

## Chemical Reactivity of Chlorambucil in Organic Solvents: Influence of 4-Chloro Butyronitrile Nucleophile to Voltammogram Profiles

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Chlorambucil is one of anticancer drug that has been mainly used in the treatment of cancer disease. It is an alkylating agent and can be given orally. Interaction of chlorambucil in the presence of 4-chloro butyronitrile has not been studied electrochemically. We presented the investigation of electrochemical reduction and oxidation of chlorambucil in the presence of 4-chloro butyronitrile as a nucleophile in acetone and acetonitrile using cyclic voltammetry technique. The results showed that the value of chemical reactivities ( $K_f$ ) of chlorambucil in such solution system was higher than that without the presence of 4-chloro butyronitrile as a nucleophile. We found that at working potential range of 0.8 V to 1.5 V and scan rates range of 0.1 Vsec<sup>-1</sup> to 1 Vsec<sup>-1</sup>, the values of chemical reactivity of chlorambucil in acetone and acetonitrile, in the presence and absence of 4-chloro butyronitrile were 0.2715sec<sup>-1</sup>, 0.2676 sec<sup>-1</sup> and 0.2248 sec<sup>-1</sup>, 0.0245 sec<sup>-1</sup>, respectively. These results indicated that the values of chemical reactivity of chlorambucil can be affected by the type of solvent used. In addition, the shift of these values were influenced by the presence of another nucleophile in solution i.e. 4-chloro butyronitrile.

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**Keywords:** Chemical reactivity, chlorambucil, cyclic voltammetry, organic solvent

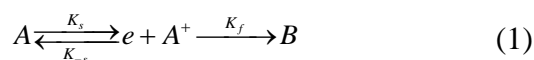
### 1. INTRODUCTION

Alkylating agent is a popular anticancer drugs that work through inhibition of cancer cell at DNA alkylation stage. Although these agents work effectively to cure cancer disease but the side effect

often arise by the appearance of the secondary cancer. The secondary cancer is the result of mutagenic activity of cells as the cumulative reaction of alkylating agent with normal cells [1, 2]. Alkylating agent reactivity is usually predicted using *in vitro* and *in vivo* method. Unfortunately, both methods are time consuming, and the result depends on the cell or objects condition which is not usually homogeneous. Recently, interest on bioelectrochemistry has been increasing, as well as Wen et al. performed on colchicine analysis in the presence of bovine serum albumin [3]. In this study, we developed the electrochemical method to measure the alkylating ability of an anticancer drug i.e. chlorambucil. In this method, we add a nucleophile i.e. 4-chloro butyronitrile in the solution system in order to reach a similar effect as the alkylating reaction occurred in the real cancer cells.

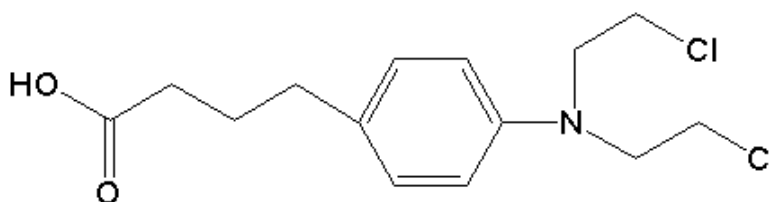
Alkylating ability of chlorambucil was measured by kinetic measurement i.e. reaction rate in different solvent (in the presence and absence of 4-chloro butyronitrile). Previous studies showed that most alkylating reactions of anticancer drugs followed  $SN_1$  mechanism, in which carbocation play an important role in the reaction. Since this mechanism analog to  $E_rC_i$  mechanism, the rate of alkylating reaction can be calculated using Nicholson Shain method [4, 5]. In this method, carbocation formed electrochemically and the solvent will play role as a nucleophile. Therefore, the influence of the nucleophile can be calculated using this method.

The reaction model is as follow:



Since in Nicholson Shain method, solvent play role as a nucleophile, hence the alkylating ability of anticancer drug with different nucleophiles can also be determined using various solvent i.e. acetone and acetonitrile. In order to complete determination of alkylating ability, we also determined the reaction rate constant ( $K_f$ ) with different levels of basicity.

Chlorambucil (Figure 1), is an alkylating agent that widely be used for the treatment of lymphocytic leukemia, lymphomas and ovarian carcinoma [6]. Application of chlorambucil had been used since 1964, but until now the study of chlorambucil is still intensively performed [7].



**Figure 1.** The structure of chlorambucil [6].

The side effects of chlorambucil are nausea, vomit, pulmonary fibrosis, spermatogenesis and jaundice [8, 9]. In genotoxic, the toxicity of chlorambucil was found in a DNA alkylation stage.

