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OPTIMIZATION OF THE ANALYTICAL CONDITIONS FOR AMINO ACID PROFILING USING A SHEATHLESS CE/ESI-MS

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Abstract: We report a robust strategy for amino acid profiling method based on capillary electrophoresis-electrospray ionization-mass spectrometry (CE/ESI-MS) that offers a convenient platform for the direct analysis of underivatized amino acids from dried blood spot without any chemical derivatization. Sample preparation steps were simplified without derivatization. The analytical conditions with respect to pH and concentration of background electrolyte were optimized for 11 amino acids. The mean recoveries of amino acids for intra- and inter-day assays were found to be 96.9–102.1%. When compared with HPLC-IDMS for blood spot samples, highly agreeable results were obtained with the proposed CE-IDMS method.

Keywords: CE-MS, sheathless ESI interface, amino acid profiling, dried blood spot.

1. INTRODUCTION

Determination of amino acid (AA) composition for AA profiling is an important component of many medical, scientific and industrial processes, and the identification of AAs in body fluids such as urine and blood is particularly helpful in the diagnosis, prognosis and treatment of diseases [1,2]. Since AAs are highly polar, nonvolatile compounds and have little chromophore, AA analysis represents one of the most challenging forms of analytical research. Although the CE-MS technique has been employed in various fields, and the merits derived from CE separation and MS detection have been demonstrated, the on-line interfacing of CE with MS remains a challenging issue [3]. A sheathless interface has been developed to avoid the dilution effect of the sheath liquid in sheath-flow supported interface. As a sheathless interface operates without an external flow, electrical contact to the spray tip or its vicinity should be established without the assistance of the sheath liquid. For the widespread application of such sheathless ESI interfaces, stability of operation and durability for long term use are essential. Easy fabrication and quick replacement would also be advantageous in routine applications. In the previous papers, we reported on the development of a two-capillary sheathless CE/ESI-MS interface with an ionophore membrane-packed electro-conduction channel with stable operation and improved sensitivity [4,5]. Fabrication and replacement of capillary tubing were readily achieved. More importantly, the interface operated stably and can last long enough for repeated analysis in routine applications. In this work, we demonstrate the potential of a CE-MS system equipped with our sheathless ESI interface for routine analysis of AAs profiling for clinical use. Most publications in this area have reported success utilizing sheath-flow interface systems combined with acidic electrolytes. Alternatively, we use an alkaline buffer to maintain sufficient fast electroosmotic flow, so that the electrospray should not be in shortage of flow.

The dried blood spot (DBS) is an effective way to allow simple collection of blood, easy transport, high-volume storage and long-term stability [6]. DBS can be obtained from heel or finger pricks and spotted onto filter paper, which absorbing certain quantity of blood in it. The use of DBS in neonatal screening was introduced by Dr. Guthrie for the detection of phenylketonuria in early 1960s. Nowadays, DBS is applied to many other fields, including therapeutic drug monitoring, toxicokinetics and pharmacokinetics [7].

In this report, we investigated the sensitivity of CE-MS with a sheathless interface in addition to the stability and durability of the proposed interface during routine applications. Repeatability and reproducibility of analysis were also of great concern. The validity of CE-MS in the direct analysis of AAs was to be confirmed through comparison with analytical results by HPLC-IDMS. Finally, the method was applied to the quantification of AAs in DBS as a trial for possible clinical use.

2. EXPERIMENTS

1. Interface fabrication: The main body of the interface was machined with an accurate realization of the designed structure (Figure 1). A separation capillary was joined to an emitter capillary inside the channel to create a 400-μm diameter “capillary channel” in the center of the interface body. At both ends of a capillary channel, receptacles of finger-tightened fittings for standard capillary tubing were implemented, which facilitated easy replacement of capillaries. Where the capillaries adjoined, an electro-conduction channel referred to as the “conduction channel” was prepared. Nafion tubing was inserted into this conduction channel through-hole and forcefully pushed. A piece of platinum (Pt) wire was wrapped around the interface body as an electrode. A piece of sponge was then placed over the Pt wire and soaked with an electrolyte to provide an electrical contact between the conduction channel and the Pt wire.

