High Performance Liquid Chromatography of Amino Acids Using Potentiometric Detector With A Tungsten Oxide Electrode

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Abstract— High Performance Liquid Chromatography (HPLC) method combine with potentiometric detector was applied as a method for analysis of amino acids (aspartic acid, glutamic acid and arginine). The separation was carried out on a C18 column with isocratic elution of a mixture of acetonitrile and phosphat buffer. Potentiometric detector using a tungsten oxide electrode as working electrode and Ag/AgCl as refference electrode. The detection method was based on the presence of H+ ions from amino acids. This application was a development of previous studies that was succesfully applied to detect the presence of amino acids in batch and flow systems. Amino acid determination was characterized by its retention time. Retention time of aspartic acid, glutamic acid and arginine were as follow: 8,46; 13,0; and 15,21 minutes. The optimized separation conditions obtained at a flow rate of 1,2 mL/min with 15% acetonitrile concentration, buffer pH 6,5 with phosphat concentration of 5×10^{-4} M. Detector performance tested by the recovery test of samples, and the results obtained for glutamic acid, aspartic acid and arginine respectively: 89,1%, 94,9%, and 110%. Linear range obtained at 10^{-3} M to 10^{-7} M. Detection limit were $1,58 \times 10^{-7}$ M for glutamic acid, $6,58 \times 10^{-8}$ M for aspartic acid and $6,51 \times 10^{-8}$ M for arginine.

Key words— amino acid, HPLC, tungsten electrode, potentiometry

INTRODUCTION

Liquid chromatography has become a widely used method for amino acids separation. Liquid chromatographic method often used spectrophotometric, photometric, fluorometric, mass spectroscopy and electrochemical detector [1].

UV detector was the most popular and most widely used detector. This detector are less suitable for the determination of several components that have lack of strong group chromophore, however the detection of analyte must be at high enough concentration [2]. This detector also less selective because some compounds that have the same chromophore group will also be detected by generating adjacent peaks in chromatogram. Most of amino acids do not emit light and contain a little group chromophore. Its allows the use of a UV detector directly only at low wavelengths that can affect the sensitivity and interference. Therefore, using UV spektrophotometer as a detector required more complex treatment such as reaction with other compounds, hydrolysis and derivatization of amino acids [3]. Other commonly used detector is mass spectroscopy detector. This detector is relatively expensive, although quite selective in detecting analytes.

Recently, potentiometric detection has also been used in conjunction with various method for determination of amino acid. The use of this detection method due to their extensive application areas and reliability, short analysis time, high sensitivity and selectivity [4,5,6]. Potentiometric technique has been applied using ion-selective electrodes. The use of antimony wire electrode for the determination of carboxylic acids in FIA and HPLC had also been published. Masruroh and Dewi [7,8]also published a potentiometric using tungsten oxide electrode for detecting amino acids in batch and flow system. Tungsten, has been known as one of metal that can serve as a pH indicator [9]. Chen was conducted to detect carboxylic acids with tungsten oxide is highly prospective for the potentiometric detection in flow system [10]. The use of potentiometric detector in conjunction with liquid chromatography for carboxylic acids analysis has also been published [10].

Experimental

a. Instrumentation

pH meter (Jenway), ultrasonic degasser, HPLC instrument: Waters associates models, Waters 515 pump, Rheodyne 7125 injection valve with a sample loop 20 µL (Waters Associates, Miliford, MA). Column µ-bondapak C-18, 3,9 x 300 mm with a particle size of 10 µm (Waters Associates), potentiometric detector: tungsten oxide electrode (working electrode), Ag/AgCl electrode (refference electrode), and the potential differences between electrode monitored by a digital voltmeter Sanwa PC 500 connected to a PC.

b. Reagen and Solution

All reagen used were analytical grade and dissolved with distilled water. Buffer solution prepared by mixing 5×10^{-4} M $Na_2HPO_4.2H_2O$ and 5×10^{-4} M $NaH_2PO_4.2H_2O$ to obtain pH 5,5, pH 6,0, pH 6,5, pH 7,0 and pH 7,5 measured by Jenway pH meter. Carrier solutions were prepared by dissolving acetonitrile with distilled water and filtered by whatman membrane. Stock solutions of amino acids were prepared by dissolving aspartic acids (Merck), glutamic acids (Merck) and arginine (Merck) and adjusted with distilled water to make 1×10^{-1}M. The solutions then filtered with Whatman filter with a pore size of 0.2 µm, and diluted to the required concentrations when necessary.

