjective because it is based on the observation of a red band. Second, the Wayne assay can only detect relatively high POA concentrations of more than 0.5 mM (P. Sheen personal communication). Therefore, it requires several MTB colonies to be harvested from a preliminary agar culture. More recently, it has been demonstrated that the measurement of accumulated POA in the supernatant of a 1–2 days PZA exposed liquid culture can be used as a quantitative biomarker of PZA resistance.\textsuperscript{17,18}

A variety of methods exist that are used to both detect and quantify the presence of PZA. These include the characterization of PZA by voltammetry using polyhistidine modified electrodes.\textsuperscript{19} PZA detection through UV–V is spectrophotometry using multivariate calibration,\textsuperscript{20} as well as several chromatographic approaches. All of these methods have a low limit of detection for PZA, (approximately 8 nM-0.4 µM). However, the aforementioned methods require expensive equipment and nonreusable components. These factors prevent the possibility of their use in low-resources settings. More importantly, though is the fact that these methods have not been previously used for the identification of POA.

In this study, we set out to develop an affordable method for POA detection that can be utilized in resource-poor settings. To achieve this, we created two inexpensive reusable sensors consisting of two working electrodes and a portable potentiostat that are capable of measuring low concentrations of POA in solution. To the best of our knowledge, this is the first reported economical POA electrochemical sensor.

**Methods**

**Development of the electrochemical sensors**

The sensors developed were based on a two-electrode electrochemical cell as reported in the literature.\textsuperscript{21,22} Two working electrodes were made from gold (Premion®, 99.985% pure by Alfa Aesar, Tewksbury, Massachusetts, USA) and platinum (99.95% pure by Word Precision Instruments, Sarasota, Florida, USA) wires. In addition, a counter electrode was made from stainless steel cylinder. To fix these wires at the center of the stainless steel cylinder, a dielectric acrylic polymer (Valux™ Plus by 3M ESPE, St. Paul, Minnesota, USA) was used to fill the internal space [Figure 2]. Using this approach, we built two sensors of different sizes: A large sensor [Figure 2a] with a 13.2-mm external diameter and a 9.5-mm internal diameter with two working electrodes (gold and platinum) of 1-mm diameter each and a small sensor [Figure 2b] with 4-mm external diameter and 3-mm internal diameter with two working electrodes (Gold And Platinum) of 0.5-mm diameter each. For the rest of this article, we will refer to the small and large electrochemical sensors based on the working electrode that is selected for use (i.e., small gold, small platinum, large gold, or large platinum). The large sensor was designed to enable the measurement of a 5 ml sample contained in a 10 mL beaker, while the small sensor was made to enable the measurement of a 150 µL sample in the well of an ELISA plate. The material cost of each sensor was approximately $80.

**Detection of pyrazinoic acid in water-based solutions**

Water-based solutions of varying concentrations of POA (1 µM, 2 µM, 4 µM, 8 µM, 10 µM, 20 µM, 40 µM, 70 µM, and 100 µM) were tested using the electrochemical sensors. These solutions were created using chemically pure POA obtained from Sigma-Aldrich (St. Louis, Missouri, USA). A portable potentiostat (Uniscan PG581 potentiostat by Uniscan Instruments Limited, Buxton, Derbyshire, United Kingdom; approximately $5000 at time of purchase) was used with

![Figure 1: Conversion of pyrazinamide and water (H₂O) into pyrazinoic acid and ammonia (NH₃) by the enzyme pyrazinamidase](image)

**Figure 1:** Conversion of pyrazinamide and water (H₂O) into pyrazinoic acid and ammonia (NH₃) by the enzyme pyrazinamidase

![Figure 2: (a) Large electrochemical sensor with a 13.2-mm external diameter; (b) Small electrochemical sensor with a 4-mm external diameter; (c) end on view of the large (13.2 mm) electrochemical sensor showing both its gold (Au) and platinum (Pt) working electrodes](image)

**Figure 2:** (a) Large electrochemical sensor with a 13.2-mm external diameter; (b) Small electrochemical sensor with a 4-mm external diameter; (c) end on view of the large (13.2 mm) electrochemical sensor showing both its gold (Au) and platinum (Pt) working electrodes
our electrochemical sensors to perform linear and cyclic voltammetry measurements of all of the POA solutions. The voltage setting for these experiments was 0.01 V/s from −2 V to +2 V for linear voltammetry and 0.01 V/s from −2 V to +2 V for cyclic voltammetry. The potentiostat was used to measure the current produced by the variation of voltage in the POA solutions. All voltammetry was performed at a working temperature of 22°C. All measurements of current were performed with both sensors and with both the platinum and gold working electrodes. Six repetitions of voltammetry were undertaken for each POA solution.

