HPLC DETERMINATION OF PRIMARY AMINO ACIDS USING ELECTROCHEMILUMINESCENCE OF RUTHENIUM COMPLEX

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A precolumn derivatization method combined with a tris(2,2'-bipyridine)ruthenium(II) [$Ru(bpy)_3^{2+}$] electrochemiluminescence(ECL) was developed for the determination of primary amino acids. They were reacted with divinyl sulfone(DVS), and separated with reversed-phase column using a mobile phase containing 15 mM NaH_2PO_4-Na_2HPO_4(pH 6.5). The eluted DVS-primary amino acids were mixed with 0.5 mM $Ru(bpy)_3^{2+}$ reagent within flow tube and then the solution passed through a thin layer flow cell equipped with glassy carbon electrode and was oxidized to $Ru(bpy)_3^{3+}$ at +1.30 V(vs. Ag/AgCl). $Ru(bpy)_3^{3+}$ reacted with DVS-primary amino acids to emit light. The detection limit ranged from 50 fmol for glutamic acid to 1 pmol for serine at a S/N ratio of 3.

Keywords Electrochemiluminescence, high performance liquid chromatography, postcolumn derivatization, tris(2,2'-bipyridine)ruthenium(II), amino acid, divinyl sulfone

In recent years, the determination of amino acid is an important subject in the field of biochemistry. The chemiluminescence(CL) methods have been reported for the determination of amino acid[1-7]. Amino acids were usually derivatized with a chemiluminescent compound like phthalhydrazide[1] analogous before reversed-phase HPLC separation. This procedure needs ferricyanide and hydrogen peroxide as catalyst for the formation of luminescence. The detection limit of this method was at the fmol level.

On the other hand, the chemiluminescent procedure using $Ru(bpy)_3^{2+}$ has been becoming an attractive detection method for underivatized[2-6] and derivatized[7] amino acids, due to low detection limits and wide linear working ranges using relative simple instrumentation. Brune and Bobbit[5] reported the chemiluminescent reaction sequence shown as scheme 1 for ruthenium/ amino acid system.

The CL reaction of selected nitrogen compounds such as mono-, di, and trialkylamines with $Ru(bpy)_3^{3+}$ has also been reported by Danielson and Noffesinger[8]. The luminescence intensity for aliphatic amines follows the order of tertiary>secondary> primary compound. A cycloaddition reaction of primary amines with divinyl sulfone(DVS) for the ECL has been reported by Uchikura *et al* [9]. The detection limit reported about 30 pmol for propylamine and 1 pmol for 3-aminopentane, respectively.

The objective of this paper is to describe the method for the HPLC determination of some primary amino acids after pre-column cycloaddition reaction of DVS. The several factors influencing ECL intensity for DVS-amino acid derivatives and the conditions for cycloaddition reaction are precisely discussed.

Experimental

Chemical and Standard Solution

$Ru(bpy)_3Cl_2 \cdot 6H_2O$ was obtained from Sigma Chemical Co.(St. Louis, MO, USA) and used without further purification. Amino acids were purchased from Wako Pure Chemical Industries. LTD(Osaka, Japan). Divinyl sulfone(DVS) was obtained from Aldrich Chemical Co. Inc.(WI, USA). All of

the other chemicals were of guaranteed grade. The deionised water was twice-distilled. All stock standard solution(2 mM) of amino acids were prepared by dissolving them in water. A working solution was prepared by appropriate dilution of the stock solution before use.

Apparatus

The luminescence intensity was observed by modifying the commercially available "Chemiluminescence Analyzer" (Soma Kodak, Co.,). The electrolysis cell for ECL observation was designed and assembled in our laboratory. The main body of the well was composed of Diflon and stainless steel block tightly fixed to each other. Since luminescence intensity has close relationship with the orifice shape and thickness of the Teflon-sheet spacer, a spacer sheet having the thickness of 50 μm has been chosen. The volume of thin layer cell was 1.5 μl. The experimental setup is shown in Fig.1. A three-electrode system was used for the potentiostatic control of the electrolytic system. The working electrode was a glassy carbon disk(22.1 mm^2). The counter electrode at the outlet consisted of a stainless steel pipe and the reference electrode was Ag/AgCl[10]. The potentiostat(Prinston Appl. Res., Co., type 174A) was used for controlling the potential of working electrode.

