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Potentiometric Method for Resistance's Measurement of Pyrazinamide in Mycobacterium Tuberculosis

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***Abstract*— An experimental research of the drug pyrazinamide is analyzed and tested with potentiometric method. By understanding the biomechatronic system, consisted of a mechanism of action of the drug Pyrazinamide in a biological system, and an electrical signals, voltametric measures, a method to detect resistance to this specific drug is developed and tested firstly in vitro, then in cultures and finally in decontaminated sputum.**

I. Introduction

Tuberculosis (TB) is a very mortal disease in the world, approximately each 20 seconds a person die by this disease. And in the american continent, Peru is the second country with most incident, 31000 cases in 2010, behind Brasil with 85000. The TB in Peru hasn't been controlled effectively and has caused be more resistant (TB MDR) and even extremely resistant (TB XDR). One factor of an effectively control is a fast, cheap and trustable diagnostic.

Important facts to research in TB are that the used drugs have been discovered more than 50 years ago and the studied schemes are from 1960-1970. It is one of the eight commitments of the world in this millennium to reduce it for the year 2015. It is a declared priority of Peru. WHO declared an emergency world state by the TB. 1/3 of the world population is estimated infected. TB causes 11-50% of the death of patients with VIH. In 2009, the 65% of new cases is concentrated between Brazil, Peru, Haiti and Mexico. It is estimated that 50% of patients with VIH will develop TB.

Actually between the fast tests for diagnostic, the most limiting disadvantages are high cost, highly prepared technicians and high risk. By the other hand, the time to detect if a person has the disease is decreased from 60 days (biochemist methods) to 6 hours (molecular methods) with more than 90% of sensitivity and specificity. A method developed in the laboratory that have good performance, low cost and 9 days of cultivation is Microscopic Observation Drug Susceptibility (MODS) assay [1, 2, 3 and 4].

Studies found that Pyrazinoic acid (POA) is an indicator of resistance to the drug pyrazinamide (PZA), therefore by analyzing the concentration of POA in the sample indicates the effectiveness of the drug PZA. The first part explains the mechanism of action of PZA, the drug PZA, the enzyme which converts the PZA, the resistance's mechanism and the potentiometric method. The second part of this study consists of experiments with POA, reduction of the size of the sensor and finally experiments with cultures of strains H37Rv and decontaminated sputum.

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II. Mechanism's action of Pyrazinamide

The mechanism of action of the pyrazinamide has been studied very extensively [5, 6, 7] and the following diagram shows a schematic representation.

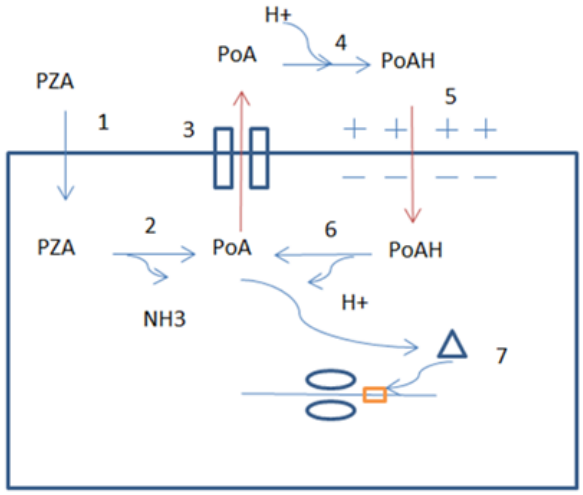

Fig. 1. 1. Passive diffusion. 2. Ammonia is liberated. 3. Ejection by a pump. 4. Protonate. 5. By gradient. 6. Deprotonate. 7. POA inhibit Ribosomal Protein S1 (RpsA)

1. *Passive diffusion*

It is a movement of molecular substances across membranes.

2. *Liberation of ammonia*

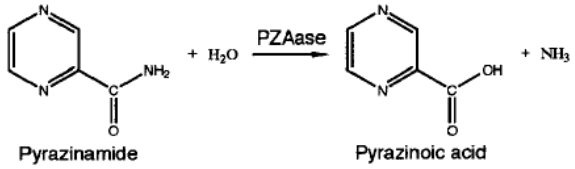

Fig. 2. Pyrazinamide is transformed to Pyrazinoic Acid by the enzyme Pyrazinamidase.

3. *Ejection by pump*

The ion is evacuated by a transporter, a molecular pump which is a protein embedded to the membrane.

4. Protonate

The stoichiometric reaction is:

$$POA^- + H^+ \rightarrow POAH$$

In this type of reaction, no electron is released and no voltage of reduction and oxidation occurs.

