Analyst

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

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Baseline separation of amino acid biomarkers of hepatocellular carcinoma by polyvinylpyrrolidone-filled capillary electrophoresis with light-emitting diode-induced fluorescence in the presence of mixed micelles

Yen-Chu Chen and Po-Ling Chang*

Department of Chemistry, Tunghai University, Taichung, Taiwan

*To whom correspondence should be addressed.

E-mail: poling@thu.edu.tw Tel: +886-4-23590121#32246

Author address: No.1727, Sec.4, Taiwan Boulevard, Xitun District, Taichung 40704, Taiwan

Keywords: amino acid, biomarker, capillary electrophoresis, mixed micelle, polyvinylpyrrolidone

Abbreviations: AAs, amino acids; AA-CBIs, amino acid-cyano[*f*]benzoisoindoles; CE, capillary electrophoresis; CE-LEDIF, capillary electrophoresis-light emitting diode induced fluorescence; NDA, naphthalene-2,3-dicarboxaldehyde; PVP, polyvinylpyrrolidone; IP, isopropanol; SDS, sodium dodecyl sulfate

Abstract

Physiological amino acids (AAs) are important indices for monitoring various diseases, including cancer. This study proposes a polymer-based separation method in the presence of mixed micelles for the determination of AAs by capillary electrophoresis with light-emitting diode-induced fluorescence. The separation of 18 amino acid-cyano[*f*]benzoisoindoles (AA-CBIs) was successfully achieved using a solution of polyvinylpyrrolidone (PVP, 5% w/v, M_{avg} 1,300,000 Da). In addition, we demonstrated that mixed micelles composed of sodium dodecyl sulfate and isopropanol may affect the migration order of the AA-CBIs and greatly improve the speed of separation. With the exception of proline, 21 plasma AA-CBIs, including high isoelectric point AAs (lysine, ornithine, and arginine), were identified by using optimized separation conditions with minimal matrix effects. The results of this study demonstrated the distinct advantages of the proposed method, such as simplicity, high efficiency, and cost-effectiveness. This method has great potential for the diagnosis of several important diseases, including carcinomas, aminoacidopathies, and neurotransmission disorders.

Introduction

Amino acids (AAs) are zwitterions that play an important role in maintaining normal physiological functions. Several severe diseases have been shown to be associated with metabolic dysfunctions, resulting in abnormal quantities of AAs in body fluids. For example, primary aminoacidopathies such as arginase deficiency, citrullinemia, cystinuria, maple syrup urine disease, phenylketonuria, and tyrosinemia are well-known inherited AA disorders. Secondary aminoacidopathies such as hyperammonemia, lactic acidosis, organic acidurias, and transient tyrosinemia of newborns can also cause serious metabolic disorders.[1] In addition, some amino acids may act as biomarkers for the diagnosis of malignant tumors, such as branched chain amino acids (leucine, isoleucine, and valine) for hepatocellular carcinoma,[2] and glycine and tyrosine for colorectal cancer.[3] More recently, it has been demonstrated that free amino acids in plasma could be used for early detection of five cancer types.[4] Therefore, developing a useful and cost-effective method for the analysis of AAs is an important topic in clinical research.

The determination of physiological AAs is typically performed using reverse-phase HPLC,[5] LC/MS,[6] or ion exchange chromatography[7] coupled with absorption detection, and is already used in clinical analysis in hospitals. These processes are time-consuming, have a high per-sample cost, and require considerable specimen

volumes, which greatly limit the clinical application of amino acid analysis.[8] Capillary electrophoresis (CE) has been widely utilized in bioanalysis because of its several advantages, including a small sample size, high separation efficiency, and high throughput feasibility.[9-12] These characteristics enable the practical use of CE for clinical applications,[13-16] and make it suitable for large-scale screening using the capillary array electrophoresis format.[17-19] Several CE-based techniques have been established for the analysis of AAs, employing various separation strategies.[20-22] Among these techniques, capillary zone electrophoresis in the presence of electroosmotic flow (EOF; uncoated capillary) has been widely utilized in the development of methods for AA analysis. For example, Zunic et al. separated 13 AAs and peptides with sodium carbonate (pH 10.2)-buffered p-aminosalicylic acid by indirect absorbance detection.[23] Lorenzo et al. separated 15 AAs from human plasma using a borate buffer (pH 10.25) prepared with 12.5 mM β-cyclodextrin, by applying laser-induced fluorescence detection with 4-fluoro-7-nitro-2,1,3-benzoxadiazole labeling.[24] Moreover, Nouadje et al. successfully separated fluorescein isothiocyanate-labeled AAs using a mixture of sodium dodecyl sulfate (SDS)/boric acid, and identified 21 AAs from cerebrospinal fluid specimens.[25] Subsequently, Siri et al. compared the use of high-performance liquid chromatography (HPLC) and micellar electrokinetic chromatography (MEKC) for the determination of

naphthalene-2,3-dicarboxaldehyde (NDA)-labeled AAs and catecholamines.[26] The authors also demonstrated that the separation efficiency of MEKC (N ≈ 200000) for AA-CBIs is superior to that of HPLC (N ≈ 5000). Furthermore, Jaworska et al. used an MEKC background electrolyte to successfully separate twenty 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate-derivatized AAs from medicinal products for parenteral nutrition.[27] In addition to free solution electrophoresis, capillary electrochromatography has been useful in AA analysis, yielding straightforward results with enantiomeric separation.[28, 29] The separation of AAs can be accomplished by chip electrophoresis, which has proven useful for *in vivo* chemical monitoring of a microdialysis probe.[30]