The HPLC was performed using a LC-6A(Shimadzu Co., Japan) liquid chromatography equipped with a Rheodyne 7125 sample injector(Cotati, CA, USA) and a $5C_{18}$ AR reversed-phase column(250 × 4.6 mm id., waters). The mobile phase was 15 mM NaH_2PO_4-Na_2HPO_4 buffer solution(pH 6.5), and the flow rate was 0.5 ml/min. The reagent solution was prepared by dissolving 0.5 mM $Ru(bpy)_3Cl_2$ in 0.4 M sodium acetate and 0.015 M sodium hydroxide(pH 13.0). The flow rate for Ru complex was 0.3 ml/min. A VP-6537A pen recorder(National, Japan) was used for recording the luminescence intensity.

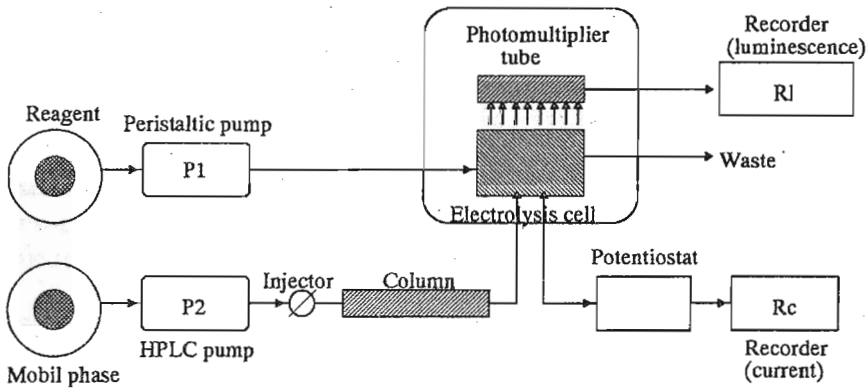


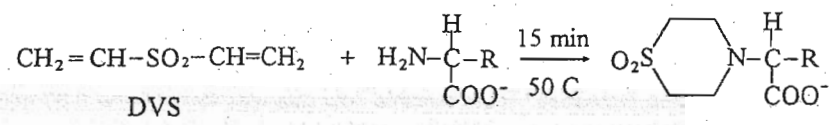
Fig.1 Experimental setup for the ECL-HPLC

Results and Discussion

Derivatization reaction

The derivatization procedure reported by Uchikura *et al.*[9] was not adequate for our analytical purpose. Since the use of higher concentration of DVS and methanol interfered with the determination of aspartic acid, a procedure using acetonitrile and lower concentration of DVS were chosen as a results of

our investigation. The reaction conditions were examined by using 20 μM glycine standard solution. As the amount of DVS is proportional to the emission intensity from 0.5 mM to 8 mM range(Fig. 2), 10 mM DVS was adopted in the following experiments. As the reaction in scheme 2 was accelerated in the presence of a base, the effect of pH in the formation of DVS derivatives was examined. It was found that the emission intensity increased with increasing pH, though the luminescence intensity again decreased above pH 8.6(Fig. 3). The effect of reaction temperature(30 ℃ - 80 ℃) and time(15 min- 1 hr.) on the derivatization reaction were then investigated. The luminescence intensity of the derivatized compound attained a maximum value after 15 min of reaction at 50℃ , and the reaction time over 15 min at 50℃ was useless for increasing ECL intensity. Thus, the optimum derivatization conditions were 10 mM DVS, pH 8.5 borate buffer, and reaction time of 15 min at 50℃(Scheme 1).