c. Preparation of Tungsten Oxides Electrodes

Preparation of tungsten oxide electrode referred to [10] as well as [9] with the following steps: tungsten rod with a diameter of 1 millimeter washed with acetone than heated in a furnace with a temperature of 500° C for 1 hour until the tungsten surface become yellow-green. Tungsten electrodes than cooled up and were stored in a solution of 1×10^{-3} M NaOH for 24 hours and before use, the tungsten electrode was washed with distilled water.

d. Optimization of HPLC Condition and Characterization of Tungsten Oxides Electrodes

Flow rate: the effect of flow rate was analyzed by changing the flow rate at 0,4; 0,6; 0,8; 1,0 and 1,2 mL/min. Composition of eluent (carrier) and pH of the solution was maintained constant. The optimum flow rate was obtained by plotting the variation of the flow rate of the potential (mV).

Eluent (carrier) Composition: Determine the effect of eluent composition by varying the eluent composition of 5%, 10%, 15% and 20% acetonitrile, with constant pH and flow rate. The optimum eluent composition was obtained by plotting the variation of the eluent composition on the potential (mV).

Buffer pH: Determine the effect of pH buffer solution by varying buffer pH solution at pH 5,5; 6,0; 655; 7,0 and 7,5, but constant eluent composition and flow rate. The optimum pH was obtained by plotting the variation of pH on the potential (mV).

Calibration curve: a series of standart solution with concentration of 10^{-3} M, 10^{-4} M, 10^{-5} M, 10^{-6} M, and 10^{-7} M was measured at optimum condition of flow rate, buffer pH and eluent composition

Reproducibility: standard solution of amino acids (glutamic acids, aspartic acids and arginine) with concentration of of 10^{-3} M, 10^{-4} M, 10^{-5} M, 10^{-6} M, and

10^{-7} M were measured by HPLC-potentiometric detection using tungsten oxide electrode.

Recovery test: the sample used was a mixture of standard solution of glutamic acid, aspartic acid and arginine. Measurements were conducted using the optimum conditions of optimization flow rate, eluent composition and pH buffers.

RESULT AND DISCUSSION

The selectivity of separation in High Performance Liquid Chromatography (HPLC) reversed phase can be altered by changing various parameters, such as the flow rate, buffer pH, and carrier composition.

Flow rate was one of parameters that has a correlation with the retention time, so it can be used as an optimized parameter for components analysis in the sample. Retention time will be inversely proportional to the flow rate of the carrier. The slower the flow rate of the carrier, the greater the retention time. The smaller the retention time in this case did not mean the better the signal would be generated. The flow rate in the system can be used to improve the chromatographic retention time, resolution and selectivity of a separation

a. Development of the Optimum Flow rate

The results of potential difference measurements for six different flow rates used, can be seen in Figure 1. The potentiometric responses of tungsten oxide electrode and Ag/AgCl showed the optimum potential difference at flow rate of 1,2 mL/min. At this flow rate, almost all H+ ions from amino acids were eluted and detected at the same time. The higher the H+ ions detected at the same time, the higher the response.
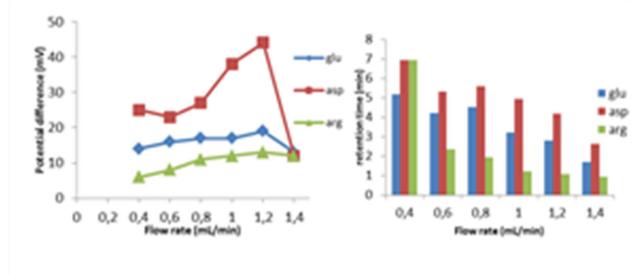

Figure 1. Effect of flow rate on potential differences and retention time of amino acids

Mobile phase (carrier) composition also affecting the separation process in HPLC. Different composition of acetonitrile-water would make different separation. Optimum separation and condition obtained by analyzing retention time and potential differences.

