

Development of noninvasive method of measuring copper and zinc by passive diffusion: an *in vitro* model

Queenie Charisse Landrito¹, Gianina Krisha Rodymae Menor¹, Fortunato Sevilla III², Leah Tolosa³ and Cristina Tiangco^{1,2,*}

¹Department of Chemical Engineering, Faculty of Engineering, University of Santo Tomas, España Boulevard, Manila 1008, Philippines

²Research Center for the Natural and Applied Sciences, University of Santo Tomas, España Boulevard, Manila 1008, Philippines

³Center for Advanced Sensor Technology, University of Maryland Baltimore County, MD, USA 21250

Abstract. There has been increasing interest in the determination of essential or toxic elements in human body fluids in relation to health and nutrition. The human body is composed of almost every natural element found in nature. This includes copper and zinc that are both essential if obtained at a right amount and toxic when exceeds. This study aims to develop a noninvasive method for measuring trace amounts of copper and zinc by passive diffusion. A cellulose acetate membrane was used as a model representing human skin. Different amount of permeant concentration of copper and zinc solutions such as 0.1, 0.5, and 1mM were placed inside the membrane and the samples were collected and analyzed using Atomic Absorption Spectroscopy. The amount of copper and zinc diffusing shows proportionality with time. The permeability and steady state flux were computed. Permeability of -2.39×10^{-7} cm/s and 6.67×10^{-7} cm/s were computed for copper at 0.1 mM and 0.5 mM concentrations respectively and 3.11×10^{-7} , 4.61×10^{-7} and 1.75×10^{-6} cm/s were computed for zinc at 1, 0.5 and 0.1mM permeant concentrations respectively. For steady state flux, the values computed are 1.22×10^{-3} , 9.03×10^{-4} and 6.84×10^{-4} $\mu\text{M}/\text{cm}^2\text{min}$ for zinc at 1, 0.5 and 0.1 mM respectively and -7.84×10^{-4} and 1.27×10^{-3} $\mu\text{M}/\text{cm}^2\text{min}$ for copper at 0.5 and 0.1 mM permeant concentrations. The values gathered are all acceptable taking into the account previous studies regarding skin diffusions. This method presents a noninvasive alternative method to track copper and zinc trends.

1 Introduction

Copper is a naturally produced metal and can be obtain through ingestion of food and water containing copper. It is an element that is responsible for metabolism, production of hemoglobin, myelin, melanin and it also helps thyroid gland to keep its functionality. Zinc on the other hand, is responsible for cellular metabolism, proper sense of taste and smell, normal growth, development of baby of a pregnant woman, and helps to avoid aging acceleration. Copper and zinc are both micronutrients that are essential due to its effect on the organs functionality. World Health Organization (WHO) has set limits for trace metal for a normal function of body, hence higher or lower concentration adversely affects normal biological function. These include copper and zinc imbalance that leads to several diseases like Alzheimer's disease and Wilson's disease or worst death.

Trace element deficiencies are associated to chronic diseases or to problems with its absorption. These have been shown to predispose a person to promote diabetes and complications. It was found out that mean values of zinc, manganese and chromium were significantly reduced in blood and hair samples and high mean values

of copper and iron were detected in diabetic patients [1]. Ratio of zinc to copper has been associated with the risk of developing coronary heart disease [2].

Current method in copper and zinc determination in human body includes analysis of media such as hair [1,3], fingernails [4], sweat [5-7], urine [8] and serum [9]. However, these media have each of its limitations, like for hair and fingernails, trace metals measurement could differ depending on the individual's ability to excrete toxins. Moreover, use of urine and serum to determine amount of metal in the body can vary depending on several factors affecting its level of toxins such as metabolism and environment. This study focuses on transdermal zinc and copper determination by passive diffusion. The advantage of considering skin as matrix is this is simpler and could be done without interference like oxygen, carbon dioxide and moisture. Transdermal glucose was measured by passive diffusion [10] and biosensor like glucose binding protein was immobilized to measure the micromolar levels of transdermal glucose [11]. Cellulose acetate dialysis membrane was used as model for human skin.