Previous studies have demonstrated that linear polymer solutions are effective in improving the separation efficiency of AAs from cerebrospinal fluid,[31] ascites,[8] tea leaves,[32] red wine,[33] and tobacco extracts.[34] These studies used polyethylene oxide as the separation matrix in the presence of EOF, but failed to achieve baseline resolution for AAs with neutral isoelectric points. Moreover, control of EOF is critical, since capillary exchange may lead to misidentification of peaks, especially if the AAs are not fully separated. To improve the electrophoretic separation of AA-CBIs, we propose a novel polymer-based technique for the separation of NDA-derivatized AAs, followed by light-emitting diode-induced fluorescence. The separation of AAs was

implemented using polyvinylpyrrolidone (PVP), a well-known low viscosity linear polymer that is effective in suppressing EOF.[18, 35, 36] Additionally, we optimized separation efficiency and speed by utilizing additives such as organic solvents isopropanol (IP) and surfactants (SDS). The detailed experimental conditions and their effect on the separation of AA-CBIs are described in the main text.

Experimental

Chemical solution and plasma preparation

The D/L-AA standard package, NaCN, PVP (M_{avg} 1,300,000 Da), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), IP, and SDS were obtained from Sigma-Aldrich (St Louis, MO, USA). Acetonitrile was obtained from J. T. Baker (Phillipsburg, NJ, USA), and NDA was obtained from Tokyo Chemical Industry (Tokyo, Japan). The PVP solutions were prepared in HEPES (10 mM, pH 7.0) at various concentrations (w/v%). The NDA stock solution was dissolved in methanol, and the stock AA solutions were prepared in double deionized H_2O (ddH_2O). The stock solution of NaCN (10 mM) was prepared in deionized water. All stock solutions were stored at 4°C. Human plasma was collected from the donor (first author) using EDTA blood collection tubes (BD Vacutainer; Becton, Dickinson & Co., Franklin Lakes, NJ, USA). The collected whole blood was centrifuged at 2000 ×*g* for 10 min and the plasma was separated from the blood cells. The upper layer plasma was transferred to a 15-mL plastic tube and stored at −20°C. To remove the obstructing proteins from the plasma, the sample was incubated in boiling water for 10 min, followed by centrifugation at 18,000 ×*g*. The suspension was then transferred to a 1.5-mL plastic vial and stored at −20°C.

Derivatization of AAs with naphthalene-2,3-dicarboxaldehyde

The derivatization of AAs with NDA was performed in 0.2-mL centrifuge tubes using a previously published modified procedure.[37] Briefly, the AA mixtures (4.5 μM each, 5 μL) were added separately to solutions comprising sodium tetraborate (10 mM, pH 9.3, 5 μL), ACN (99%, 20 μL), NaCN (10 mM, 5 μL), NDA (1 mM, 5 μL), and deionized H_2O (final volume of 50 μL). For the derivatization of plasma AAs, the concentrations of NaCN and NDA were increased to 100 mM and 10 mM, respectively. For derivatization of the AAs of untreated plasma, a 5-μL sample was used instead of the AA stock solutions. To reduce the interference of proteins during derivatization, 200 μL of the plasma was heated at 95°C for 10 min and subsequently centrifuged at 10,000 ×*g* for 5 min. The suspension (protein-free plasma) was utilized for derivatization of AAs according to the procedure mentioned above. Once all the components of the derivatization reaction were added, the reaction mixture was gently mixed by brief vortexing and allowed to stand at ambient temperature for 30 min. The resulting reaction products were stable for up to 12 h when stored at −20°C.

Capillary electrophoresis with light-emitting diode-induced fluorescence

A capillary electrophoresis light-emitting diode-induced fluorescence (CE-LEDIF) system built in-house was used in this study; the system was built as previously reported, with slight modifications.[37] Briefly, a violet light-emitting diode (LED)

(InGaN; maximum output at 405 nm with a range of 390–420 nm and a radiant power of 2 mW) was used for excitation. The emitted light of the LED was filtered using a 405-nm interference filter with a radiant power of 0.25 mW for excitation. Fluorescence was collected through a 10× objective (numerical aperture: 0.25). A single interference filter (486 nm) was used to block scattered light before the emitted light reached the photomultiplier tube (R928, Hamamatsu Photonics K. K., Shizuoka-Ken, Japan). The fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) were 35 cm in length (effective length, 28 cm) with an inner diameter of 75 μm and an outer diameter of 365 μm. Prior to analysis, the capillaries were treated overnight with 5% (w/v) PVP (in H_2O) to coat the walls. The PVP solution (1%, 2%, or 5%) prepared in 10-mM HEPES buffer at pH 7.0 was introduced into the capillary from the cathode end using a manual syringe pump. IP (5%, 10%, 15%, 20%, and 40%) and SDS (10, 20, and 30 mM) in HEPES buffer were used for the preparation of PVP solutions. The NDA amino acid-cyano[*f*]benzoisoindoles (AA-CBIs) were introduced by electrokinetic injection (10 s at −10 kV) into a capillary filled with PVP. Separation was conducted at −20 kV. The currents altered during separation, eventually reaching a maximum of 25 μA, suggesting negligible Joule heating. Subsequent to each run, the used PVP was pushed forward to the outlet of the capillary using nitrogen gas, and fresh PVP solution was added using a manual

syringe pump prior to injection of the next sample. Teflon tubing with a 300-μm inner diameter (Supelco Inc., Bellefonte, PA, USA) was used to connect the capillary and a No. 25_G syringe needle (TERUMO, Tokyo, Japan) as well as a high-pressure nitrogen cylinder.