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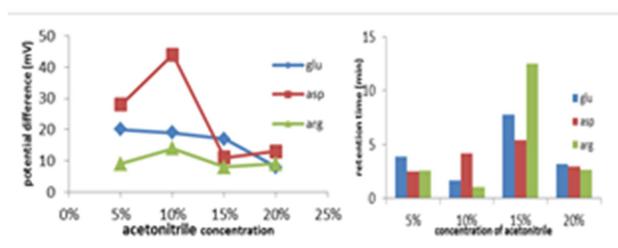

Figure 2: Effect of acetonitrile concentration on (a) potential difference and (b) retention time

Based on Figure 2, that potential differences decreased when acetonirile concentration increased and

optimum potential difference at 10% acetonitrile. At this composition, the potential differences was high, but the retention time of aspartic acid and arginine at this concentration were close to each other. In this concentration, aspartic acid and arginine were difficult to be separated, so that the concentration of acetonitrile used was that having a large enough difference in retention time for each amino acid was at a concentration of 15%, although at different concentrations of 15% was not the optimum potential but the retention times for the three amino acids at these concentrations differ greatly.

c. Optimum PH

The influence of pH buffer on the electrode responses was investigated using a different pH of phosphat buffer (5,5 to 7,5) at concentration 5×10^{-4} M. Buffer solution can maintain the pH of the environtment from the effect of the addition of a litle acid/strong base, or by dilution. Buffer also can maintain the stability of the sample. In solution, amino acids are able to ionized to produce H+ ions. The process is depends on pH of the solution.
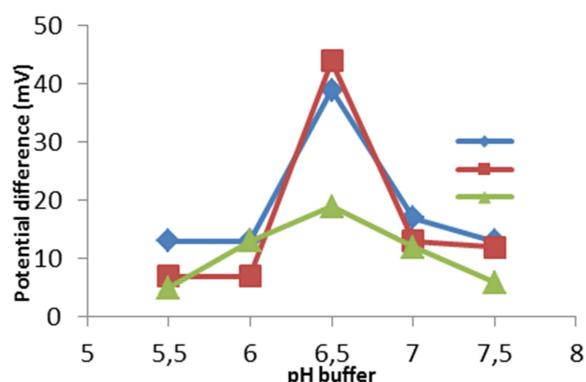

Figure 3: Effect of pH buffer to potential differences

The characteristic of an amino acid is determined by its R group. Each amino acid has a different pKa value for its R group. For aspartic acid pKa was 3,86, glutamic acid 4,25 and 12,48 for arginine. Ka shows the ionization constants of the amino acids. The greater the Ka of amino acids, the stronger the amino acid. The stronger amino acids, the greater the H+ ions generated. Based on the data obtained, the more produce H+ ions were aspartic acid, glutamic acid, and then the last one was arginine. So that the potential differences generated by aspartic acid was higher when compared with glutamic acid and arginine, because electrodes can detect more H+ ions .

All of the peaks of amino acids emerge earlier and sharper if the pH is too high, and peaks the chromatograph later if the pH is too low. Optimization results show that at pH 7,0 and pH 7,5, potential differences all three amino acids decreased. At the buffer of pH 5,5, pH 6,0 and pH 6,5 the amount of H+ ions in solution increased. Electrode will detect total H+ ions from the buffer or from amino acids. At pH 7 and pH 7,5 buffer contained more OH- ions than the number of H+ ions, so that the H+ ions are ionized from the three amino acids that will be drawn by the negative force of the buffer. As a result of H+ ions in solution becomes slightly, so that the potential differences decreased.

d. Potentiometric Detection in HPLC Analysis

Amino acids dissociated in water and produce H+ ions. Tungsten coated with tungsten oxide would be very sensitive to the changes of H+ ions concentration in solution. This pH difference will lead to different responses that occur as a result of reactions in the tungsten oxide layer, which is followed by changes in the level of oxidation of tungsten.