2. CE-MS: A home-built CE system was interfaced with a triple-quadrupole mass spectrometer (Quattro Ultima; Micromass). The CE-MS interface described above was installed on a commercial nanospray mounting system PV-300 (New Objective). Two independent bipolar high-voltage supplies were used for electrical connections in the CE separation and ESI. Employment of the sheathless CE-MS system for the analysis of underivatized AAs was performed using uncoated fused-silica capillaries with 50-μm id, a 95-cm separation capillary and a 4-cm emitter tip. The BGE used comprised 5 mM NH_4Ac, adjusted to pH 10.8 with NH_4OH. A voltage of 25 kV was applied for CE separation, where 2.6 kV was applied to the interface for positive mode ESI. Samples were electrokinetically injected at 25 kV for 3 s.

3. DBS sample and sample preparation: The DBS samples for AA profiling were prepared in our lab. Whole blood was collected in EDTA vials from a healthy male adult, comprising AAs of unknown concentration. The blood were dispensed in 50-μL portions onto S&S 903 filter paper with dashed-line 13-mm printed circles (903™ Protein saver card, lot no. W102; Whatman, Sanford, ME, USA). The DBS samples were dried overnight on the dry rack at room temperature and stored in a zippered plastic bag with desiccant at 4℃. For method validations, DBS calibrator from commercial source for phenylketonuria screening kit were purchased from Bio-Rad Diagnostics Group (Quantase™ Neonatal phenylalanine screening; Hercules, CA, USA), and the concentrations of phenylalanine in each blood spot on a Guthrie filter paper were 1.56, 3.82, 7.34 and 15.15 mg/dL.

For CE-MS analysis, a 3.2-mm (1/8'') diameter disc was punched out of the 13-mm diameter spotted DBS with a paper punch (Whatman, lot no. 9238030) and placed into a conical-bottom tube. One hundred microliter of internal standard solution were also gravimetrically added to each tube. The extraction was carried out by gentle agitation on a shaker for 30 min. For sample deproteinization, the extracts were transformed to centrifugal unltrafiltrarion unit (MWCO, 10,000), and then centrifuged for 20 min at 12,400 g. The filtrates were injected into the CE-MS system.

4. Intra- and inter-day assays: The precision and accuracy of intra-day assays were determined using four different concentrations of DBS calibrators in five independent experiments. To determine inter-day variation, the level of phenylalanine in the DBS calibrator was measured on five consecutive days.

5. Method comparison: DBS samples were prepared according to the sample preparation procedure detailed above. Exact matching double ID-MS quantification was adopted to improve quantification accuracy, where the concentrations of AAs are calculated using a single-point calibrator at concentrations very close to that of the unknown sample. Therefore, the amounts of isotope-labeled AAs used as the working internal standards were chosen so as to provide a comparison of results from replicated tests. The filtrates after sample preparation were analyzed using both CE-MS and CE-UV to assess the extent of uneliminated compounds. Finally, the concentrations of 6 AAs in serum were determined by both CE-IDMS and LC-IDMS.

3. RESULTS AND DISCUSSION

1. Stability and durability of the ESI interface: In sheathless ESI, the formation of electrospray is especially dependent upon the CE conditions as the CE effluent is the sole liquid source of the electrospray. When 25 kV was applied for the electrophoresis using 5 mM NH_4Ac as the BGE, a stable Taylor cone was observed at 2.4 kV applied for the electrospray [5]. Under these conditions, the flow-rate was to be ~23 nL/min. A strong EOF due to the alkaline BGE maintained a flow stream across the emitter capillary, and good repeatable results of migration time and peak area were obtained through twenty-time repetition of runs of the same sample (Figure 2). The RSDs were < 1.3% in migration time and < 5.9% in peak areas. As is common in microfluidic channels, the formation of bubbles must be avoided. In order to avoid the formation of a bubble, the BGE needs to be filtrated and degassed as thoroughly as possible prior to use. The stability and durability of the CE-MS interface, in addition to its ease of handling, would allow application of the CE-MS system to routine analysis.