The objectives of the study were to measure the amount of transdermal copper and zinc by passive diffusion and to measure the parameters following the

* Corresponding author: cetiango@ust.edu.ph

diffusion of transdermal copper and zinc from the skin model.

2 Materials and methods

2.1 Materials and reagents

The cellulose acetate dialysis membrane was bought from Thermo Fisher Scientific. CuSO_4 , ZnSO_4 , NaH_2PO_4 , $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$, NaCl and NaOH are all analytical grade and supplied by Sigma-Aldrich. All aqueous solutions were prepared with distilled water.

Hitachi Z230 Atomic Absorption Spectrophotometer (AAS) was used during the experiment to measure the absorbance of copper and zinc standards and the collected samples.

2.2 Experimental set-up

2.2.1 Preparation of copper solution

For the preparation of 200 mM Copper (II) sulfate stock solution, 1.5955 g of Copper (II) sulfate was dissolved in 50 mL of distilled water. The stock solution was serially diluted to prepare three standard solutions with different concentrations of 0.1, 0.5 and 1.0 mM, which were used as the main solution placed inside the dialysis tubing during the experiment.

2.2.2 Preparation of zinc solution

For the preparation of 200 mM Zinc sulfate stock solution, 1.6147 g of Zinc sulfate was dissolved in 50 mL of distilled water. The stock solution was serially diluted to prepare three standard solutions with different concentrations of 0.1, 0.5 and 1.0 mM, which were used as the main solution placed inside the dialysis tubing during the experiment.

2.2.3 Preparation of buffer solution

A 10X Phosphate-buffered saline (PBS) was prepared by diluting 82.3 g NaH_2PO_4 , 23.5g $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$ and 40.0g NaCl in 1000mL distilled water. The buffer used per experiment was prepared by diluting 100 mL of 10X PBS in a 900 mL of distilled water to make it 1X PBS. A Sartorius pH meter was used to determine and maintain the pH of the buffer within the range of 7.0-7.4.

2.2.4 Passive diffusion set-up

A simple passive diffusion cell set-up was prepared with the copper and zinc solution placed inside the dialysis tubing and 1X PBS buffer was placed inside the 1 L beaker. Eight (8) samples of 20 mL each were collected from the 1 L buffer solution at 15 minutes followed by 30-minute interval. The collected samples were analyzed by using Atomic Absorption Spectrometry (AAS).

2.2.5 Determination of the calibration curve

The absorbance of standard solutions of Copper sulfate and Zinc sulfate were measured using AAS. The stock solution was serially diluted to prepare eight standard solutions with concentrations of 0.5, 1, 2, 4, 6, 8, 10 and 20 ppm. Calibration curve of the standard solutions were plotted and used in determining the concentration of the amounts of samples diffused over time.

2.2.6 Measurement procedure

The collected samples from buffer were also measured using AAS. Concentrations of copper and zinc diffused per unit time were calculated using the equation of the standard calibration curve.

2.2.7 Data analysis

Results from the diffusion cell experiments are reported as the amount of copper and zinc (ppm) that permeated the cellulose acetate dialysis membrane in each 15 and 30-minute interval, and the cumulative amount of copper and zinc permeation through the skin model plotted as a function of time. Passive diffusion experiments were performed to determine the skin diffusion experiments of steady-state flux (J_{ss}) and permeability coefficient (K_p).

Steady-state flux (J_{ss}) is the amount of permeant crossing the membrane at a constant rate and occurs after the lag phase. The flux can be calculated using **Eq. 1**.

$$J_{ss} = \frac{Q}{A \times t} \quad (1)$$

where Q is the quantity of compound transported through the membrane at time t , and A is the area of exposed membrane in cm^2 .

The permeability coefficient corresponds to the rate at which permeant penetrates the skin. The permeability coefficient can be calculated using **Eq. 2**.

$$K_p = \frac{Q}{[A \times t(C_o - C_i)]} \quad (2)$$

C_o and C_i are the concentrations of the compound on the outer side and the inner side of the membrane respectively.