Chemical computation on AAs and AA–CBIs

The partition coefficient (logP), net charge, partial charge, and structure of the AAs and AA-CBIs were calculated and drawn by using MarvinSketch (ChemAxon, Budapest, Hungary). Briefly, the structure of AAs can be determined using the "Name to structure" function in the software toolbox and attaching the CBI onto the primary amine of the AAs to form the AA-CBIs.[38] The logP was then obtained by selecting the partitioning option shown in the calculation function. The net charge of the AA-CBIs was obtained from the isoelectric point calculation, followed by selection of the suitable net charge according to the pH value of the buffer. The partial charge of the atoms of the AA-CBIs could be obtained from the "charge calculation" option. All software-simulated data are listed in Table S1.

Results and Discussion

Improvement of separation efficiency using PVP buffer solution

We predicted that PVP might play an important role in the separation of AA-CBIs, similar to polyethylene oxide.[8] Therefore, we tested various PVP concentrations (Fig. 1) in order to optimize the separation conditions. As shown in Fig. 1a, no AA-CBIs could be differentiated in the capillary zone electrophoresis mode. However, when 1% PVP was added to the HEPES buffer, a noticeable improvement in resolution was observed (Fig. 1b). This improvement could be attributed to the hydrophobic interaction between AA-CBIs and PVP molecules. For example, Glu-CBI and Asp-CBI have net charges of −2.000 and −1.998, respectively; however, the hydrophobicity (partition coefficient, log P) shows that Glu-CBI (logP 2.73) is more hydrophobic than Asp-CBI (logP 2.44) (Table S1). Therefore, Asp-CBI migrated faster than all other AAs. Similarly, tryptophan (Trp)-CBI migrated at the end of the electropherogram (Fig. 1b), since it possesses the highest hydrophobicity (logP 4.83) among the AAs. However, the PVP polymer is a hydrophilic molecule (logP −0.81 for PVP monomer) that easily dissolves in water or buffer solution due to the oxygen (logP −0.14) and nitrogen (logP −0.36) atoms that it contains. The partial negative charge of the oxygen on PVP is −0.44 (Fig. S1), and may attract the partial positive hydrogen atom of the AA-CBIs, resulting in the formation of a hydrogen bond. Both van der Waals forces and hydrogen bonds affect the interaction of AA-CBIs and PVP molecules, and play an important role during electrophoretic migration. For instance,

the logP of AA-CBIs Trp, phenylalanine (Phe), and tyrosine (Tyr) are 4.83, 4.73, and 4.43, respectively, and they carry a net charge equal to −1.000 (Table S1). If the separation of AA-CBIs was governed solely by hydrophobicity, the migration order would be: Trp-CBI > Phe-CBI > Tyr-CBI. However, the electropherogram indicates that the migration time of the three aromatic AAs is Trp-CBI > Tyr-CBI > Phe-CBI. Thus, additional molecular interactions between PVP and AA-CBIs must affect the separation process. As shown in Figs. S1a and S1b, the partial positive charge of hydrogen on the highly electronegative atoms (oxygen and nitrogen) of Trp and Tyr is upwards of 0.19 and 0.22, respectively; this results in the formation of hydrogen bonds with the PVP molecules. Trp-CBI and Tyr-CBI migrated slower than Phe-CBI, since Phe-CBI does not efficiently form hydrogen bonds with PVP molecules. This result demonstrates that hydrogen bond interactions may also play an important role during AA-CBI separation except to the charge to mass ratio of the AA-CBIs and their hydrophobic interaction with PVP.

As shown in Fig. 1c, increasing the PVP concentration to 2% led to improved resolution, enabling the differentiation of serine (Ser)-CBI, alanine (Ala)-CBI, and asparagine (Asn)-CBI (Fig. 1c). Similarly, norvaline (Nva)-CBI was also separated from methionine (Met)-CBI and histidine (His)-CBI; however, the isomeric AAs, leucine (Leu)-CBI and isoleucine (Ile)-CBI, remained unresolved under these

experimental conditions. Leu (logP 4.33) and Ile (logP 4.41) reached baseline resolution when the PVP concentration was increased to 5% (Fig. 1d). Moreover, the overlapping peaks of Met-CBI and His-CBI; and the peaks of Ser-CBI, Ala-CBI, and Asn-CBI were also fully separated using 5% PVP. The baseline resolution of 18 AA-CBIs is demonstrated in Fig. 1d. The high level of separation of AA-CBIs obtained by using 5% PVP is suitable for the quantitative analysis of acidic and neutral AAs.