2. Optimization of background electrolyte for amino acid profiling analysis: AAs possess an overall positive charge at pH values below their isoelectric point (p*I*) and overall negative charge at pH values above their p*I* (Table 1). Separation of AAs as ions requires either an acidic BGE or an alkaline BGE. An acidic BGE with weak EOF causes instability of ESI with a sheathless interface [5]. Therefore, an alkaline buffer with strong EOF was the first criterion that needed to be satisfied. Since pKa_2 values of AAs are mostly around pH 9, the AAs are negatively charged above pH 10. At this pH, a strong EOF that dominates the backward electromigration of negatively charged AAs is expected. The effective mobilities (μ_e) of AAs at different pH were calculated using equation (1) using dimethyl sulfoxide as a neutral marker (Figure 3). A pH of around 10.8 was sufficient to separate underivatized 11 AAs. BGE concentration was 5 mM for effective resolution and sensitivity in CE separation and MS detection. Possible degradation of the BGE in consecutive CE runs was avoidable by replenishment every 5 runs. CE-MS detection of 11 amino acids under optimized conditions is shown in Figure 3. MS detection was carried out in multiple reaction monitoring (MRM) mode with collisionally activated dissociation. Selected m/z of precursor and product ions are summarized in Table 1. This combination of CE separation and MS filtered detection would allow for the selective detection of most AAs.

Equations: equation (1)

$$\mu_e = L^2 / t_a V - L^2 / t_{EOF} V [cm^2 V^{-1} s^{-1}] \qquad (1)$$

L: length of the separation capillary;
V: applied potential;
t_a: migration time of an AA;
t_{EOF} : migration time of a neutral marker, DMSO

3. Homogeneity testing of DBS sample: The homogeneity analysis of DBS has been performed on 7 discs from different location of one 13-mm diameter spot (Figure 4). Results of phenylalanine and tyrosine from the homogeneity testing are shown in Figure 4. DBS samples demonstrated reliable stability in various locations of blood spot. The RSDs of measured concentration were < 1.3% in phenylalanine and < 2.2% in tyrosine.

4. Recovery, precision and accuracy: Recoveries of phenylalanine from the inter-day and intra-day assays on four DBS calibrators are shown in Table 2. Mean recoveries and the RSDs were highly stable in the phenylalanine concentration range from 1.56 to 15.15 mg/dL.

5. Determination of AAs in DBS: The six AAs were identified in DBS samples using our optimized CE-MS method. As described in the Experiments section, a 3.2-mm punched disc of DBS containing AAs were extracted in 100 µL distilled water. Organic solvents or acids have frequently been employed for sample deproteinization (protein precipitation) [8]. However, the addition of organic solvents or acids can cause current instability and abnormal peak shape, and this approach is not suitable for CE analysis without pretreatment strategies such as evaporation or neutralization. To avoid such complexities, we employed simple filtration using a filter with a MWCO of 10,000.

Figure 5 represents result comparisons of the concentrations of 6 AAs in DBS of whole blood from healthy male adult determined using CE-IDMS and LC-IDMS. Results from the two different methods were in tight agreement (96.9 to 102.0%). Therefore, the CE-IDMS method employed with a sheathless ESI interface was shown to be as reliable as LC-IDMS in the determination of AAs in DBS.

4. Conclusions: In this study, we describe a high sensitivity CE-MS method for the direct analysis of amino acids in DBS for clinical use. High stability and durability were achieved with a sheathless ESI interface that also featured convenience suitable for routine analysis. A stable electrospray was achieved using a volatile alkaline buffer that exerted a strong EOF to supply sufficient fluid to the emitter tip. Negatively charged amino acids were eluted within a short run-time using the alkaline buffer. Six amino acids in serum and DBS were determined with accuracy and precision by IDMS. The results of CE-IDMS were highly agreeable with those of LC-IDMS. The CE-MS method employing a sheathless ESI interface featured high sensitivity, robustness and convenience, and could be broadly applied to the routine analysis of biological samples with limited availability.