3 Results and discussion

3.1 Absorbance of copper from passive diffusion

Results gathered from the experiment were reported as the amount of copper and zinc that permeated through the cellulose acetate membrane (ppm) in each 15 to 30-minute intervals, and the cumulative amount of copper and zinc permeation through the cellulose membrane was plotted as a function of time.

Fig. 1 shows that the relationship between the absorbance and time is directly proportional. It was observed that the highest absorbance recorded was from

0.5 mM. As time proceeds, the absorbance of the sample increases.

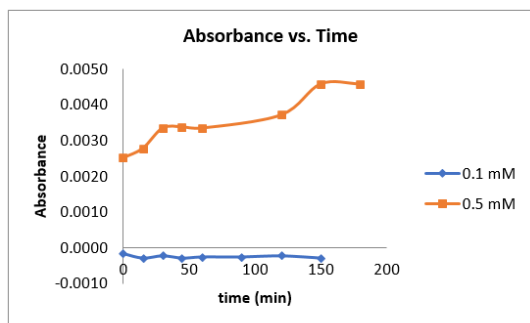


Fig. 1. Absorbance of copper from passive diffusion set-up.

Fig. 2 shows the calibration curve using serial dilutions of 200 mM CuSO₄ from the experiment. The linear range of the copper from standards is from 0.5 – 8.0 ppm, described by $y = 0.0146x - 0.0097$. The value of R^2 is 0.946, demonstrating a linear relationship between the absorbance and different concentration of copper standard solutions. This concentration is suitable for detecting the ppm range of transdermal copper.

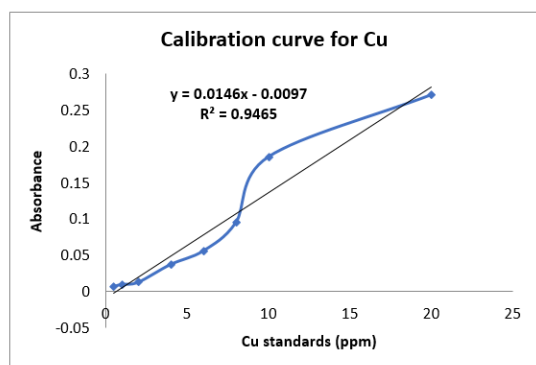


Fig. 2. Calibration curve of copper standard solution.

3.2 Transdermal copper concentrations

The transdermal copper concentrations were below 1 ppm. It was observed that the concentration increases as time increases. At 0.1 mM, the concentration diffused demonstrates a constant reading in the 180 min run, suggesting that the absorption of copper was minimal across the membrane. The results reported attest to the study made by Pirot et al [12] that the increase of copper concentration in the skin may be due to the build-up in the epidermis thus forming a reservoir following the slow diffusion through the dermis. In addition, the simple changes in the valence of copper as reported by Hostynek and Maibach [13], plays an important role for the rate of transfer across the cell membrane and skin. **Fig. 3** shows the copper concentrations from the passive diffusion experiment.

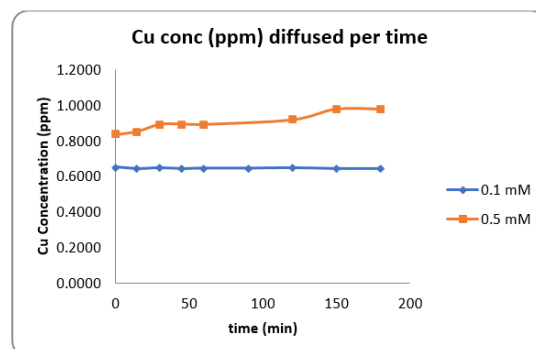


Fig. 3. Copper concentration diffusing from the cellulose membrane with respect to time.