Effect of IP on separation of AA-CBIs

The separation time for hydrophobic AA-CBIs such as Trp-CBI and Tyr-CBI is extremely long (Fig. 1d). Therefore, various concentrations of IP were added to the 5% PVP solution to improve the velocity of the AA-CBIs. The partition coefficient of PVP is −1.86, whereas the logP of IP is 0.25, which implies that IP is more hydrophobic than PVP. The addition of 5% (Fig. 2b) and 10% (Fig. 2c) IP to the PVP solution resulted in a decrease in the velocities of the aromatic AA-CBIs, particularly that of the highly hydrophobic Trp-CBI. This could be attributed to the increased hydrophobic interaction caused by the IP adsorbed onto the PVP molecules, forming an intermolecular hydrogen bond (Fig. S1c). Interestingly, the migration time of these three AA-CBIs shortened when the IP concentration was increased from 15% to 20%

(Figs. 2d & 2e). Under conditions of increased IP, the PVP serves as the polar stationary phase and the IP serves as the non-polar mobile phase; the separation of AA-CBIs by capillary gel electrophoresis in the presence of sufficient IP can be considered as normal phase chromatography. Additionally, the velocity of AA-CBIs that migrated slower than methionine (Met)-CBI was also improved (Figs. 2c–2e). The order of migration was determined by the differences in hydrophobicity; all AA-CBIs with a logP higher than that of Met-CBI migrated slower than Met-CBI (logP 3.73), except for histidine (His)-CBI (logP 2.65). His-CBI is a relatively hydrophilic molecule compared with Met-CBI. However, the net charge of His-CBI is only −0.740 at pH 7.0, which leads to an increased m/z and slower migration than Met-CBI. Conversely, threonine (Thr)-CBI migrated faster than citrulline (Cit)-CBI due to its smaller m/z of Thr-CBI (Fig. 2a). However, the migration orders of Thr-CBI and Cit-CBI were reversed when the IP concentration was increased (Figs. 2b–2d). Similar to Tyr-CBI, the structure of Thr-CBI also contains a hydroxyl group that can form hydrogen bonds with PVP molecules and IP. Consequently, IP increases the velocity of Cit-CBI, but decreases the migration of Thr-CBI; hydrogen bonds also play an important role in the apparent mobility of the -OH groups in the AA-CBIs. Similar results were also observed for serine (Ser)-CBI, which exhibited increased retention time upon addition of IP, compared with Alanine (Ala)-CBI and Asparagine

(Asn)-CBI.

Effect of SDS on separation in the presence of PVP and IP

Fig. 2 demonstrates the improved migration speed of aromatic AA-CBIs. However, the basic AAs, including Lys, Orn, and Arg, did not appear in the electropherograms (Fig. 3a) despite their partial negative net charge at pH 9.3 (Table S1). Therefore, various concentrations of SDS were added to the 5% PVP solution in an attempt to enhance the migration of the basic AAs (Fig. S2). Unfortunately, SDS micelles did not accelerate the migration of the basic AA-CBIs, but rather increased the retention of most AA-CBIs. Moreover, the broadened Trp-CBI peak in Figs. S2a–S2d indicates that 5% PVP in the presence of SDS alone could not improve the separation speed. The hydrophilic head of SDS was adsorbed onto the pyrrolidone unit of PVP, while the hydrophobic portion of SDS shifted away from the PVP molecules.[39] Consequently, the stationary environment within the capillary became more hydrophobic, thus decreasing the migration of AA-CBIs with increasing concentrations of SDS. However, the migration of the highly hydrophobic Trp-CBI was greatly improved by the IP/SDS mixed micelles (Figs. 3b & 3c), compared with 5% PVP-containing IP (Fig. 3a) or SDS alone (Fig. S2). The enhanced AA-CBI migration could be attributed to the alternation to hydrophobicity caused by the

presence of both IP and SDS. In a previous study, Dan et al. demonstrated that a mixture of IP/SDS can form small mixed micelles that adsorb onto PVP molecules.[40] Unlike the hydrophobic environment of SDS adsorbed to PVP (Fig. S2), the van der Waals interactions between AA-CBIs and PVP/mixed micelles may be reduced in the presence of IP, particularly if the IP concentration (20%) is greater than that of SDS. In other words, the free solution within the capillary is more hydrophobic than the mixed micelles adsorbed to the PVP molecules, resulting in a decrease in the retention of AA-CBIs. Moreover, the basic AAs appeared in the electropherograms when the polymer solution consisted of 20% IP, and the cathodic container contained 10 or 20 mM SDS (Figs. 3b & 3c). These results indicate that acidic, neutral, and basic AAs can be effectively separated using capillary electrophoresis with PVP, IP, and mixed micelles as the separation matrix. When the concentration of SDS reached 30 mM (Fig. 3d), the retention of Trp-CBI was slightly increased compared with that observed in Fig. 3c. This is due to the presence of large mixed micelles that formed with the increase in SDS concentration.[40] Unlike the small mixed micelles that adsorbed on the PVP molecules, the large mixed micelles remain in the free solution and decrease the hydrophobicity of the buffer solution. Consequently, the interaction of hydrophobic Trp-CBI with PVP and adsorbed small mixed micelles were increased. Thus, the speed of AA-CBI separation using IP/SDS can be improved when the SDS

concentration is limited to the formation of adsorbed small micelles. Interestingly, the migration of His-CBI exhibited a reversed trend with increasing concentrations of SDS (Figs. 3b & 3c), compared with that of Trp-CBI. His-CBI is a hydrophilic molecule that carries a partial positive charge (net charge: −0.74). Electrostatic interactions may also play an important role in the retention of His-CBI with increased concentrations of SDS (Figs. 3b & 3c). This could be attributed to the adsorbed small mixed micelles with negative charges. However, this effect decreased as the concentration of SDS reached 30 mM (Fig. 3d). The free large mixed micelles competed with the adsorbed small micelles for the electrostatic interaction with His-CBI, thereby increasing the migration of His-CBI compared to that observed in Fig. 3c.