Chlorambucil induced chromosome breakages in the culture of human peripheral lymphocytes [10]. Pregnant women are strictly prohibited to use this agent due to the high risk in inducing the fetal abnormalities [11, 12]. Reports on the teratogenic effect was found in intra peritoneal administration of chlorambucil in rats [13, 14], as significant excess of lungtumors, lymphosarcomas and ovarian tumors arise [15, 16]. In addition, in the presence of croton oil, chlorambucil induces papillomas [17]. Schmahl et al. reviewed the occurrence of second tumor after chemotherapy using chlorambucil. In conclusion, 50% of second tumors were acute leukemia [18]. The individual case report was published by Reimer et al. which found leukemia and others tumor in chlorambucil treated patient [19, 20]. Catovsky and Galton also found myelomonocytic leukemia in a chlorambucil-treatment [21], and Steigbigel et al. reported an acute myeloproliferative disorder following longterm chlorambucil treatment [22].

## 2. EXPERIMENTAL

### 2.1. Apparatus and Procedures

Voltammetry measurements were conducted using BASi Epsilon Electrochemical analyzer. A standard three-electrode electrochemical cell was used for all electrochemical measurements with a platinum electrode (1 mm diameter) as working electrode, a platinum (Pt) wire as an auxiliary electrode and a Ag/AgCl (KCl 3 M) as a reference electrode. Cyclic voltammetry measurements were conducted in a homemade 10 mL glass cell. Working electrode was polished with an alumina suspension.

Measured solution was made by dissolving a certain amount of chlorambucil in solvents containing 0.1 M sodium perchlorate. Phosphate buffer solution was added to the solution before measurements were conducted. All data in the calculation were subtracted to the blank.

### 2.2. Chemicals

All Chemicals used in this study are in p.a grade. Chlorambucil was obtained from Sigma while acetone, acetonitrile, sodium perchlorate, potassium dihydrogen phosphate and sodium hydroxide were obtained from Merck.

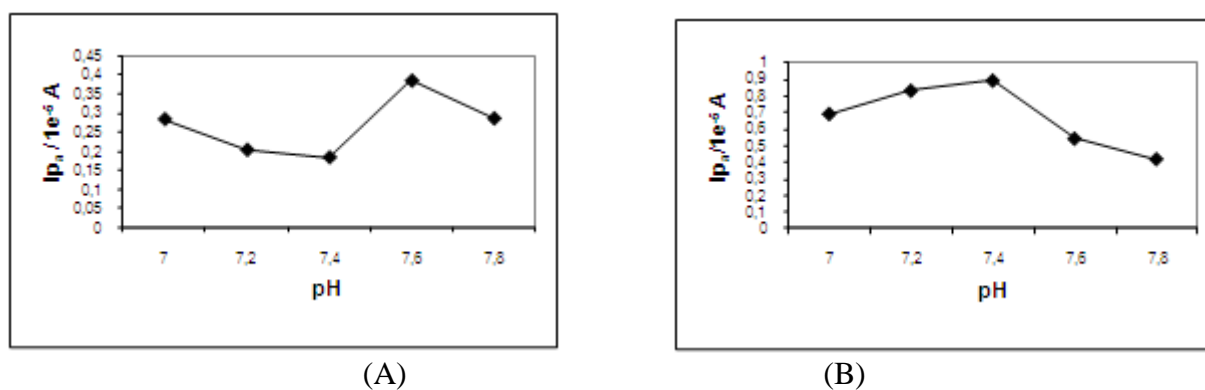
## 3. RESULTS & DISCUSSION

### 3.1. The Selection of supporting electrolyte solution and effect of the addition of pH buffer

The supporting electrolyte solutions were chosen carefully from four different solutions i.e. potassium chloride, potassium nitrate, sodium chloride and sodium perchlorate. The selection was made based on a curve analysis. The sharpest and highest peak of currents from the supporting

electrolyte solution were used as the selection parameters. The results of the analysis was found that sodium perchlorate reacted more favourably compare to the others.

The effective pH to activated chlorambucil in the cation form was found in the range of 6.0 – 8.0 [23]. Addition of phosphate buffer was performed to examine its influence to the peak at the current response in organic solution, although pH of the solution was still following organic solution system, instead of the buffer solution system. Determination of an optimum pH was chosen carefully in the range of pH 7.0 – 8.0. The selection parameter used was the highest peak current response from the  $I_p$  - pH curve (Figure 2). We found that in acetone and acetonitrile system,  $\text{KH}_2\text{PO}_4\cdot\text{NaOH}$  buffer solution gave the best response at pH 7.6 and 7.4, respectively.