3.3 Diffusivity of copper

As shown in **Table 1**, the flux and permeability coefficient depend on the concentration diffused per time. As the concentration increases, the flux and permeability coefficient increases. This is due to the particles moving from higher concentrations to lower concentrations. As the number of particles from the high concentration decreases, the particles contained in the lower concentration increases, thus promoting rapid diffusion rates. The permeability coefficient computed in the study is within the 10^{-7} - 10^{-3} cm/hr or 10^{-11} - 10^{-7} cm/s range of the permeability coefficients given by Hostynek and Maibach [13]. Deviations may be attributed to the errors made in the dilution of the sample. The concentration greatly affects the absorbance readings.

Table 1. The steady state flux and permeability coefficient for different concentrations of copper across the membrane.

Starting copper concentrations (mM)	Copper permeation after 180 minutes (ppm)(±SD)	J _{ss} , Steady state flux (μM/cm ² /min)	K _p , Permeability coefficient (cm/sec)
0.5	0.905± 0.75	1.27×10^{-3}	6.67×10^{-7}
0.1	0.647± 0.65	-7.84×10^{-5}	-2.39×10^{-7}

3.4 Absorbance of zinc from passive diffusion

As expected, from the three different permeant concentrations it was observed that the highest absorbance recorded was from 1 mM concentration. However, those three follows the same trend, which is in increasing order with respect to time. As time proceeds, the absorbance also increases. **Fig. 4** shows the increasing absorbance of zinc with time.

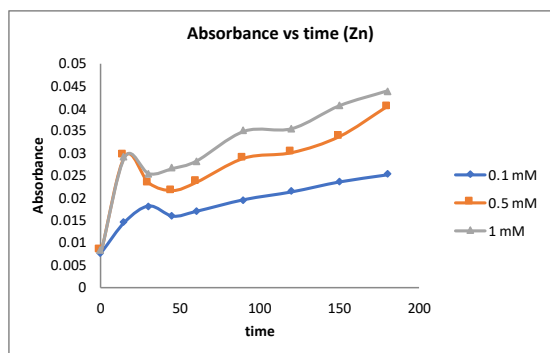


Fig. 4. Absorbance of zinc from passive diffusion set-up.

Fig. 5 shows the calibration curve using serial dilutions of 200 mM ZnSO₄ from the experiment. The linear range of the zinc from standards is from 0.5 – 4.0 ppm, described by $y = 0.2214x - 0.1018$. The value of R^2 is 0.998, demonstrating a linear relationship between the absorbance and different concentration of copper standard solutions. This concentration is suitable for detecting the ppm range of transdermal zinc.

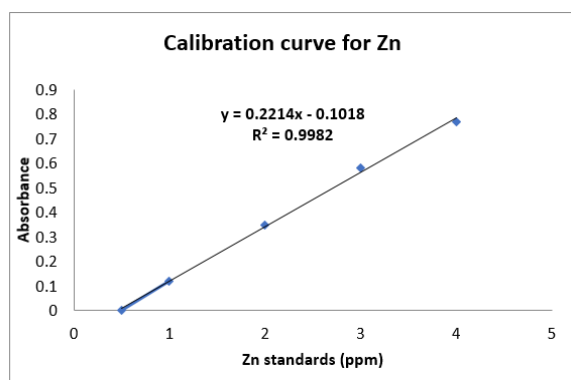


Fig. 5. Calibration curve of zinc standard solution.

3.5 Transdermal zinc concentrations

Zinc concentrations in 30-minute samples collected at eight sampling times for 0.1, 0.5 and 1.0 mM zinc solution were measured using AAS. The transdermal zinc concentrations were also below 1 ppm. The method of AAS is capable of detecting the zinc concentrations. **Fig. 6** shows the zinc concentrations from the passive diffusion experiment.

Through the linearity equation apprehended in calibration, the concentrations for the samples were computed. As shown in **Fig. 6**, the concentrations of the samples show the same trend as of that the absorbance. Therefore, the obtained sample data are the same with the standard samples when it comes to trend. The increasing concentration for the samples show that the zinc diffusing from the cellulose membrane is correlated with its absorbance, time of diffusion and the concentration of the permeant. The higher the permeant concentration, the faster the rate of diffusion, according to Markings [14]. Several factors affect the rate of diffusion including temperature, density of the diffusing substance, medium of diffusion and concentration gradient. Among those factors only the permeant

concentration is different for all the experimental runs. The larger the difference of concentration of the permeant and the buffer leads to greater probability of molecular collision resulting to increasing rate of diffusion [14]. Also, a good correlation was obtained after plotting transdermal zinc concentrations with zinc concentration (0.1-1.0 mM) inside the membrane ($R^2 = 0.9218$) as shown in **Fig. 7**.

