Determination of Aromatic and Branched-Chain Amino Acids in Plasma by HPLC with Electrogenerated $Ru(bpy)_3^{3+}$ Chemiluminescence Detection

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An HPLC method is described for the electrochemiluminescence (ECL) detection of amino acids, following cycloaddition reaction of their amino groups with divinyl sulfone (DVS), using electrogenerated tris(bipyridine)ruthenium(III). The derivatization reaction conditions were examined, with the optimum conditions found to be 40 mM DVS (pH 8.0) at 50 °C for 15 min. Detection limits for the 15 amino acids examined varied greatly (0.04—8.0 pmol) using a standard solution by flow injection analysis (FIA). These optimized conditions were used for HPLC determination of the amino acids in human plasma. A linear relationship was obtained up to 100 pmol on a column for aromatic and branched-chain amino acids. Recoveries of Tyr, Met, Val, Leu, Ile, Phe and Trp when added to human plasma (1 μmol/10 ml plasma, n=5) were 101.5±1.1, 99.0±1.2, 98.0±1.4, 101.1±1.6, 95.1±1.6, 99.2±1.5 and 97.7±1.3 % (mean±S.D.) respectively. The concentrations of the amino acids in the plasma are in good agreement with other published data.

Key words electrochemiluminescence; divinylsulfone; tris(bipyridine)ruthenium(II); amino acid; HPLC; plasma

Several types of inborn errors are known in the metabolism of aromatic amino acids phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp), the branched-chain amino acids valine (Val), leucine (Leu), and isoleucine (Ile), and the sulfur-containing amino acid methionine (Met), and these can cause phenylketonuria (PKU), tyrosinemia, tetrahydrobiopterin deficiency and hypermethioninemia. The determination of Phe, Tyr, Trp, Val, Leu, Ile and Met levels is important in the diagnosis of these inborn metabolism disorders. PKU is one of the disorders most widely screened for newborn babies.[1,2] Several techniques such as the Guthrie test,[3] the microplate fluorimetric system,[2] HPLC with fluorescence[4] and mass spectrometry (MS),[5] capillary electrophoresis[6] and enzymatic assay[7] have been used to screen PKU in newborn blood.

A variety of pre- and post-column derivatization HPLC techniques have been developed for the determination of amino acids. Those reagents include *o*-phthalaldehyde,[8] dansylchloride[9] and 4-(dimethylamino)-azobenzene-4′-sulfonyl chloride,[10] all of which form UV absorbent or fluorescent species with amino acids. All of these, however, suffer from one or more of the following disadvantages: lack of quantitative reaction with certain amino acids, complex coupling precedure or instability of the products or reagent.

On the other hand, a few chemiluminescence HPLC methods have been reported for the determination of amino acids.[11,12] Kobayashi and Imai[12] reported HPLC separation of dansyl amino acid derivatives combined with peroxyoxalate chemiluminescence detection. Detection limits reported were at the several fmol levels. However, the bis(2,4,6-trischlorophenyl) oxalate system is itself unstable. Recently, a selective and sensitive tris(bipyridine)ruthenium(II) ($Ru(bpy)_3^{2+}$) electrogenerated chemiluminescence (ECL) system has been applied for the detection or determination of oxalate with HPLC[13] or flow injection analysis (FIA),[14] indoles with HPLC[15] or FIA,[16] and tertiary amines with HPLC[17] or FIA.[18] We previously reported that alicyclic tertiary amines can be detected at the pmol level using this FIA system.[19] The ECL intensities of aliphatic primary amines were very low compared with those of the tertiary amines; they could only be detected at levels about 1000-times lower than those of tertiary amines.[18] We also reported the detection of primary amines using a cycloaddition reaction of the amines with divinylsulfone (DVS).[20] In contrast, the chemiluminescence procedure using $Ru(bpy)_3^{2+}$ has become an attractive method with HPLC for the detection of underivatized[15,21—24] and derivatized amino acids,[25,26] due to low detection limits, wide linear dynamic range and relatively simple instrumentation. Though we previously reported the determination of tryptophan in human plasma using the $Ru(bpy)_3^{2+}$-indole system,[15] the $Ru(bpy)_3^{2+}$ ECL method has not been applied to the determination of amino acids in human plasma samples.