According to Fenster, et.al, 2008, reaction to the dependence of concentration of H+ in tungsten oxide electrode was:

$WO_3 + xH^+ + xe \rightarrow HxWO_3 (0 < x < 1)$ ....(1)

Potential difference (ΔE) obtained when the amino acids were injected based on the following reaction (Chen, *et al*, 1996):

$WO_3 + 6H^+ + 6e \rightarrow W + 3 H_2O$ . ...(2)
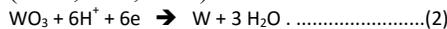
$$\Delta E = E_2 - E_1 = const. + \frac{RT}{F}\log\left(\frac{H^+_{solute}}{H^+_{bufer}}\right) \quad ....(3)$$

Where E_2 is the peak of the potential of solute (amino acids), E1 is the peak of the potential of buffer (phosphate buffer), H^+ buffer is the concentration of H^+ from the buffer (phosphate buffer) and H^+ solute is the concentration of H^+ from the amino acid is injected, R is the gas constant, T is temperature and F is the faraday constant. At constant pH and buffer composition, equation 3 can be simplified to:

$$\Delta E = E_2 - E_1 = const + 0,059\log\left(H^+_{solute}\right) ......(4)$$

Based on equation 4, tungsten oxide electrode with electrode Ag/AgCl as refference provide potential difference changes linearly to the changes in the concentration of amino acids through changes in the concentration of H^+

e. Separation and Electrode Characteristics

Sensitivity based on the measurement results obtained by the slope of 4 mV / decade for glutamic acid, 8,8 mV /decade for aspartic acid and 4.3 mV /decade for arginine. Detection of $1,58 \ 10^{-7}$ M for glutamic acid, $6,58. \ 10^{-8}$ M for aspartic acid and $6,51. \ 10^{-8}$ M for arginine.
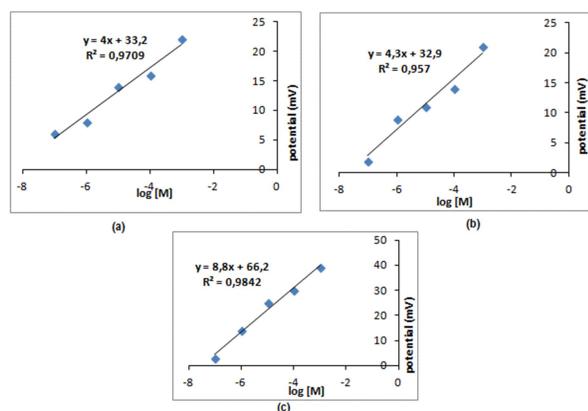

Figure 5. Calibration curve of (a) glutamic acid (b) Aspartic acid and (c) Arginine

Reproducibility is a repetition that performed between measurements in order to produce the smallest possible limit or generated data to be precise, and expressed by KV (coefficient of variation) that indicates the level of measurement error due to repetition.

Table 1 Coefficient of variation of the amino acids at different concentration

Amino acids concentration (M)	KV Arginine	KV Glutamic acids	KV Aspartic acids
10^{-7}	3,5	11,7	0,0
10^{-6}	7,7	8,7	5,0
10^{-5}	6,4	0,0	2,8
10^{-4}	0,0	4,4	2,3
10^{-3}	3,3	3,1	0,0

Recoveries of the analyte in a sample were expected above 80% (80-110%). Recovery test was used to know the sensitivity of method for separation and detection the amino acids in HPLC.
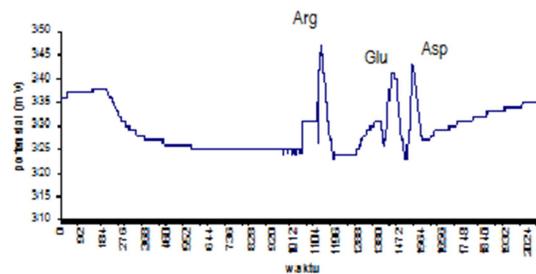

Fig 6. Chromatogram of separation of amino acids

Based on the results of the recovery test, glutamic acid recovery value was 95.9% while for aspartic acid 89.1%, and 110% for arginine. Recoveries of the analyte was affected by the sample injection technique and the separation effectiveness.

CONCLUSION

Potentiometric detector using tungsten oxide electrode was a potential detector for amino acids. Analysis of the amino acids aspartate, glutamate and arginine has a linearity of 0.9842 for aspartatic acid, 0.9709 for glutamic acid and 0.957 for arginine. Detection limit of 6.58×10^{-8} M for aspartic acids, 1.58×10^{-7} M for glutamic acid and 6.51×10^{-8} M for arginine. Flow rate, pH buffer and acetonitrile concentration affected the separation and detection of amino acids.

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