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4. FIGURES AND TABLES

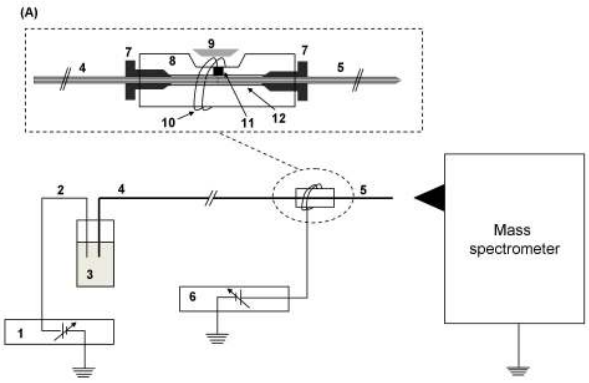

Figure 1.
Schematic diagram of the CE-MS system (not to scale): (1) power supply for CE; (2) CE anode; (3) buffer reservoir; (4) CE capillary; (5) emitter; (6) power supply for ESI; (7) capillary fitting; (8) interface body; (9) liquid-retaining sponge; (10) Pt electrode; (11) conduction channel; (12) capillary channel. Expanded view of the interface (A).

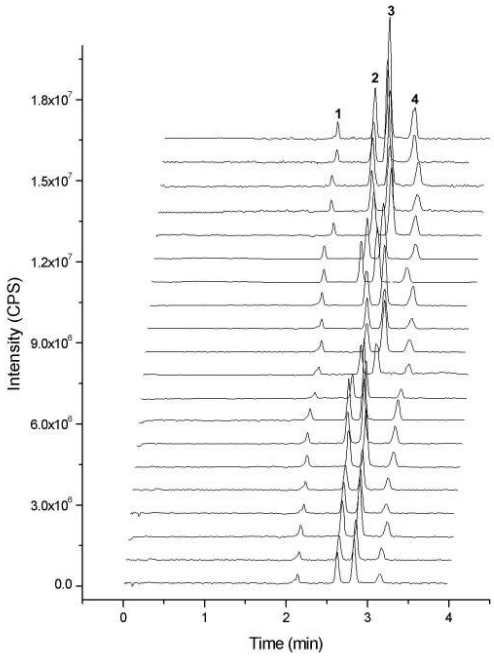

Figure 2.
Total ion electropherograms of four amino acid standards mixture with 20-times repeated injections. Peaks:

1, Pro; 2, Ile; 3, Phe; 4, Tyr. Conditions: capillary, uncoated fused-silica (40-cm separation capillary and 4-cm emitter tip x 50-μm id); electrolyte, 5 mM NH_4Ac (pH 10.8); separation voltage, 25 kV; injection, 3 s at 25 kV with sample concentration of 10 nmol/g; detection, MRM mode..

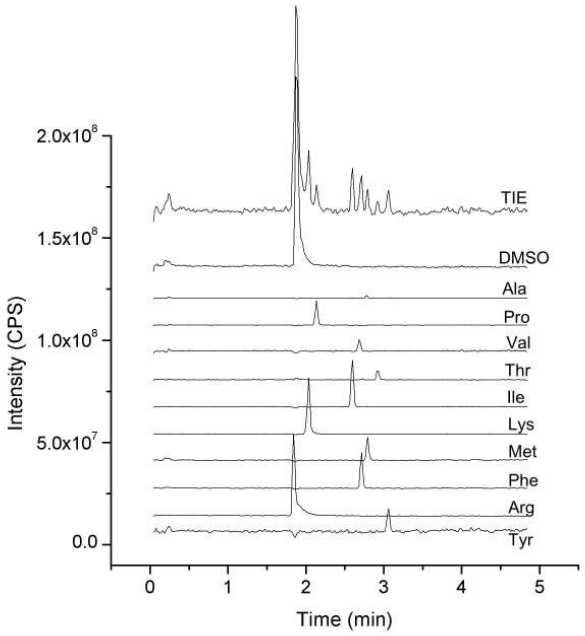

Figure 3.

Electropherogram of amino acid standard mixture by CE/ESI-MSMS. Conditions are same with Figure 2. All amino acids were at a concentration of 10 nmol/g. Details of the detection conditions are shown in Table 1. DMOS; dimethyl sulfoxide as a neutral marker, TIE; total ion electropherogram.

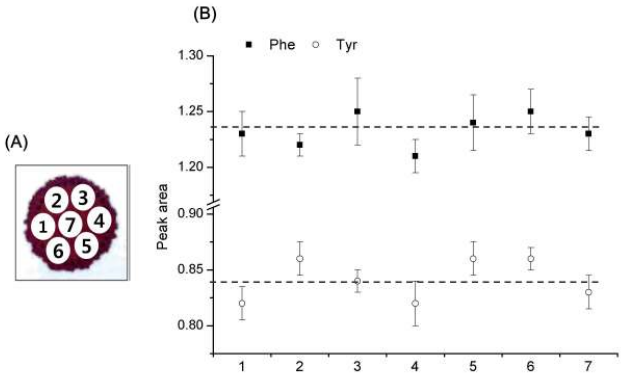

Figure 4.