Detection of pyrazinoic acid, pyrazinamide, and a combination of pyrazinoic acid/pyrazinamide in the supernatant of a negative liquid-based microscopically observed drug susceptibility culture

To test our electrochemical sensor under a more clinically relevant biochemical environment, we undertook a control microscopically observed drug susceptibility (MODS) culture. Following incubation, we collected the supernatant of the negative MODS culture and added POA and PZA to make two distinct stock solutions with final concentrations of 6 mM of POA and PZA, respectively. Using these stocks, we prepared solutions with different concentrations of POA or PZA through further dilution with MODS supernatant (5 µM, 25 µM, 50 µM, and 75 µM of POA or 575 µM, 600 µM, 625 µM, and 645 µM of PZA). In addition, we also prepared combined solutions with varying ratios of POA/PZA in MODS supernatant to create the final concentrations of 5/645 µM, 25/625 µM, 50/600 µM, and 75/575 µM. Note that, due to the limited availability of MODS culture reagents, it was only possible to perform this experiment once.

Data analysis
The data collected was considered to be nonparametric given the small sample sizes present. The mean of the repeated current readings measured during voltammetry was calculated and then plotted to make a graphical representation of the change of current for a given concentration of POA. This was repeated with a log transformation of POA concentration. These processes were undertaken for both cyclical and linear voltammetry as well as for each of the configurations of the two electrochemical sensors. In addition, the difference between the current detected by each of our electrochemical sensors and...
each of the concentrations of POA was assessed using the Kruskal–Wallis Test. A subsequent post hoc analysis was performed with Dunn’s test. These statistical tests were undertaken using GraphPad Prism version 7.03 software (GraphPad Software, La Jolla, California, USA). A significance level of 5% was set for these statistical tests.

RESULTS
Detection of pyrazinoic acid in water
The most negative currents detected in POA solutions occurred with the use of the large platinum sensor when exposed to a potential difference of 2 V [Figures 3 and 4]. We found that a linear as opposed to a logarithmic trend better appreciated the relationship of an increasingly negative current with increasing POA concentration from approximately 10 µM to 100 µM. Descriptive statistics of the currents recorded by each sensor for each concentration of POA are shown in Table 1. The Kruskal–Wallis test was used to analyze the differences between the current detected and the concentration of POA for both the electrochemical sensors with either the gold or platinum working electrode. The small gold, large gold, small platinum, and large platinum electrochemical sensors all demonstrated statistical significance with regard to the detection of an increasing POA concentration ($\chi^2 = 41.28, P < 0.0001$; $\chi^2 = 43.73, P < 0.0001$; $\chi^2 = 46.68, P < 0.0001$; $\chi^2 = 46.68, P < 0.0001$, respectively). Post hoc analysis revealed that the small electrochemical sensor with either a gold or platinum working electrode could at a minimum differentiate between 1 µM and 70 µM of POA ($P = 0.0051$ and $P = 0.0103$, respectively; Figure 5). Whereas, the large electrochemical sensor with either a gold or platinum working electrode could discriminate a lower concentration of 40 µM POA from 1 µM [$P = 0.0250$ and $P = 0.0190$, respectively; Figure 5].

Detection of pyrazinoic acid, pyrazinamide, and a combination of pyrazinoic acid/pyrazinamide in the supernatant of a negative MODS culture
Negative MODS culture fluid containing only PZA at a concentration ranging from 575 to 645 µM was found to generate a low-negative current (~2.5 mA) when a potential...
A difference of 2 V was applied. On visual inspection, there was no evidence of a relationship between PZA concentration and detected current [Figure 6]. However, it was found on visual inspection that both solutions containing POA (i.e., solely POA and a POA/PZA combination) showed a similar graphical trend and were also associated with a more negative current (range of −4 to −9 mA; Figure 6).