Separation of AA-CBIs from human plasma

As mentioned above, many important AA biomarkers are present in body fluids such as urine, cerebrospinal fluid, ascites, serum, and plasma. Such complex biological samples contain considerable amounts of proteins, metabolites, and salts that may cause serious matrix effects during NDA derivatization. In addition, successful AA derivatization should be performed with a concentration of NDA and nucleophiles that is higher than the total concentration of AAs. Sample dilution is the most

effective and convenient strategy to overcome the challenges of matrix effects and the high concentrations of physiological AAs within plasma[41] during the NDA derivatization of AAs. As demonstrated in Fig. S3a, untreated plasma was directly processed for NDA derivatization, and only a few peaks appeared in the electropherogram. Derivatization could be greatly improved by removal of plasma proteins (Fig. S3b) and use of a 10-fold dilution of plasma (Fig. S3c). The use of protein-free plasma resulted in higher peak intensities than those shown in Fig. S3c, demonstrating that proteins are strong inhibitors of the NDA reaction. When the protein-free plasma was further diluted (Fig. S3d), the number of electropherogram peaks increased. These results indicate that proteins are not the sole inhibitors of AA NDA derivatization, and that inhibition can be minimized by the combination of heat-induced protein precipitation and dilution.

Subsequent to sample pretreatment, 5% PVP (Fig. 4a) was determined to be suitable for baseline separation of AA-CBIs from human plasma, except for the basic AAs (Arg, Lys, and Orn). Arg and Orn have been demonstrated to play an important role in the urea cycle of the nervous system; thus, these basic AAs can be considered as important biomarkers of neurochemistry.[42, 43] Therefore, mixed micelles combined with PVP-filled capillary electrophoresis may serve as powerful tools for the separation of AA-CBIs, in spite of the diminished resolution between Ala and Gln. As

shown in Fig. 4b, all physiological AA-CBIs, in addition to a number of unidentified peaks, were clearly separated, and most AA-CBIs achieved baseline resolution. Furthermore, the NDA-derivatized AAs exhibited enhanced stability beyond 12 h (Fig. S4). Repeated experiments examining the separation of AAs from plasma by using CE-LEDIF (Fig. S5) also indicated that our proposed method is highly reproducible. For example, the relative standard deviation (RSD) of the Arg-CBI migration time was 0.46% after ~2 h of separation (Fig. S5). However, the RSD values of the peak area of Arg-CBI and His-CBI were 22.8% and 7.9%, respectively, when α-aminoadipic acid was used as an internal standard. The quantitative results of plasma Val, Leu, Ile, and Tyr were 246 ± 10.8 μM, 162 ± 7.33 μM, 56 ± 4.2 μM, and 186 ± 23.5 μM, respectively (n = 5). The normal ranges for the Val, Leu, Ile and Tyr are 179–335 μM, 113–205 μM, 46–90 μM, and 33–77 μM, respectively.[41] The high Tyr RSD values of 12.7% and abnormal CE-LEDIF quantitative results also indicated that α-aminoadipic acid is not an appropriate internal standard. Therefore, an internal standard that is closer to Tyr-CBI might be a better option for the quantitation of Tyr-CBI. Under these optimized conditions, the LOD for Val, Leu, Ile, and Tyr (liver cancer biomarkers) are 5.3 nM, 5.7 nM, 16 nM, 6.2 nM; and LOQ are 18 nM, 19 nM, 54 nM and 21 nM respectively. These results indicate that the sensitivity of CE-LEDIF is sufficient to detect physiological AAs[41] and suitable for clinical

diagnosis using AAs as biomarkers. An obvious disadvantage of this method is the excessive separation time required, which might limit its further clinical applications. Interestingly, up to 2 hours of separation time is required for the examination of AAs by ion-exchange chromatography, which is extensively used in hospitals.[7, 44] Therefore, our proposed method still remains a viable option due to the natural superiority of CE.[45, 46] Further improvement in the time required for AA analysis could be achieved using pressure-assisted capillary electrophoresis[47] or by shortening the length of separation using chip electrophoresis.[48-50]

Conclusions

In this study, we demonstrated a powerful method for the separation of AA-CBIs from plasma using capillary gel electrophoresis in the absence of EOF. The low viscosity polymer solution is an influential factor in the baseline resolution of 18 acidic and neutral AA-CBIs from plasma. By using mixed micelles (IP and SDS) together with a polymer solution, 21 plasma AAs (including three basic AAs) were identified in the electropherogram. This high level of resolution provides the ability to accurately quantify AAs in complex biological specimens without the need for protein filtration. The stability of the AA-CBIs also demonstrates that the products of NDA derivatization are stable for up to 12 h, which is suitable for clinical examination. Compared to conventional liquid chromatography, this technique provides distinct

advantages, including simplicity (ease of preparation of the polymer solution), satisfactory sensitivity, low cost (less consumed reagents and lower power excitation by LED), minimal sample volume requirements, reduced matrix effects, and high-level resolution. Therefore, this proposed method should be more suitable for larger-scale AA profiling required for metabolomics and/or cancer studies, compared with IC or HPLC of body fluids combined with capillary array electrophoresis.