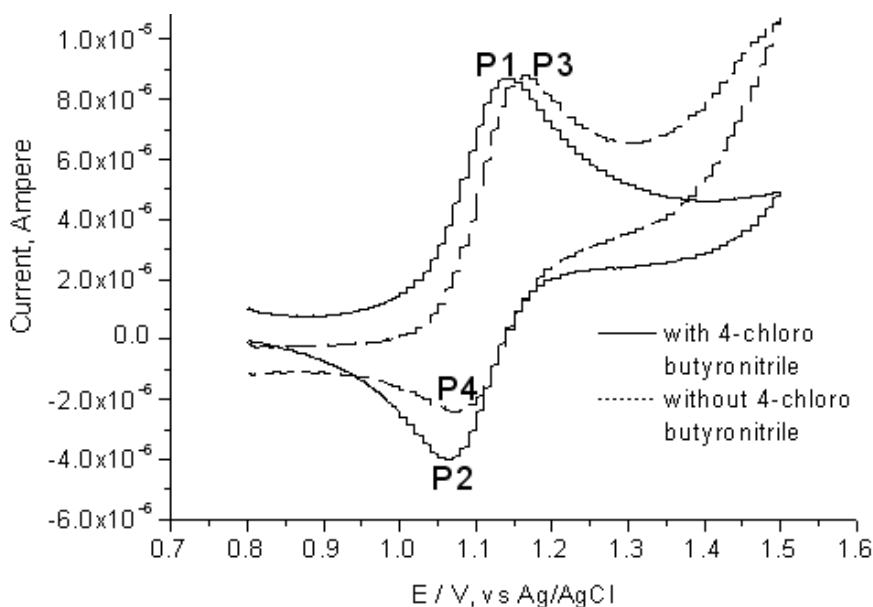
**Figure 2.** Effects of pH for 0.1M  $\text{KH}_2\text{PO}_4\cdot\text{NaOH}$  buffer solution in acetone (a) and acetonitrile (b).

The acid dissociation constant ( $\text{pK}_a$ ) of chlorambucil is 5.8 in aqueous solution. The value showed that chlorambucil is a weak acid. So, normally, if chlorambucil was measured in the range of pH 7.0-8.0, the current value will decreased along with the increasing of pH. If the value of pH increased, the concentration of hydrogen ion ( $\text{H}^+$ ) will be lower, thereby give a lower current during the formation of carbocation. In contrast, in the organic solution system, pH value was determined based on pH of the system solution, instead of the added buffer. Figure 2 showed that the highest current values were found at pH 7.6 and 7.4 for chlorambucil in acetone and acetonitrile, respectively. Those values reflect the existence of interactions among chlorambucil, water and organic solvent [24]. These interaction influenced the amount of carbocations formed. Further investigation is still needed, however especially for the interaction mechanism between two solutions medium. We also found that there was only a small amount change in the value of peak potential in different pH i.e. 0.001 – 0.01 V. Therefore, we strongly suggest that the addition of pH buffer do not influence the peak potential value.

### 3.2. The cyclic voltammogram profiles of chlorambucil in the presence and the absence of 4-chloro butyronitrile

Figure 3 showed the cyclic voltammograms of chlorambucil in the presence and the absence of 4-chloro butyronitrile in acetonitrile. A pair of reversible redox peaks (P1 and P2; P3 and P4) at the