In an acid medium, another stoichiometric reaction can be:

$$POA^- + 3H^+ + 2e^- \rightarrow POAH + H_2(g)$$

This reaction absorbs 2 electrons and released hydrogen gas.

5. By gradient

It occurs when a substance moves across the membrane from a place with high concentration of the substance to a place with lower concentration.

6. Deprotonate

It is the inverse of protonate. A hydrogen ion is released.

7. Inhibition of Ribosomal Protein S1 (RpsA)

Recently studies on POA, suggest that POA inhibit the trans-translation mechanism. By this inhibition, the bacteria die due to the inability to produce nucleoriboproteins [8].

8. Acidification

Due to the cycle of ejection, protonate, diffusion by gradient and deprotonate. The inner medium becomes acidified, and the bacteria die.

III. PYRAZINAMIDE

The pyrazinamide (PZA) is an antibiotic, fundamentally bacteriostatic. With rifampicin and isoniazid causes the death of a large amount of bacillus during the initial phase of the treatment, reducing the treatment from 9 to 6 months. In spite of their in vivo activity, PZA is not active against Mycobacterium Tuberculosis (MTB) in cultures near to neutral pH.

A. History

In 1936, Dalmer and Walter synthesized PZA before the discovery as an antituberculosis drug in 1952. In 1945, Chorine discovered that nicotinamide (vitamin B3) had inhibitory activity against MTB. Later studies found that PZA is a synthetic analogue of nicotinamide, more active in a murine model of tuberculosis. PZA is poorly soluble in organic solvents; in contrast, POA is lightly soluble in water and more soluble in hot water.

B. Antituberculous activity

The minimum inhibitory concentration (MIC) of PZA for MTB has been reported in the range of 6.25-50 μg/ml in a 5.5 pH. Using the medium BACTEC 7H12B (pH 5.6), found that PZA kills 0, 33, 60, 68, 57 and 72% of bacterial population in 31, 62, 125, 250, 500 and 1000 μg /ml during 2 weeks of treatment. The minimum bactericide concentration (MBC), defined as the death of 99% of the bacterial population, cannot be reached because even the highest concentration used (1000ug/ml) of PZA kills only 72% of the bacterial population.

C. Factors that affect the PZA activity

The pH in the medium is the most important factor which determines the activity of PZA. A big size of the inoculum reduces the PZA activity. Recent studies show that large inoculum sizes cause an increase in pH from 5.5 to neutral. The increment in pH would be due to de production of ammonia, product from the deamination of PZA by the bacterial pyrazinamidase. The Bovine Seric Albumin (BSA 0.5%) is a supplement in the culture medium of MTB. The BSA 5-10% increases the pH of the medium (pH 5.6) in one pH unit. It is found that BSA binds to POA significantly, but not to PZA.

IV. PYRAZINAMIDASE

The pyrazinamidase (PZase) is a hydrolase that convert the prodrug PZA to the active form POA as shown in figure 2. Due to the structural similarity, the nicotinamide and PZA are converted by the same enzyme nicotinamidase, called PZase, to acidic forms POA and nicotinic acid.

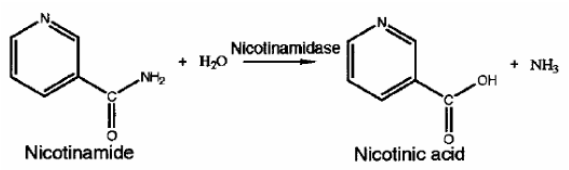

Fig. 3. Conversion of the nicotinamide and pyrazinamide to acidic forms by the pyrazinamidase/nicotinamidase

The nicotinamidase/PZase of MTB hydrolyses nicotinamide more efficiently than pyrazinamide. The enzyme is localized in the cytoplasm and is expressed constitutively. The nicotinamidase is a ubiquitous enzyme present in prokaryotes.

The gene product of Pyrococcus Horikoshii 999 (PH999) has a great sequence homology with the PZase of MTB. The three dimensional structure of PZase of PH999 revealed a potential binding site of a metal in the active site and confirmed that Zn2+ increased the enzymatic activity [9, 10].

V. PZA RESISTANCE MECHANISM

Clinical isolations of MTB resistant to PZA are often defective in the activity of PZase and there is a good correlation between the PZA resistance and the losing of enzymatic activity [11].

Strains of MTB resistant to PZA lack both of nicotinamidase and PZase, are resistant to nicotinamide also, but not to others antituberculosis drugs. Mutations in the gen pncA are the principal mechanism of resistance to PZA.