Acknowledgment

The authors are grateful to the Ministry of Science and Technology of Taiwan (102-2113-M-029-002-MY2) for providing financial support for this work.

Figures and Captions

Fig. 1 Improvement of the resolution of AA-CBIs in the presence of a) 0%, b) 1%, c) 2%, and d) 5% PVP. Peak identities: 1. Asp, 2. Glu, 3. Gly, 4. Ser, 5. Ala, 6. Asn, 7. Thr, 8. Cit, 9. Met, 10. His, 11. Nva, 12. Val, 13. Nle, 14. Leu, 15. Ile, 16. Phe, 17. Tyr and 18. Trp

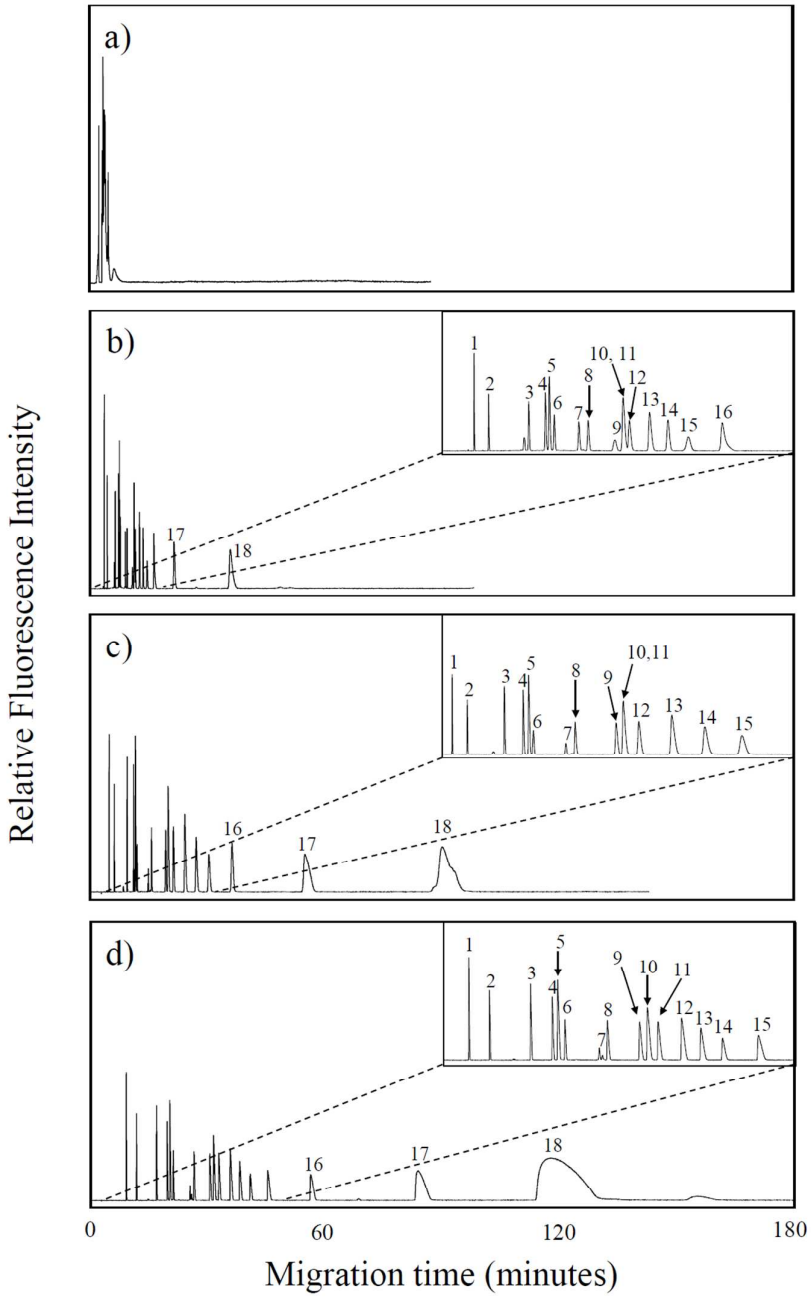

Fig. 2 Effect of IP on the separation of AA-CBIs. The separations were performed by a) 5% PVP containing b) 5%, c) 10%, d) 15%, and e) 20% IP. Peak identities: 1. Asp, 2. Glu, 3. Gly, 4. Ser, 5. Ala, 6. Asn, 7. Thr, 8. Cit, 9. Met, 10. His, 11. Nva, 12. Val, 13. Nle, 14. Leu, 15. Ile, 16. Phe, 17. Tyr and 18. Trp