In this paper, we describe the HPLC separation of aromatic and branched-chain amino acids combined with cycloaddition reaction of primary amine function with DVS, and their $Ru(bpy)_3^{3+}$ ECL detection. This method has also been applied to determine amino acids in human plasma.

Experimental

Chemicals and Standard Solution $Ru(bpy)_3Cl_2$ was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and was used without further purification. DVS was obtained from Aldrich Chemical Co. Inc. (WI, U.S.A.). L-Amino acids were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals and solvents were of guaranteed grade. Water was deionized and distilled using a hard-glass vessel. A stock standard solution (2 mM) of amino acids was prepared by dissolving them in water–methanol (1 : 1, v/v). A working solution was prepared by appropriate dilution of the stock solution before use.

Apparatus and HPLC Conditions The HPLC system consisted of a PSU-2.5T micro pump (Seishin Pharm., Tokyo, Japan), a Rheodyne 7125 sample injector with a sample volume of 10 μl (Cotati, CA, U.S.A.), a homemade electrochemical reactor,[27] an HA-101 potentio-galvanostat (Hokuto Denko, Japan), an LC30-DPC10 chemiluminescence detector (JEOL, Japan) and a U-228 Unicorder (Pantos, Tokyo). An electrochemical oxidation mode was used for controlled-current electrolysis with the current maintained at 80 μA. The column used was an L-column ODS (150×4.6 mm i.d., Kagakuhin Kensa Kyokai, Japan) which was maintained at 50 °C using a Minder Ace water bath (Taiyo Scientific, Japan). HPLC was performed using a flow system, as previously described.[15] The eluent consisted of acetonitrile and 10 mM KH_2PO_4 (15 : 85, v/v); the flow rate was 1.0 ml min^{-1}. The reagent solution was prepared by dissolving $Ru(bpy)_3Cl_2$ to 0.30 mM in 10 mM H_2SO_4; the flow rate was 0.3 ml min^{-1}. This reagent solution was stable for two months.

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Derivatization of Amino Acids The derivatization reagent solution (1 M) was DVS dissolved in methanol. In a tube was added 5 ml of a mixture of 50 mM borate buffer (pH 8.0) and methanol (1 : 1,v/v). Two hundred microliters of 1 M DVS methanol solution was combined with 100 μl of deproteinized plasma sample and then mixed by vortexing. The mixture was allowed to stand at 50 °C for 15 min and then cooled to room temperature. A 10 μl aliquot was injected directly into the HPLC. If necessary, the reaction mixture was diluted with water prior to being injected into the HPLC.

Sample Preparation Blood was obtained from healthy volunteers and collected in siliconated vacutainer tubes containing Na2-EDTA (1 mg of Na2EDTA per ml of blood) as anticoagulant. Plasma was obtained by centrifugation at 4000 ***g*** for 20 min at 4 °C. Before analysis 30 μl of 60% perchloric acid was added to the plasma sample (300 μl) and mixed by vortexing. The precipitate was removed by centrifugation at 15000 ***g*** for 15 min and the supernatant (100 μl) was used for derivatization.

Results and Discussion

Optimization of the Derivatization Reaction The derivatization reaction for an amino acid with DVS is shown in Chart 1.

The reaction conditions for primary amines with DVS were examined previously.[15)]

In this study, optimization of the derivatization reaction conditions for the amino acids were examined using Val, Leu, Ile and Phe. The DVS concentration and reaction time were examined in terms of conversion of amino acids. The amount of DVS-amino acids formed was considered to be proportional to their ECL intensity, which, in twin was measured using HPLC. The conditions were the same as those described in the experimental section. Firstly, the effect of DVS concentration within the range of 5—60 mM on the formation of DVS-amino acids was examined. Within the range of 30—60 mM formation was constant. Examination of the effect of reaction time showed that the amount of DVS-amino acids formed reached a constant level after incubation at 50 °C for 10 min. Therefore, from these studies the optimized derivatization conditions employed were 40 mM DVS (pH 8.0) at 50 °C for 15 min.