**Discussion**

The concentration of POA detected in the supernatant of a PZA exposed liquid MODS culture of *M. tuberculosis* is inversely associated with PZA resistance, and hence, is a marker of importance.[17,18] Methods for POA detection exist including mass spectrometry[24] and spectrophotometric quantitative Wayne assay;[25] however, these require expensive equipment and materials. Other studies have reported the development of electrochemical sensors that can detect PZA as opposed to POA;[19,20] however, these are not of use in detecting PZA-resistant *M. tuberculosis* given the mechanism by which these microorganisms develop resistance to PZA. Accordingly, here, we report the development of inexpensive and reusable electrochemical sensors that is capable of detecting POA.

We developed two electrochemical sensors for a material cost of approximately $80 each. Both electrochemical sensors contain two working electrodes, gold, and platinum, of which
In the laboratory setting, the supernatant of a PZA exposed MODS culture for 

MTB strains in active MODS cultures.

**Conclusion**

This study presents the proof of concept of a new and inexpensive means for measuring low concentrations of POA through voltammetry. This method can detect concentrations of POA as low as 40 µM and represents a quantitative assay for POA. Further studies are needed to assess the role of POA electrochemical sensors in the detection of PZA-resistant *MTB* strains in active MODS cultures.

**Financial support and sponsorship**

This research was funded by the Wellcome Trust (Ref: 099805/Z/12/Z), the Grand Challenge Canada (GCC Number 0687-01-10), and the LOREAL-UNESCO-CONCYTEC 2014 award.

Patricia Sheen was supported by a Wellcome Trust Intermediate fellowship. Daniel Rueda was supported by a fellowship from the French-Peruvian Doctoral School in Life Sciences (National Council of Science, Technology and Innovation of Peru).

**Conflicts of interest**

There are no conflicts of interest.

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**Figure 6:** Graph showing the relationship between current and the concentration of pyrazinoic acid, pyrazinamide, or a combined solution (pyrazinoic acid/Pyrazinamide) in the supernatant of a negative microscopically observed drug susceptibility culture. This relationship was assessed with the solutions being exposed to a potential difference of 2 V and with the use of the small (4 mm) electrochemical sensor set up with a platinum working electrode.

In water, we demonstrated that both our small and large sensors, with either the gold or platinum working electrodes, were able to detect POA concentrations as low as 40 µM and 70 µM, respectively, in a statistically significant manner [Figure 5]. This is markedly lower than the Wayne assay[25] which is often used in resource-poor settings and can only detect POA at concentrations above 0.5 mM (P. Sheen personal communication). It is important to note though that at present our detection limit of 40 µM is higher than that of quantitative spectrophotometrical colorimetric Wayne assay.[25] However, the benefits of our electrochemical sensors over this method are that they are more affordable and have the potential for miniaturization. These features are both keys for the development of alternative techniques for the detection of PZA-resistant *MTB* strains in resource-poor settings.

In the laboratory setting, the supernatant of a PZA exposed MODS culture for *MTB* does not initially contain POA but other molecules. Accordingly, we tested the use of the small platinum electrochemical sensor in the supernatant of a negative MODS culture. A similar graphical trend and level of detected current were observed in both the sample containing only POA as for the sample containing a combination of POA/PZA [Figure 6]. In contrast, the current detected in the MODS supernatant containing only PZA was both lower in amplitude and did not show the same increasingly negative trend as PZA concentration increased [Figure 6]. This observation suggests that in the supernatant of a negative MODS culture that our electrochemical sensor can detect POA electrical signals even in the presence of other molecules, including PZA. Note though that due to limited access to MODS culture supernatant, we were unable to collect a sample size sufficiently large enough to undertake a statistical analysis of this observation. In addition, this study did not test our electrochemical sensor in the supernatant of a MODS culture containing active *MTB* due to sample access issues. Therefore, further studies are needed focusing on these areas.

**Figure 6:** Graph showing the relationship between current and the concentration of pyrazinoic acid, pyrazinamide, or a combined solution (pyrazinoic acid/Pyrazinamide) in the supernatant of a negative microscopically observed drug susceptibility culture. This relationship was assessed with the solutions being exposed to a potential difference of 2 V and with the use of the small (4 mm) electrochemical sensor set up with a platinum working electrode.
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