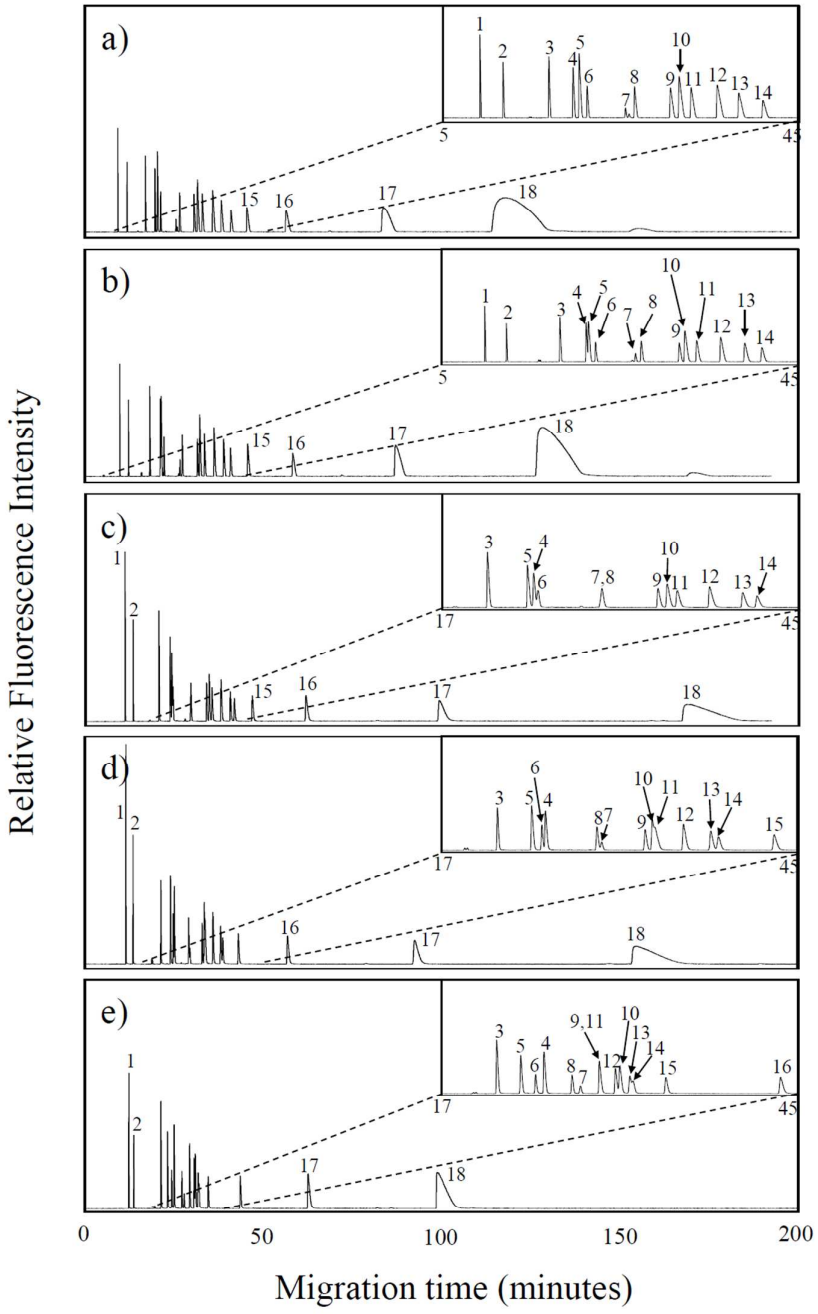

Fig. 3 Impact of SDS on the separation of AA-CBIs by PVP in the presence of IP. The separations were completed using a) 20% IP and 5% PVP containing b) 10 mM, c) 20 mM, and d) 30 mM SDS in the inlet polymer solution. Peak identities: 1. Asp, 2. Glu, 3. Gly, 4. Ser, 5. Ala, 6. Asn, 7. Thr, 8. Cit, 9. Met, 10. His, 11. Nva, 12. Val, 13. Nle, 14. Leu, 15. Ile, 16. Phe, 17. Tyr, 18. Trp, 19. Lys, 20. Orn and 21. Arg