Effect of pH and Acetonitrile Concentration on the ECL Intensity The ECL intensity is very dependent on the pH.[10,14,15)] This dependence was investigated using the HPLC system. The effects of pH on the ECL intensity were investigated for a pH range from 3.3—8.0, which was controlled by the addition of 0.1 M KH_2PO_4 buffer (pH 3—8.5) pumped at a flow rate of 0.3 ml min^{-1} into the eluent stream between the column and detector using a T-piece connector. The pH of the reaction was measured from the solution flowing out of the detector. The ECL intensity increased as the pH increased from 3.3 to 7.5 (Fig. 1); however, the baseline noise increased with increasing pH. The detection pH was selected as pH 3.5—4.5 from the results that gave the best signal to noise ratio. By a similar operation, the inclusion of an organic modifier also affected the ECL intensity. Increasing acetonitrile concentration decreased the ECL intensity (Fig. 2). This seems to be advantageous if the acetonitrile concentration is low.

Detection of DVS-Amino Acids The ECL intensity of DVS-amino acids was measured using a FIA system. The carrier solution consisted of acetonitrile and 10 mM KH_2PO_4 (15 : 85, v/v), and the flow rate was 1.0 ml min^{-1}. The relative ECL intensity and detection limits for the DVS-amino acids are shown in Table 1. The ECL intensity of Trp is the sum of the results for both the tertiary amine and indole skeleton.[11)] Detection limits (s/n=3) for the 15 amino acids varied

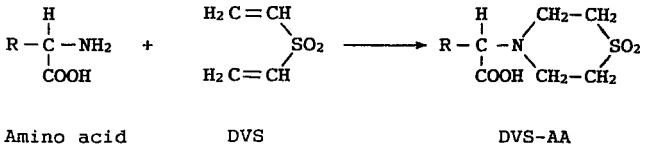

Chart 1

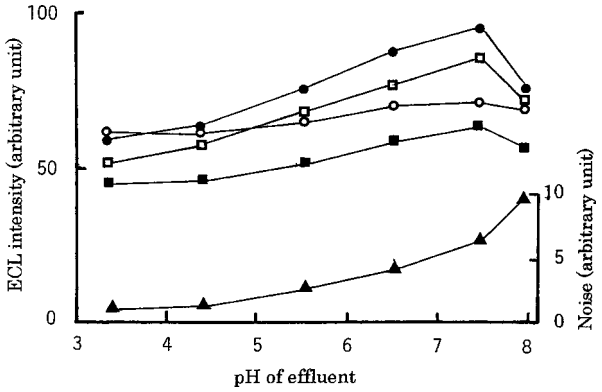

Fig. 1. Effect of pH on the ECL Intensity and Noise

Curves: (●), Val; (□), Leu; (○), Ile; (■), Phe; (▲), noise. Sample: each amino acid, 20 pmol/20 μl.

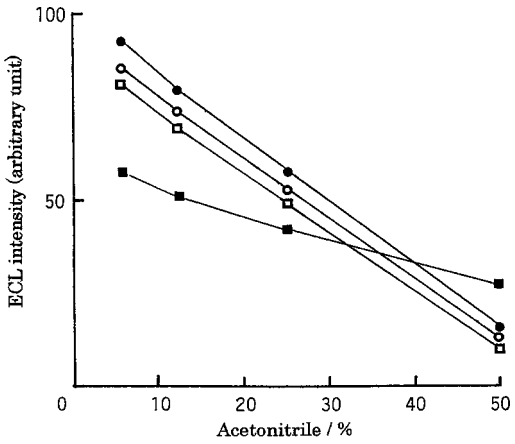

Fig. 2. Effect of Acetonitrile Concentration on the ECL Intensity

Curves: (●), Val; (□), Leu; (○), Ile; (■), Phe. Sample: each amino acid, 20 pmol/20 μl.

Table 1. Relative ECL Intensities and Detection Limits of DVS-Amino Acids

Compound (DVS-amino acid)	ECL intensity (arbitrary unit)	Detection limits (pmol)
Alanine	96.0	0.46
Aspartic acid	34.5	1.28
Cysteine	40.0	1.10
Glutamic acid	128.5	0.34
Glycine	13.0	3.38
Histidine	5.5	8.00
Isoleucine	880.0	0.05
Leucine	770.0	0.06
Methionine	330.0	0.13
Phenylalanine	440.0	0.10
Serine	18.0	2.44
Threonine	56.0	0.79
Tryptophan	40.0	1.10
Tyrosine	1100.0	0.04
Valine	660.0	0.07