Strains of MTB resistant to PZA are susceptible to POA, indicating that the resistant to PZA is caused by changes in the PZase activity due to mutations in the pncA gen or in rarely cases to mutations in the regulatory gen of pncA [12, 13 and 14].

Mutations identified in the pncA gen are mostly missense, causing substitutions of amino acids and in other cases insertion or deletions of nucleotides; and mutations nonsense located in the structural gen of pncA or in the region promoter of pncA. The mutations are highly diverse and are distributed along the gen. However there is a certain degree of "grouping" in three regions of pncA, 3-17, 61-85 and 132-142. The three regions where the mutations of pncA seem to group, correspond to three of the four loops that contribute mostly to the skeleton of the active site.

A small proportion of strains resistant to PZA have no mutations in the gen pncA, neither in the putative promoter region. A group of these strains are PZA-negative, suggesting mutations in the promoter region or in an unknown regulatory gen of pncA, indicating a possible alternative mechanism of resistance to PZA. Another possibility is a failure to the entrance of PZA or functional alterations in the efflux pump.

VI. Potentiometric Method

A. Potentiostat

The potentiostat is a device of control and measurement that holds constant the current through the electrolytic cell.

We use UNISCAN INSTRUMENT PG 581, which has the following main features: Processor of dual 16 bits @18MHz, data acquisition of 16 bits with 100 kHz, rising time 1V/µs, maximum current +/-20mA, current range of 1nA to 10mA, resolution of applied voltage of 61µV.

B. Electrochemical System

The electrochemical system consists of a working electrode, auxiliary (counter) electrode and reference electrode, and the connections are as shown in the figure 4, as studies on electronic tongue [15, 16 and 17]. In this type of system, a double electric layer appears in heterogeneous systems. Immediately after applying a voltage, a solution layer charged is formed which consist in two parts; a compact inner layer where the potential decreases linearly with the distance, and a diffused layer where the potential decreases exponentially [18].

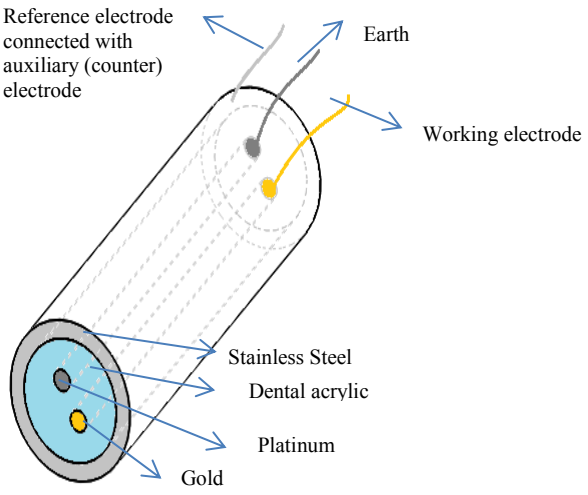

Fig. 4. Electrical connection of the electrode

C. Electrical Sensor

Electrical parameters for measurements are voltage, current, charge, resistance, capacitance, inductance, power, frequency and phase. In this study we measured linear voltammetry, and due to good reproducibility and robustness, no special shielding was needed for the small currents.

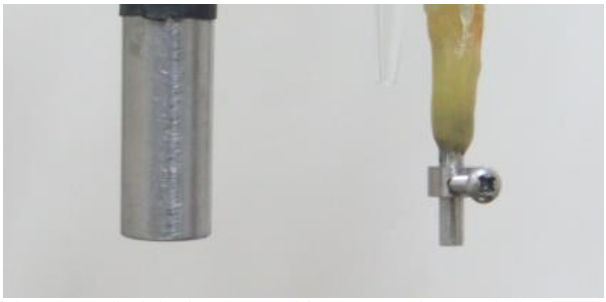

Fig. 5. In the left, the sensor with 13.2mm of external diameter is shown. In the right, the mini sensor with 4 mm of external diameter is shown.

D. Fabrication of the mini sensor

The sputum's samples have a volume of 1.5 ml, using this preliminary sensor we need a volume of 5ml. Therefore, to analyse the sample, we had to dilute the sample in water. Due to this limitation, we manufactured a mini sensor. The volume for samples reaches 100 µl.

The mini sensor is built using electrodes of gold and platinum with a diameter of 0.5mm. The external cylinder of stainless steel has an external diameter of 4mm, and an internal diameter of 3mm.