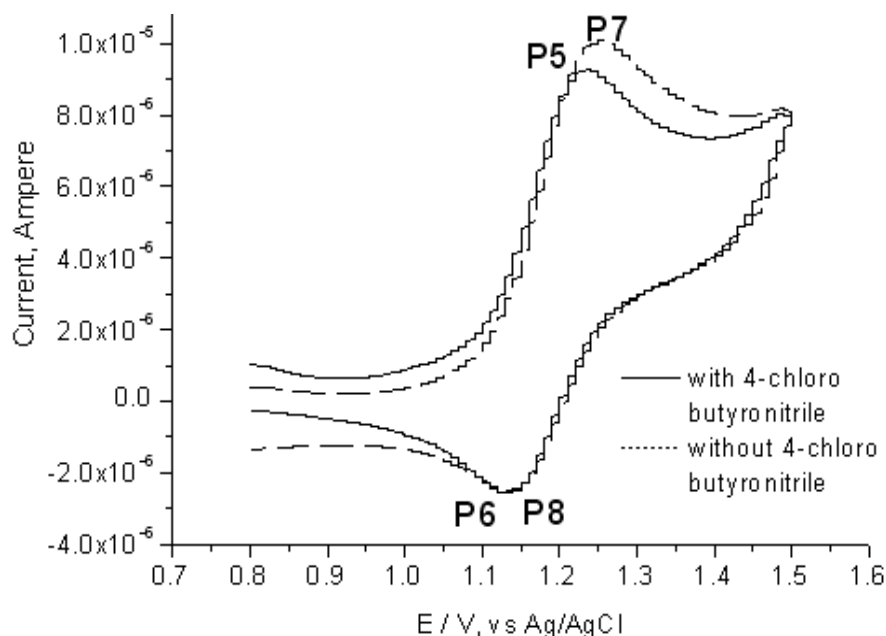
scan rate of  $1 \text{ Vsec}^{-1}$  appeared at potentials of  $E_{p_a} = 1.14 \text{ V}$  and  $E_{p_c} = 1.06 \text{ V}$  with  $E^0 (E_{1/2}) = 1.10 \text{ V}$ ;  $E_{p_a} = 1.16 \text{ V}$  and  $E_{p_c} = 1.08 \text{ V}$  with  $E^0 = 1.12 \text{ V}$ , respectively. When the reactions process followed the  $E_rC_i$  mechanism, the peak's currents of reduction will increase along with the increasing of the scan rate. The opposite effect applies to the reactions followed the  $E_rC_r$  mechanism. This is the reason why we use relatively fast scan rate i.e.  $1 \text{ Vsec}^{-1}$  [25]. Our results also suggested that chlorambucil was relatively prone to be oxidized in the presence of 4-chloro butyronitrile. The detected peak's current was higher than that in the absence of nucleophile during the reduction process on the backward of scan rate. In contrast, cyclic voltammogram of chlorambucil in acetone at  $1 \text{ Vsec}^{-1}$  showed insignificance differences in its potential peak reduction (Figure 4). The shift of peak potential in the presence and in the absence of 4-chloro butyronitrile are as follow  $E_{p_a} = 1.23 \text{ V}$  (P5) and  $E_{p_c} = 1.14 \text{ V}$  (P6) with  $E^0 (E_{1/2}) = 1.185 \text{ V}$ ;  $E_{p_a} = 1.25 \text{ V}$  (P7) and  $E_{p_c} = 1.14 \text{ V}$  (P8) with  $E^0 (E_{1/2}) = 1.195 \text{ V}$ . The oxidation's peak current of chlorambucil in the presence of 4-chloro butyronitrile was  $9.44 \mu\text{A}$ . It was lower compare to that in absence of 4-chloro butyronitrile, which was  $10.13 \mu\text{A}$ . In contrast, the reduction's peak current was almost similar both in the presence and in the absence of 4-chloro butyronitrile, which were  $2.58 \mu\text{A}$  and  $2.67 \mu\text{A}$ , respectively. These values influence the calculation of the rate constant in each condition, the interaction type and the competition between 2 nucleophiles in chlorambucil's carbocation.



**Figure 3.** Cyclic voltammograms of chlorambucil in acetonitrile in the presence and the absence of 4-chloro butyronitrile.

Acetone has a higher donor number and hydrogen bonding acceptor basicity values than acetonitrile. In order that, chlorambucil's reactivity in acetone should be higher than in acetonitrile. In cyclic voltammogram profile, one of chemical reactivity parameter which can be seen is the oxidation's peak current ( $I_{p_a}$ ) value. Value of  $I_{p_a}$  is a quantity of carbocation formed during the reaction process, which are the amount of carbocation reacted with the solvent. Figures 3 and 4 showed cyclic

voltammogram profiles of chlorambucil both in the presence and the absence of 4-chloro butyronitrile in acetonitrile and acetone, respectively. Based on Figures 3 and 4, it can be decided that the reactivity of chlorambucil in acetone with the presence of 4-chloro butyronitrile is more reactive than that in acetonitrile because the peak of P5 is higher than P1. This proposed statement is in the line with the principle stated above and can be proved in Tables 1 and 2 which showed that  $I_{p_a}$  values of chlorambucil in acetone with the presence of 4-chloro butyronitrile is higher than that in acetonitrile.



**Figure 4.** Cyclic voltammograms of chlorambucil in acetone in the presence and the absence of 4-chloro butyronitrile.

### 3.3. Effect of the applied scan rate

Based on Table 1 and Table 2, the cyclic voltammogram of chlorambucil in the presence of 4-chloro butyronitrile exhibited the shift in the oxidation's peak potential in 0.5 % of standard deviation in acetonitrile. In Table 2, there is no apparent shift in the oxidation's peak potential in acetone. In contrast, the reduction's peak potential both in acetone and acetonitrile shifted with standard deviation of 0.4 % and 0.3 %, respectively.