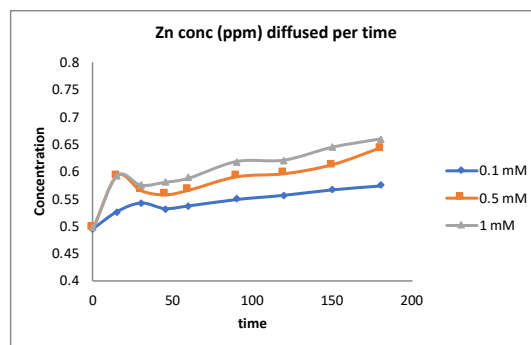


Fig. 6. Zinc concentration diffusing from the cellulose membrane with respect to time.

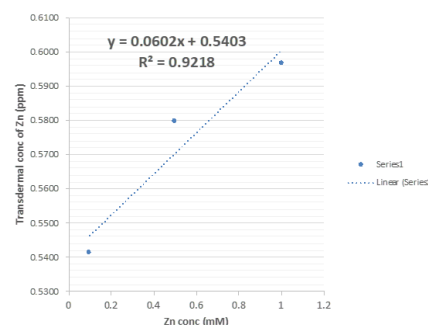


Fig. 7. Correlation between transdermal zinc and concentration of zinc inside the membrane.

3.6 Diffusivity of zinc

Table 2 shows the computed values using the given formulas. It was shown in the table that among the three the higher the permeant concentration the higher the flux. This being said, flux is proportional to the concentration gradient and the permeability is simply the constant that relates the flux and concentration gradient [15].

Table 2. The steady state flux and permeability coefficient for different concentrations of zinc across the membrane.

Starting Zinc conc. (mM)	Zinc permeation after 180 minutes (ppm)(±SD)	J _{ss} , Steady state flux (μM/cm ² /min)	K _p , Permeability coefficient (cm/sec)
1	0.597±2.15	1.22 × 10 ⁻³	3.11 × 10 ⁻⁷
0.5	0.580±4.57	9.03 × 10 ⁻⁴	4.61 × 10 ⁻⁷
0.1	0.541±0.236	6.84 × 10 ⁻⁴	1.75 × 10 ⁻⁶

4 Conclusion

This research study shows the feasibility of developing noninvasive method of measuring transdermal copper and zinc by passive diffusion. Consistent results were obtained using cellulose acetate dialysis tubing as the membrane. 0.905 and 0.647 ppm of transdermal copper while 0.597, 0.580 and 0.541 ppm of transdermal zinc were detected from the 1-0.1 mM concentration range inside the membrane, respectively. The concentration diffused is directly proportional to the concentration inside the membrane. Zinc permeated through the cellulose acetate membrane with good correlation between the external and internal zinc concentrations ($R^2 = 0.9218$).

The parameters for diffusion were determined by calculating the steady state flux and permeability coefficient. The steady state flux (J_{ss}) of transdermal copper ranges from $1.27 \times 10^{-3} - 7.84 \times 10^{-5}$ $\mu\text{M}/\text{cm}^2/\text{min}$ while its apparent permeability coefficient (K_p) ranges from $-2.39 \times 10^{-7} - 6.67 \times 10^{-7}$ cm/s. Whereas the computed steady state flux of transdermal zinc ranges from $1.22 \times 10^{-3} - 9.03 \times 10^{-4}$ $\mu\text{M}/\text{cm}^2/\text{min}$ while its apparent permeability coefficient ranges from $1.75 \times 10^{-6} - 4.61 \times 10^{-7}$ cm/s. This relatively shows that the higher the permeant concentration, the faster the rate of diffusion.

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