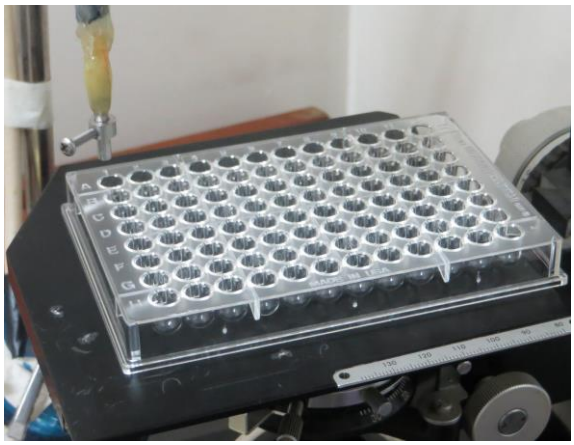

Fig. 6. The mini sensor is introduced in microplates of volumes of 100 µl.

E. Voltametric cycle

Many cycles can be researched such as cyclic voltammetry, chrono amperometry, chrono voltammetry, differential pulse voltammetry, square wave voltammetry, etc. In this study we used the linear voltammetry due to a good repeatability and stability.

VII. Results and Discussion

A. Linear Voltammetry with the sensor in Ethylene glycol

Due to the size of the sensor, it was used a 10 ml beaker with 5ml of sample to measure concentration of POA. For this experiment, we prepared POA solutions of 10µM, 100µM, 500µM and 2000µM in ethylene glycol 25% (in water). The linear Voltametric configuration was a sweep from -2 V to 2V with a sweep rate of 0.01 (V/s) and 0.001 volts per point. The Potentiostat gives us plots of voltage vs current from different concentrations of POA.

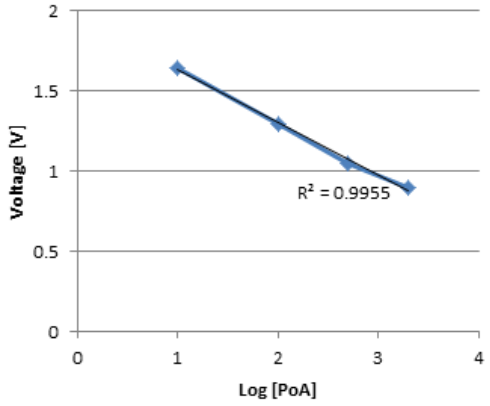

Fig. 7. Plot of the logarithm of concentration of POA in µM with the voltage where the current is -2.06µA.

The figure 7 shows a correlation of logarithm of the concentration with the voltage where the current is -2.06µA. The current of -2.06µA for this configuration has the best linear correlation from the experimental data, others currents haven't that good linear correlation.

B. Linear Voltammetry with the mini sensor

The size of the sensor allowed us to measure samples of 130µl in microplates. We tested concentrations of POA in water with of 2 µM, 4 µM, 8 µM, 10 µM, 100 µM, 500 µM, 2000 µM and 10000 µM.

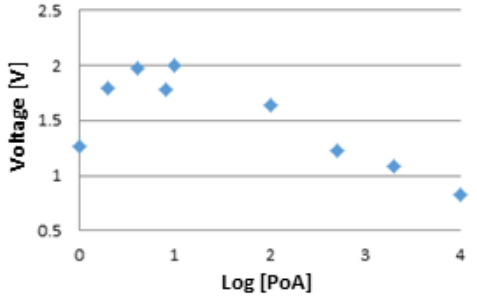

Fig. 8. Plot of logarithm of concentration of POA in µM with the voltage where the current is -0.9µA.

From figure 8, a plot of the Voltage vs logarithm of concentration of POA from plots of the Voltage vs Current obtained from the Potentiostat. It can be seen a linear correlation of logarithm of concentration of POA and Voltage from 10 µM to 10000 µM. Since the experimental results, from Wayne's procedure [19], detect at least 0.5mM of POA, we can observe that compared with the Wayne test, the potentiometric method can detect 50 times less concentration of POA, which give us a new and more sensitive method for the detection of POA and with that, the detection of resistance to Pyrazinamide in samples of M. tuberculosis.

C. Voltammetry with mini sensor in H37Rv strain's culture

We prepared 3 POA samples adding 20µl of culture to a final volume of 100µl with concentrations of 20 µM, 100 µM and 500 µM of POA

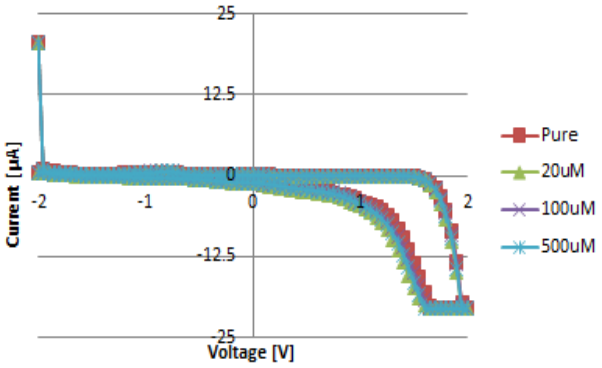

Fig. 9. Voltammetry of pure cultures (MF1 1/1C) and solutions of 20 µM, 100 µM and 500 µM of POA is shown.