Based on the plots of currents versus potentials, the cyclic voltammogram profile of chlorambucil in acetone showed a better response than in acetonitrile. Figures 5 and 6 showed that in various scan rates, the cyclic voltammogram of chlorambucil in the presence of 4-chloro butyronitrile gave significant differences. Based on the figures, the increasing of scan rate in the line along with increasing of oxidation and reduction of peak current, and increase of scan rate tend the oxidation potential value to more positive for  $E_{p_a}$  and more negative for  $E_{p_c}$ . The pair of reversible redox peaks were showed by  $P_A$  and  $P_B$ ;  $P_C$  and  $P_D$  peak's pair potential. The cyclic voltammogram did not showed significant difference when the initial potential was varied between the value of 0.8 V and 1.5 V. It

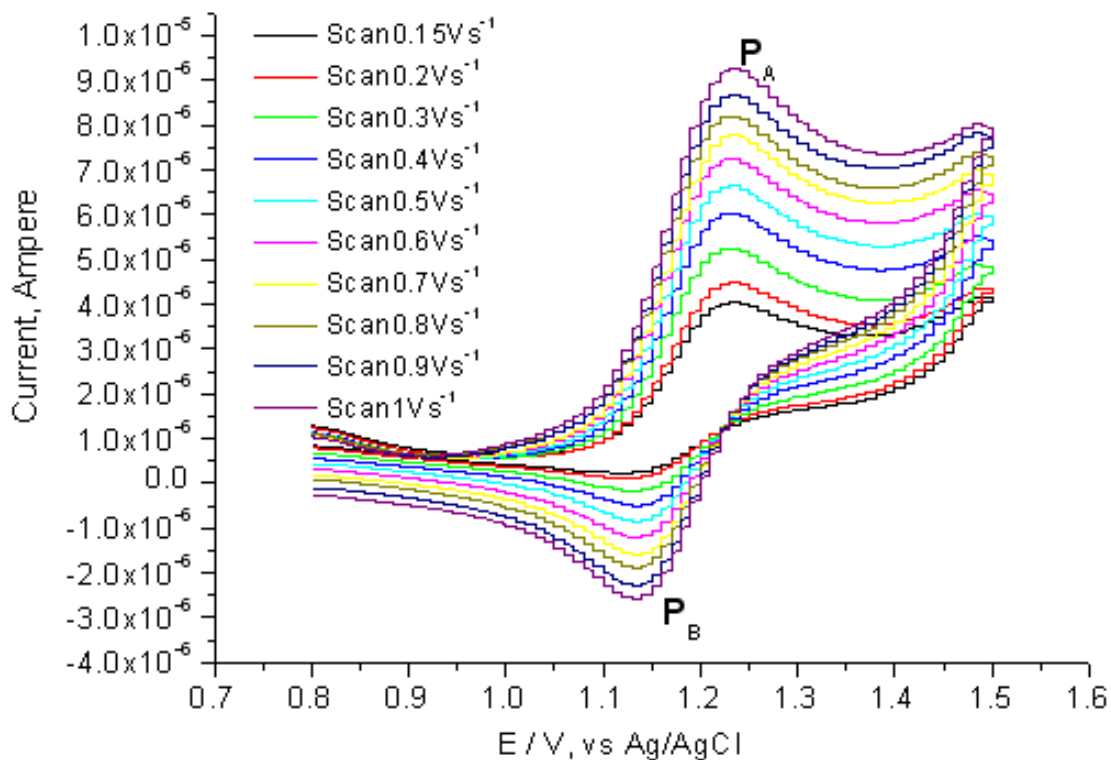
was likely that the equilibrium was established between the oxidation and reduction forms of chlorambucil.

**Table 1.** Potentials vs currents of chlorambucil in the presence of 4-chloro butyronitrile in acetonitrile in various scan rates.