Scheme 1

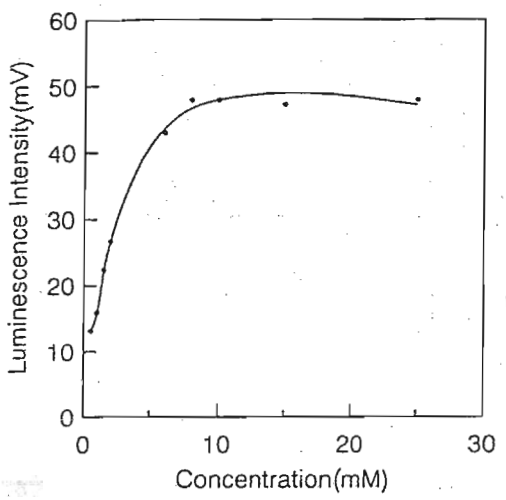


Fig.2 The relationship between concentration of DVS and luminescence of derivative. Concentration of Gly-DVS: 1 μM

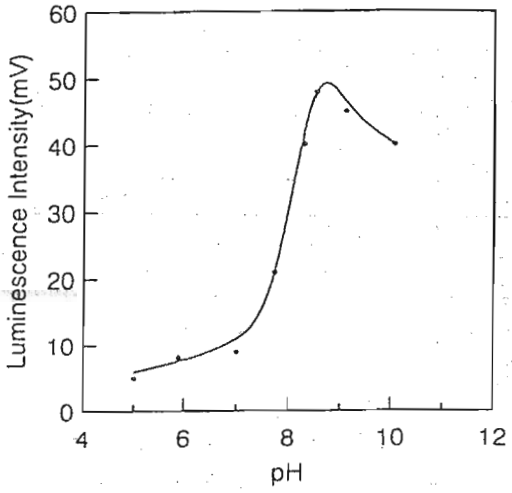


Fig.3 The relationship between pH for derivatization reaction and the luminescence intensity Concentration of Gly-DVS: 1 μM

Carrier Solution

Rubinstein and Bard[11] reported that the chemiluminescence intensity due to the reaction of sodium oxalate with $Ru(bpy)_3^{3+}$ depend on the pH of the solution, with a maximum signal at a pH 6.0. Uchikura *et al.*[9] pointed out that the ECL of DVS-derivatized primary amines increased with the pH from 2.5 to 7.0, but again decreased at higher pH. Our experimental results showed that the ECL intensity increased with increasing pH of carrier solution, and attained a constant value at above pH 8.6(Fig.4). Though the intensity slightly increased over that value of pH , but the blank value was also increased. With the increase in the concentration of sodium acetate in carrier solution, the luminescence intensity increased until the concentration was up to 0.3 M. This is probably due to the effect on electrolytic efficiency, and thus 0.4 M sodium acetate was adopted in the following experiments.

ECL responses also depended on the flow rate of carrier solution. The luminescence intensity decreased with increasing rate over 0.35 ml/min, since $Ru(bpy)_3^{3+}$ was formed as a result of the chemiluminescent reaction on gly-DVS. Although the luminescence intensity increased at lower flow rate, the blank response was also increased. Thus, suitable flow rate was found to be 0.30 ml/min. In addition, the emission was observed when the applied potential was over + 0.6V. Since the optimum potential range for the gly-DVS ECL was from + 1.1 V to + 1.4 V(vs. Ag/AgCl), the fixed potential at + 1.30 V was selected(Fig.5).