From figure 9, differences on the graphic between different concentrations are appreciated. For a deeper analysis, we plot the logarithm of concentration and voltage where the current reveals a better linear correlation.

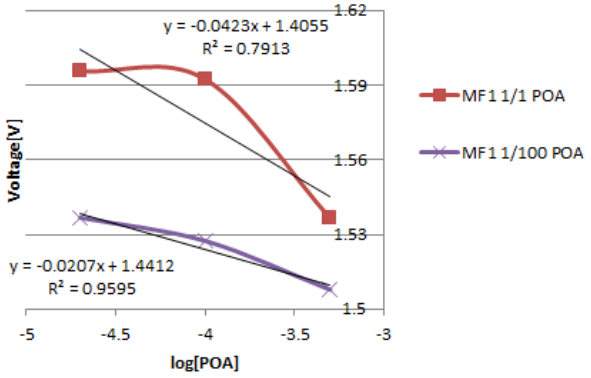

Fig. 10. Plot of logarithm of concentration of POA where the current is -20µA of two cultures (MF1 1/1 and MF1 1/100) is shown.

Figure 10 shows a good correlation between logarithms of concentration of POA with the voltage, where the current is -20µA on two cultures. MF1 1/100 is another culture where the plot has a greater linear correlation; and compared with MF1 1/1 it has 100 times less concentration of PZA added.

D. Voltammetry with mini sensor in decontaminated sputum's culture

Using 20µl of decontaminated sputum, solutions of 100ul with concentrations of 20µM, 100µM and 500µM of POA were prepared.

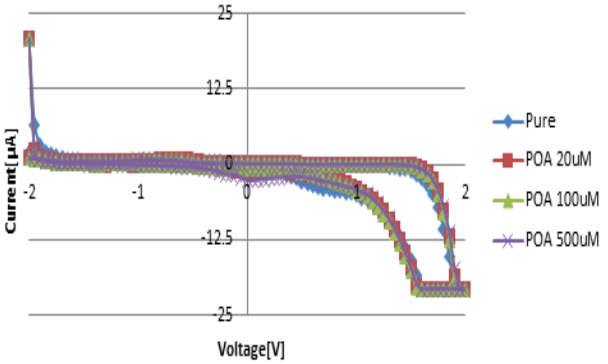

Fig. 11. Voltammetry of pure decontaminated sputum cultures and solutions of 20µM, 100µM and 500µM of POA is shown.

From figure 11, differences on the graphic between different concentrations are appreciated. For a deeper analysis, we plot the logarithm of concentration with voltage where the current reveals a better linear correlation.

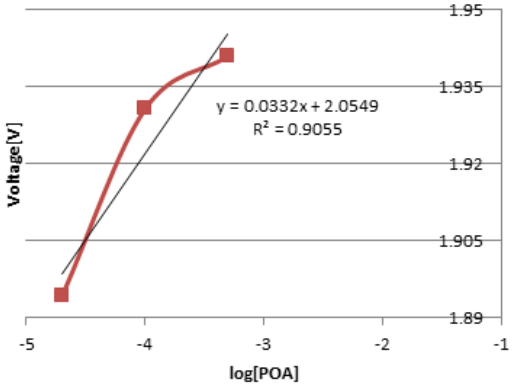

Fig. 12. Plot of logarithm of concentration of POA where the current is -20µA of decontaminated sputum is shown.

Figure 12 shows a good correlation between logarithms of concentration of POA with the voltage, where the current is -20µA. This correlation is inverse to the H37Rv strain's culture.

VIII. CONCLUSIONS

Two improvements from previous in vitro researches were achieved. The first one is that measuring of POA in Ethylene Glycol and water by potentiometric method is 50 times more sensible compared with Wayne. The second one is that by using a mini sensor we can measure volumes of 100 µl, 50 times less volume than the first sensor. Experiments with strain and sputum cultures were achieved and differentiated by concentration. And from the cultures of strain H37Rv, we found that the most difference is in the range of 1.4V to 1.7V.

By understanding the biomechatronic system, consisting of a mechanism of action of a drug in biological system, in this case the mycobacterium tuberculosis; and with the electrochemical signal, in this case voltammetry of POA, we can improve the present diagnosis of resistance to drug, such as the pyrazinamide.

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