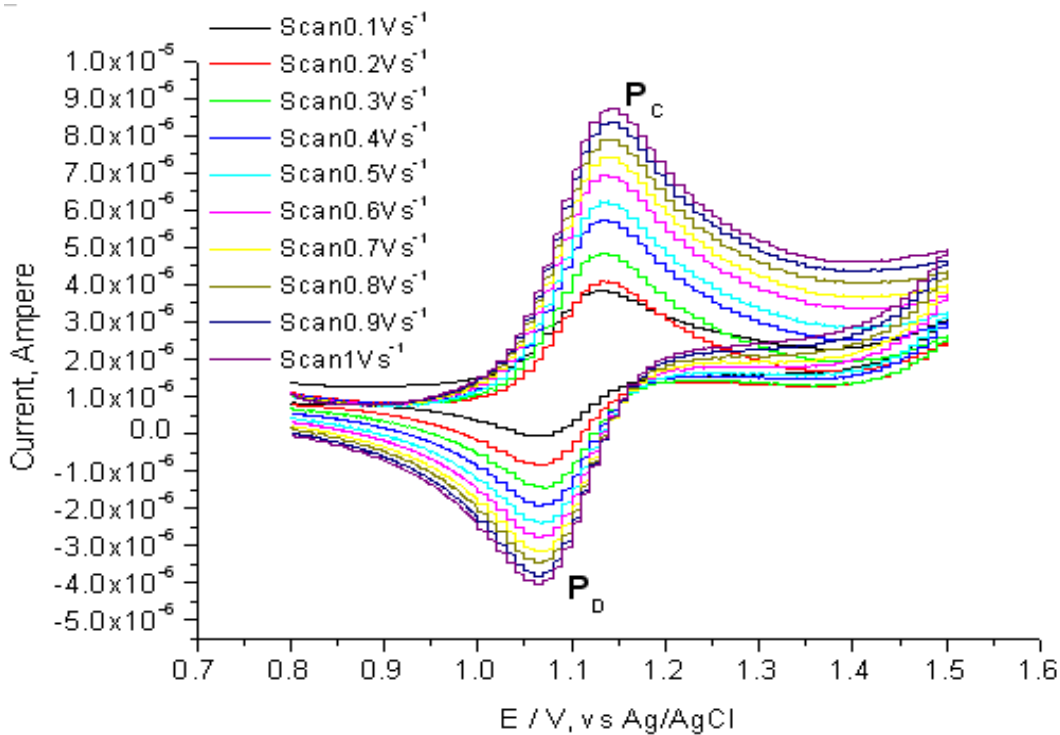
Scan Rates (V/s)	Potential (Volt)		Current (Ampere)	
	Ep <sub>a</sub>	Ep <sub>c</sub>	Ip <sub>a</sub>	Ip <sub>c</sub>
0.10	1.13	1.07	3.84E-06	-6.04E-08
0.20	1.13	1.07	4.08E-06	-8.42E-07
0.30	1.13	1.07	4.85E-06	-1.46E-06
0.40	1.14	1.07	5.75E-06	-1.94E-06
0.50	1.13	1.07	6.23E-06	-2.42E-06
0.60	1.14	1.07	6.95E-06	-2.79E-06
0.70	1.14	1.06	7.42E-06	-3.17E-06
0.80	1.14	1.07	7.89E-06	-3.49E-06
0.90	1.14	1.07	8.34E-06	-3.81E-06
1.00	1.14	1.07	8.71E-06	-4.03E-06

**Table 2.** Potentials vs currents of chlorambucil in the presence of 4-chloro butyronitrile in acetone in various scan rates.

Scan Rates (V/s)	Potential (Volt)		Current (Ampere)	
	Ep <sub>a</sub>	Ep <sub>c</sub>	Ip <sub>a</sub>	Ip <sub>c</sub>
0.15	1.23	1.14	4.09E-06	-1.10E-08
0.20	1.23	1.14	4.53E-06	-6.57E-08
0.30	1.23	1.13	5.29E-06	-1.75E-07
0.40	1.23	1.13	6.06E-06	-5.58E-07
0.50	1.23	1.13	6.66E-06	-8.86E-07
0.60	1.23	1.13	7.26E-06	-1.26E-06
0.70	1.23	1.13	7.81E-06	-1.65E-06
0.80	1.23	1.13	8.25E-06	-1.92E-06
0.90	1.23	1.13	8.69E-06	-2.30E-06
1.00	1.23	1.13	9.29E-06	-2.58E-06