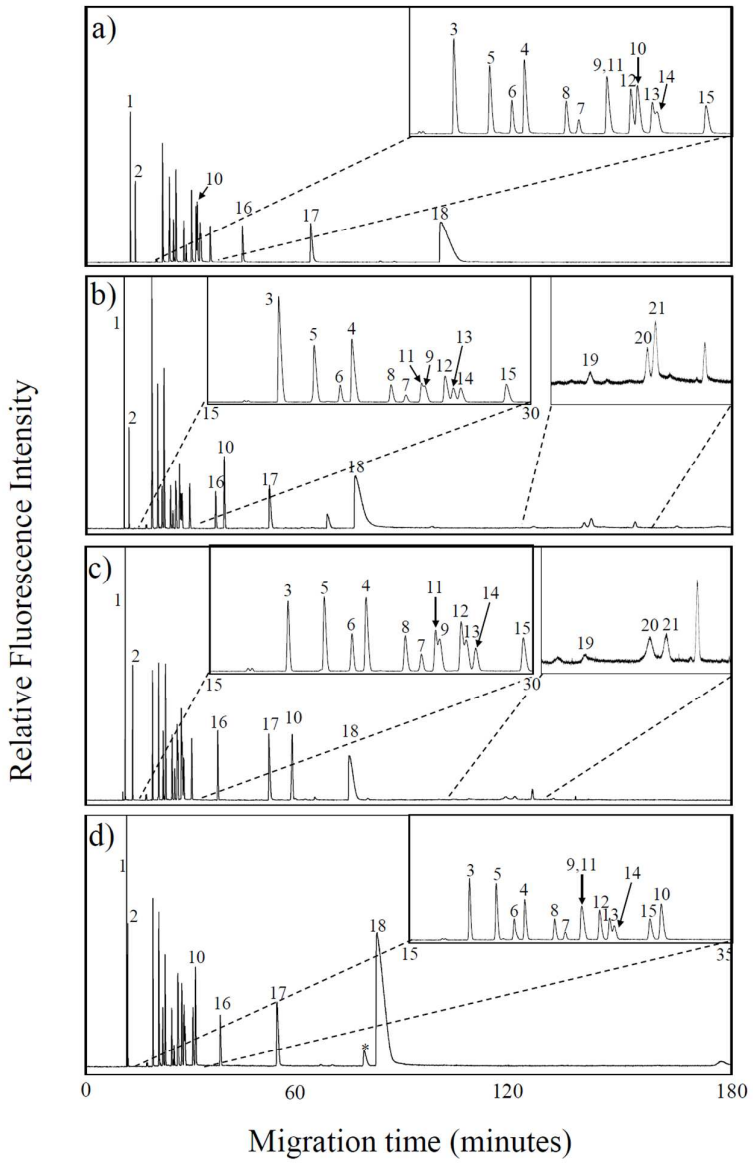

Fig. 4 Electropherograms of AA-CBIs from treated human plasma. The separations

were performed by a) 5% PVP and b) 5% PVP containing IP (20%) and SDS (20 mM). Peak identities: 1. Asp, 2. Glu, 3. Gly, 4. Gln, 5. Ser, 6. Ala, 7. Asn, 8. Thr, 9. Cit, 10. Met, 11. His, 12. Val, 13. Leu, 14. Ile, 15. Cys, 16. Phe, 17. Tyr, 18. Trp, 19. Lys, 20. Orn and 21. Arg. The asterisks indicate the unidentified compounds of the plasma.

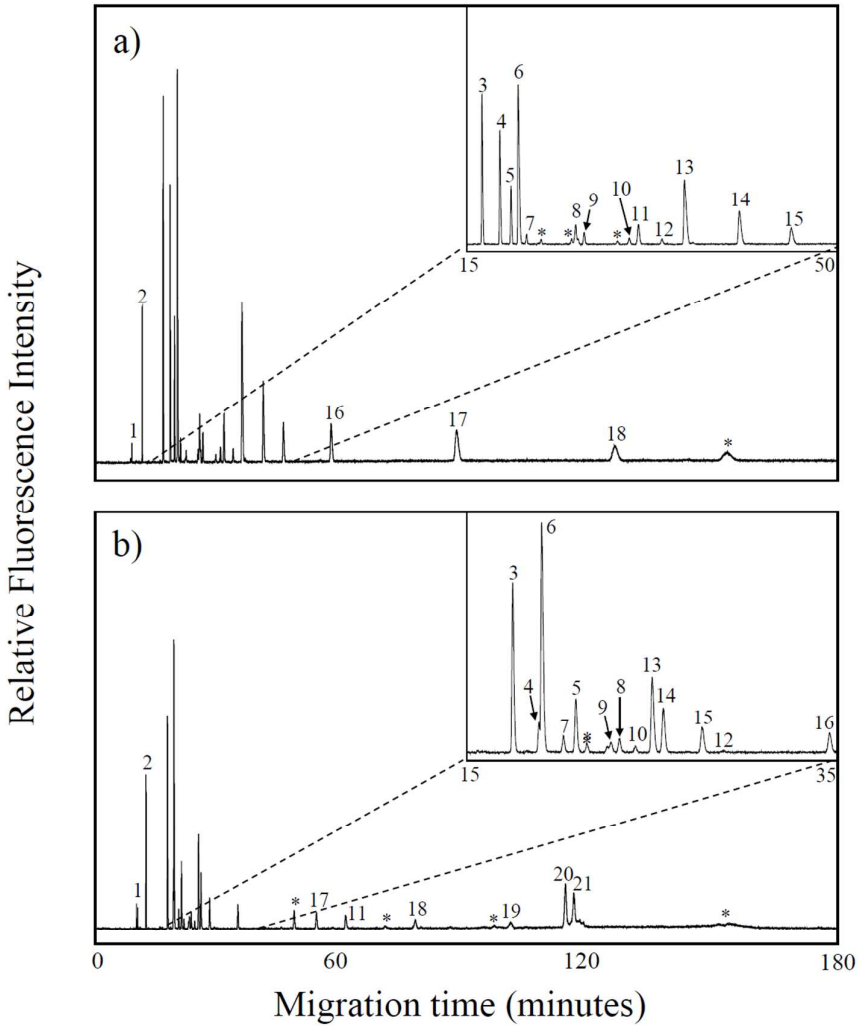

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Table of Contents

Separations of amino acid biomarkers could been performed by the polyvinylpyrrolidone-filled capillary in the presence of mixed micelles.