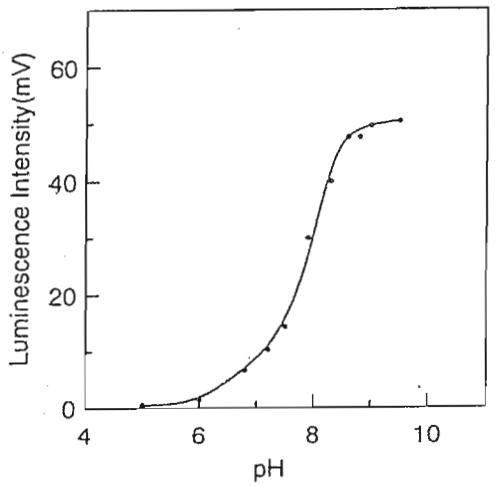


Fig.4 The effect of pH on the ECL of Gly-DVS
Concentration of Gly-DVS: 1 μM

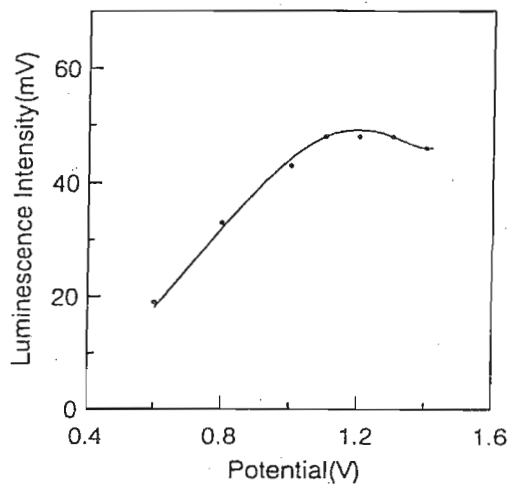


Fig.5 The effect of potentical on the ECL of Gly-DVS
Concentration of Gly-DVS: 1 μM

Separation of DVS amino acids derivatives

Lee and Nieman[7] recently reported the derivatization of six kinds of amino acid with dansyl chloride and the detection limit was to be 2 pmol for Dns-Glu. Our experimental results shown in Table 1 clearly indicate that the lower detection limits of secondary amino acids(proline, hydroxyproline, histidine and tryptophan) were compared with that of primary amino acids. According to Danielson and Noffesinger's report[8], the detection limits of amines were in the order of tertiary>secondary>primary amino acid. The detection limits for primary amino acid should be increased when they were transformed to tertiary amino acid. Table 2 shows that luminescence intensities obviously increase when primary amino acids were derivatized with DVS. The best separation of the derivatives was achieved on a reversed-phase C_{18} column by eluting with 15 mM phosphate buffer solution(pH 6.5) and a flow rate of 0.5 ml/min. Under these conditions, nine kinds of primary amino acid were efficiently separated.

Tab.1 Comparison of ECL intensity and detection limits(D.L) of HPLC for amino acids

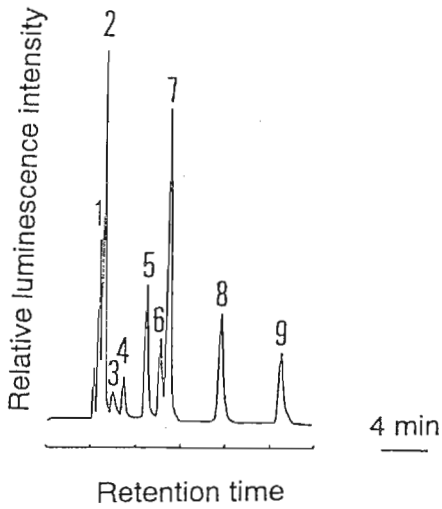
Amino Acid	Light Emission (mV)*	Detection Limit (pmol)	Retention time(min)
Proline	1035	0.3	5.65
Hydroxyproline	1020	0.3	5.05
Histidine	88	3.5	5.64
Tryptophan	76	4.8	13.96
Tyrosine	42	6.0	14.19
Methionine	39	8.0	8.12
Leucine	22	14	10.62
Isoleucine	20	16	13.19
Valine	17	18	8.16
Phenylanine	16	18	24.38
Alanine	14	20	5.04
Asparagine	12	25	7.27
Glutamic acid	9.0	35	4.93
Glutamine	7.9	40	5.15
Aspartic acid	5.8	50	4.84
Glycine	3.9	80	4.90
Lysine	3.1	100	6.14
Serine	2.5	120	6.56
Cysteine	1.7	180	8.53