Homogeneity testing for punched location in DBS sample. Punched sites (A) and measured concentrations of phenylalanine and tyrosine in the punched discs. Dashed lines indicate average value of results.

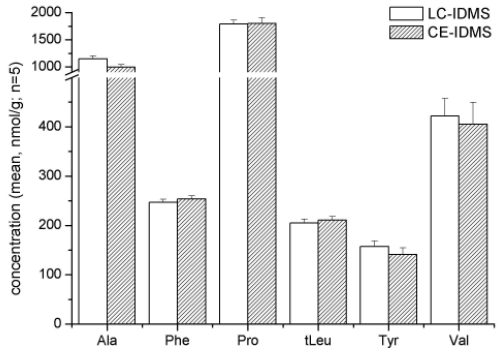

Figure 5.

Comparisons of concentration results of six amino acids in DBS using LC-IDMS and CE-IDMS. LC-MS conditions: column; Kinetex C18, 2.0×150 mm, 2.6μm (Phenomenex, USA), eluent; isocratic elution with 10% acetonitrile in 0.1% trifluoroacetic acid, flow rate; 0.2 mL/min, column temperature; 40℃, injection volume; 5 μL, run time; 5 min/sample, ESI voltage, 2.5 kV, MS system is same with CE-MS and other parameters were optimized. tLeu = Ile + Leu

Table 1.

Properties and MS/MS settings of targeted amino acids and isotope-labeled amino acids (internal standards).

Compounds	Abbreviations	pK_a values			pI	MS/MS setting *(m/z)*	
		COOH	NH_3^+	R group		precursor ion [M+H]+	product ion [M+H]+
L-alanine	Ala	2.34	9.69		6.01	89.80	90.19
L-alanine-$^{13}C_2$	Ala*					91.80	92.24
L-arginine	Arg	2.17	9.04	12.48	10.76	175.00	70.22
L-histidine	His	1.82	9.17	6.00	7.59	155.95	110.04
L-isoleucine	Ile	2.36	9.60		5.98	131.90	86.19
L-isoleucine-$^{13}C_6$,^{15}N	Ile*					139.20	92.18
L-leucine	Leu	2.36	9.68		6.02	131.90	86.19
L-lysine	Lys	2.18	8.95	10.53	9.74	147.00	130.06
L-methionine	Met	2.28	9.21		5.74	150.05	104.06
L-phenylalanine	Phe	1.83	9.13		5.48	166.20	120.04
L-phenylalanine-$^{13}C_9$,^{15}N	Phe*					176.20	129.05
L-proline	Pro	1.99	10.96		6.48	116.20	70.33
L-proline-$^{13}C_5$,^{15}N	Pro*					122.12	75.23
L-threonine	Thr	2.11	9.62		5.87	120.00	74.29
L-tyrosine	Tyr	2.20	9.11	10.07	5.66	181.90	136.13
L-tyrosine-phenyl-$^{13}C_6$	Tyr*					187.90	142.09
L-valine	Val	2.32	9.62		5.97	117.95	72.35
L-valine-$^{13}C_5$,^{15}N	Val*					124.00	77.28

Table 2.

Recovery and precision for intra-day and inter-day phenylalanine assays on DBS calibrators for phenylketonuria screening.

	Added amount (mg/dL)	Found amount (mg/dL)		
		Mean ± SD (mg/dL, n=5)	RSD (%)	Recovery (%)
Intra-day	1.56	1.51 ± 0.05	4.20	96.97
	3.82	3.89 ± 0.15	3.76	102.16
	7.34	7.27 ± 0.26	3.79	99.05
	15.15	15.14 ± 0.40	2.77	99.94
Inter-day	1.56	1.58 ± 0.04	3.37	101.66
	3.82	3.85 ± 0.13	3.15	101.02
	7.34	7.26 ± 0.21	3.04	99.49
	15.15	15.17 ± 0.07	0.52	100.18

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