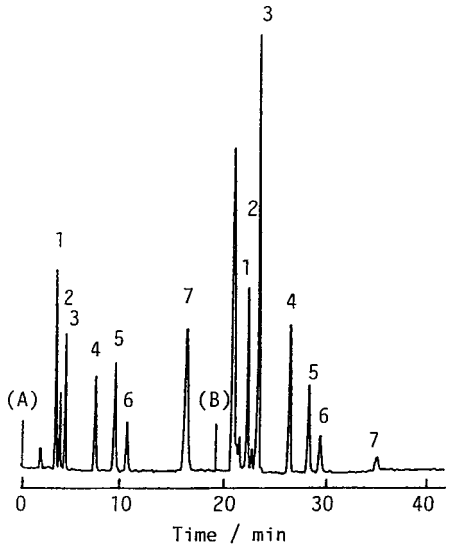

Fig. 3. Chromatograms Obtained with Authentic (A) and Plasma (B)

Peaks: 1=Tyr [(A) 10 pmol and (B) 46 μmol/l]; 2=Met [(A) 10 pmol and (B) 14 μmol/l]; 3=Val [(A) 10 pmol and (B) 160 μmol/l]; 4=Leu [(A) 10 pmol and (B) 76 μmol/l]; 5=Ile [(A) 10 pmol and (B) 40 μmol]; 6=Phe [(A) 10 pmol and (B) 36 μmol/l]; 7=Trp [(A) 50 pmol and (B) 24 μmol/l].

greatly (0.04—8.0 pmol). There have been several published reports on $Ru(bpy)_3^{2+}$ chemiluminescence detection of underivatized amino acids which have mentioned detection limits in the picomol range.[16—19] These showed that detection limits for underivatized amino acids increased when derivatized with DVS.

HPLC Determination of Amino Acids in Human Plasma We applied the DVS derivatization procedure to the determination of aromatic and branched amino acids in human plasma. A typical chromatogram obtained with a standard solution and a plasma sample is shown in Fig. 3. The chromatogram peaks were attributed to the amino acids in the plasma sample, and depend on the retention times. Each peak is completely separated and an interference peak based on the human plasma did not appear. The other DVS-amino acids, alanine, glycine and serine, eluted in advance from the peak of Tyr. A linear relationship was obtained for up to 100 pmol on the column. The recoveries of Tyr, Met, Val, Leu, Ile, Phe and Trp when amino acids were added to the plasma sample (1 μmol/l plasma, n=5) were: 101.5±1.1, 99.0±1.2, 98.0±1.4, 101.1±1.6, 95.1±1.6, 99.2±1.5 and 97.7±1.3% (mean±S.D., n=5) respectively. The concentrations of the amino acids in the plasma are given in Table 3 and are in good agreement with other published data.

This method should prove to be useful as a stable, sensitive and specific technique. Its application is anticipated to aid in the diagnosis of inborn metabolism disorders.

Table 2. Recoveries of Amino Acids Added to Human Plasma

Amino acid	Original detected value (μmol/l)[a]	Spike (μmol/l)	Detected value after spike (μmol/l)[a]	Recovery (%)[a]
Tyrosine	63±0.6	100	164±1.8	101.5±1.1
Methionine	18±0.2	100	117±1.2	99.0±1.2
Valine	180±2.1	100	278±4.1	98.0±1.4
Leucine	130±2.1	100	231±4.4	101.1±1.6
Isoleicine	51±0.7	100	146±2.2	95.1±1.6
Phenylalanine	55±0.8	100	154±2.3	99.2±1.5
Tryptophan	38±0.4	100	136±1.5	97.7±1.3

a) Mean±S.D. (n=5).

Table 3. Amino Acid Concentrations in Human Plasma

Amino acid	Concentration (μmol/l)	
	Found[a]	Normal range[b]
Tyrosine	61±12	40—80
Methionine	15±8	13—32
Valine	176±22	151—302
Leucine	99±20	77—162
Isoleucine	54±17	38—83
Phenylalanine	63±11	37—61
Tryptophan	33±9	19—45

a) Sample (n=10). *b*) C. R. Scriver *et al.*, "Amino Acid Metabolism and Its Disorders," W. B. Saunders, Philadelphia, 1973, p. 42.

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