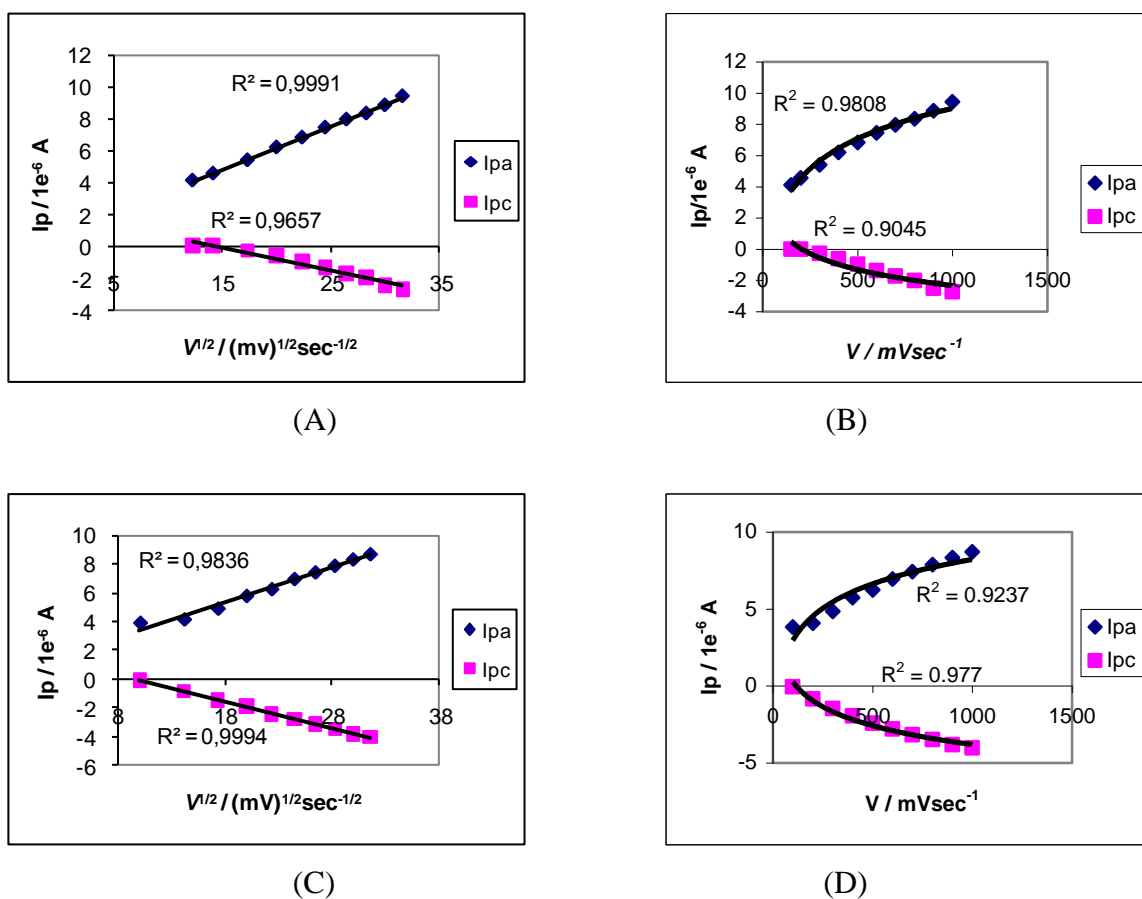
**Figure 5.** Cyclic voltammograms of chlorambucil in acetone in the presence of 4-chloro butyronitrile. The scan rates from inner to outer: 0.15; 0.2; 0.3; 0.4; 0.5; 0.6; 0.7; 0.8; 0.9 and  $1.0 \text{ Vsec}^{-1}$ .



**Figure 6.** Cyclic voltammograms of chlorambucil in acetonitrile in the presence of 4-chloro butyronitrile. The scan rates from inner to outer: 0.15; 0.2; 0.3; 0.4; 0.5; 0.6; 0.7; 0.8; 0.9 and  $1.0 \text{ Vsec}^{-1}$ .



Figures 7A and 7C showed the relationship of  $I_p$ 's peak current and the square root of scan rate in acetone and acetonitrile, respectively. The four plots of  $I_p$  versus  $V^{1/2}$  gave a straight line with the average value of  $R^2 > 0.96$ . These results strongly suggested that the reversible redox reaction was controlled by the diffusion step. In a different way, Figure 7B (acetone) and Figure 7D (acetonitrile) showed the relationship between  $I_p$ 's peak current and the scan rate ( $V$ ). The four plots of  $I_p$  versus  $V$  gave a logarithmic relationship with the average value of  $R^2 > 0.90$ . The logarithmic of the plot between  $I_p$  and  $V$  reflect a diffusion controlled of the reversible redox reaction of chlorambucil and not an adsorption-driven process.



**Figure 7.** Plots of the peak's current values ( $I_p$ ) vs the square root of scan rates ( $V^{1/2}$ ) and the peak's current values ( $I_p$ ) vs the scan rates ( $V$ ) of chlorambucil in acetone (A and B) and acetonitrile (C and D), respectively.