* Concentration of amino acid: 20μM; injection volume: 20μl

Tab.2 Comparison of the detection limits(D.L) for underivatized and DVS derivatized amino acids

Amio acid	Underivatized amino acid D.L(pmol)	DVS derivatized amino acid D.L(pmol)	Retention time(min)
Alanine	20	0.06	12.63
Arginine	30	0.2	23.38
Asparagine	25	0.3	7.33
Aspartic acid	50	0.1	5.33
Glutamine	40	0.2	10.33
Glutamic acid	35	0.05	5.78
Glycine	80	0.2	8.08
Lysine	100	0.5	11.53
Histidine	3.5	0.8	17.68
Serine	120	1.0	7.23

A chromatogram of the nine kinds of derivatized amino acid is shown in Fig. 6. The calibration curve for gly-DVS was linear between 0.2 pmol to 500 pmol. The reproducibility of the luminescence intensity of chromatograms at 1 μM was 1.2 to 3.8 %(n=5). The detection limit was 50 fmol for glutamic acid and 1 pmol for serine at a S/N of 3.



1. Aspartic acid; 2. Glutamic Acid; 3. Serine; 4. Glycine: 5. Glutamine; 6. Lysine; 7. Alanine; 8. Histidine; 9. Arginine Eluent: 0.015 M NaH_2PO_4–Na_2HPO_4, pH 6.5; Flow rate: 0.5 ml/min; Carrier solution: 0.5 mM $Ru(bpy)_3^{2+}$, 0.4 M Sodium acetate, 0.015 M NaOH, pH 13.0, Flow rate: 0.3 ml/min; Potential, + 1.30 V(vs. Ag/AgCl); Separation column: 5 C_{18} AR(250 × 4.6 mm i.d.,); Temperature: 25 ℃ Sample volumn: 20 μl; Sample concentration: 1 μM for each amino acid(20 pmol).

Fig.6 Chromatograms for derivatized amino acids.

In conclusion, the chemiluminescence reaction of DVS-derivatized primary amino acid with electrogenerated $Ru(bpy)_3^{3+}$ can provide for the sensitive and reproducible detection of amino acids after HPLC separation. This detection method has a wide dynamic range and detection limits for primary amino acids are comparable to those reported using other methods. Although underivatized amino acids can be detected with $Ru(bpy)_3^{3+}$ CL, the DVS derivatives have detection limits impoved by two or three order of magnitude for primary amino acids. Furthermore, $Ru(bpy)_3^{2+}$ has advantages of reagent stability, greater compatibility with reversed-phase HPLC solvent. It is possible to applicate this method to determinate some amino acids in practice samples. We are now doing research work on this field.

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ルテニウム錯体の電解化学発光を用いる第一アミノ酸のHPLCによる定量

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各種アミノ酸の高感度な分離分析として、アミノ酸のジビニルスルホン（DVS）誘導体を合成し、アミノ酸の混合試料をHPLCで分離後、ポストカラム的にTris-(2,2'-bipyridine)(II){$Ru(bpy)_3^{2+}$}溶液を混合し、その電解酸化体と誘導体の反応による化学発光を測定する方法を開発した。C_{18}化学結合固定相を持つ分離カラムに対して、15mM NaH_2PO_4-Na_2HPO_4(pH 6.5)の移動相を送液し、0.5 mM $Ru(bpy)_3^{2+}$ — 0.4 M NaAc — 0.5 M NaOH(pH 13.0)溶液をポストカラム的に使用すると、9種の一級アミノ酸がほぼ完全に分離できた。本法によれば、0 — 100 pmol までの範囲でアミノ酸濃度と発光強度は良好な直線関係を示し、検出下限(S/N)は、セリンは0.5 pmol、アラニンは.20 fmolであった。

(Received May 17, 1995)

(Accepted November 21, 1995)