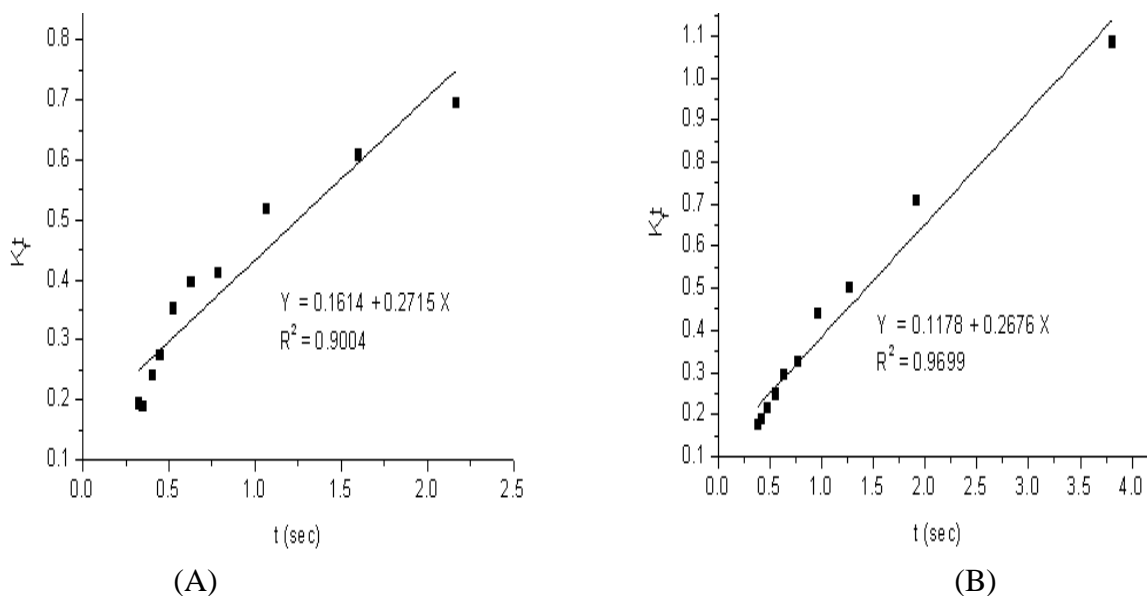
### 3.4. The Determination of forward reaction rate constant ( $K_f$ )

In order to determine the forward reaction rate constant ( $K_f$ ) of chlorambucil in different solvents, we calculated the parameters of each analyt in the cyclic voltammogram to obtain the values of  $I_{p_c}/I_{p_a}$ ,  $E^0$  and  $t$ . The determination of  $K_f t$  values was based on the working curve plots of Nicholson ( $I_{p_c}/I_{p_a}$  versus  $\log K_f t$ ) [4]. The value of  $K_f$  determined as the value of slope from the curve of  $K_f t$

versus  $t$  of each analyt. Both of reduction-oxidation peak were determined by using  $I_p/I_{p_a}$  calculation with the correction of Nicholson equation [26], i.e.

$$\frac{I_{p_c}}{I_{p_a}} = \frac{(I_{p_c})_0}{(I_{p_a})_0} + \frac{(0.485)(I_{p_c})_0}{(I_{p_a})_0} + 0.086 \quad (2)$$

Plots of  $K_f t$  versus  $t$  of chlorambucil in acetone and acetonitrile of chlorambucil with the presence of 4-chloro butyronitrile produce a linear curves with the slope of  $0.2715 \text{sec}^{-1}$  and  $0.2676 \text{sec}^{-1}$ , respectively (Figure 8). These result is greater than the calculated rate constant of chlorambucil's forward reaction without the addition of 4-chloro butyronitrile i.e.  $0.2248 \text{sec}^{-1}$  and  $0.0245 \text{sec}^{-1}$  for acetone and acetonitrile, respectively. These results showed that the chlorambucil's chemical reactivities (the forward reaction rate constant) were not gave rise to the same value in different solvents. In other word, the solvent influences directly to the chemical reactivity. In acetone, chlorambucil has a higher value of  $K_f$  than in acetonitrile. We think that it is caused by the physical properties of solvent such as the number of hydrogen bond donor and also hydrogen bonding acceptor basicity. Based on these values, it can be infered that acetone is strongly suggested to be more recommended solvent than acetonitrile, particularly for chlorambucil. The addition of another nucleophile compounds affect to the value of chemical reactivity, but did not affect to the sequence.



**Figure 8.** Plot of  $K_f t$  versus  $t$  to find values of  $K_f$  of chlorambucil in the presence of 4-chloro butyronitrile in acetone (A) and in acetonitrile (B).

#### 4. CONCLUSIONS

Cyclic voltammetry measurements provide useful information about the current and potential values in the oxidation-reduction of chlorambucil in acetone and acetonitrile. The presence of 4-chloro butyronitrile as a the nucleophile beside the solvent in the oxidation-reduction of chlorambucil, will

inevitably impact on the value of its chemical reactivity. Change in the value of chlorambucil's chemical reactivity can be seen from the trend shift occurring in the oxidation and reduction potentials in the cyclic voltammogram formed.

#### ACKNOWLEDGMENT

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