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## Development of Micellar Electro Kinetic Chromatography for the Separation and Quantitation of L-valine, L-leucine, L-isoleucine and L-phenylalanine in Human Plasma and Comparison with HPLC

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**Abstract:** Phenylketonuria (PKU) and Maple Syrup Urine Disease (MSUD) are two inborn metabolic diseases which are carried by autosomal recessive genes in man. These genetic errors result in accumulation of phenylalanine (in PKU) or valine, leucine and isoleucine (in MSUD). At high concentrations, amongst other problems, these amino acids cause mental retardation. However if detected early after birth, using special diets and other forms of therapy, mental abnormalities can be prevented. As a result in many countries screening of infants for MSUD and PKU, by measuring plasma amino acids has become a routine neonatal test. Capillary Electrophoresis (CE) assays have a number of advantages over the traditional chromatography techniques (such as GC or HPLC). These include low cost, high speed of analysis and high resolution. These characteristics, make CE an ideal method for the screening of inborn errors of metabolism. We developed a CE assay based on pre-column derivatisation of amino acids with phenylisothiocyanate. This conjugate has strong absorbance at 254 nm. CE was carried out using a Spectrophoresis 1000 instrument, fitted with 40 cm of a 25 µm capillary, at 17°C. A running voltage of 18KV was used to separate the amino acid mixture in an electrophoretic buffer containing 45 mM imidazole, 6 mM borate and 208 mM SDS, fixed at pH 9 with 2-N-morpholino ethane sulfonic acid. The assay was calibrated using various concentrations of amino acid standards. LOD, LOQ, recovery, inter-day and intra-day variations of the assay were determined. Also, levels of the 4 amino acids in normal and abnormal plasma were determined and compared with HPLC.

**Key words:** Micellar capillary electrokinetics chromatography, amino acids, phenylisothiocyanate, PKU, MSUD

### INTRODUCTION

Amino acids, as the building blocks of proteins have an especially important role in the normal operation of cells, tissues and organisms (Fiamegos and Stalikas, 2006; Ummadi and Weimer, 2002 ). In man, any problems in the absorption or metabolism of amino acids can lead to serious physiological abnormalities. Phenylketonuria (PKU) and Maple Syrup Urine Disease (MSUD) are two inborn errors of metabolism which lead to specific aminoacidopathies. In PKU, metabolism of phenylalanine becomes interrupted, leading to an increase in the levels of this amino acid in serum, while in MSUD the metabolic error leads to the accumulation of valine, leucine and isoleucine in the blood (Veledo *et al.*, 2005). If left untreated, both genetic diseases can cause irreversible damage to the nervous system, leading to mental retardation. Early detection of PKU and MSUD and use of appropriate diets can help prevent neurological defects

associated with these genetic disorders (Veledo *et al.*, 2005; Paez *et al.*, 2000; Deng and Deng, 2003). In order to limit the effects of PKU and MSUD on individuals, in many countries, screening programmes for the newly born have been implemented.

A number of analytical methods may be used for the detection/quantitation of amino acids in biological matrices. These range from simple spectrophotometric quantitation of phenylalanine to elaborate HPLC separation of plasma amino acids with tandem mass spectrometric quantitation. Although these assays vary considerably in specificity, sensitivity and reproducibility, there are a number of criteria which may make their use, in screening for aminoacidopathies, appropriate. These include: assay selectivity, minimum quantitation limit of the assay in comparison with the minimum normal level in patients, the range over which the response of the assay is linear, cost of performing the assay and speed of the procedure. Usually selection of the assay is a compromise

between these factors. Spectrophotometric assay of phenylalanine although cheap and fast, suffers from interference and lack of sensitivity. TLC assays of amino acids are cheap but are labor intensive and lack sensitivity. HPLC assays (Fernandez-Figares *et al.*, 2004; Hackl *et al.*, 2006; Allard *et al.*, 2004) have good sensitivity and selectivity, but usually, have long run times and are expensive to perform. GC assays have good selectivity and sensitivity, but in our experience without a mass detector, lack reproducibility.

Capillary Electrophoresis (CE) has a number of properties which make it inherently appropriate for the screening of amino acids, such as: low cost, speed of analysis and high resolution. Shen *et al.* (2002) developed a method for the assay of 16 amino acids, which although sensitive and rapid, could not completely resolve leucine from norleucine and valine from cysteine. Boulat *et al.* (2001) developed a CE method for the fluorescent detection of 3-(4-carboxybenzoyl) quinoline-2-carboxaldehyde derivatised amino acids. Although the assay was sensitive, the run time for amino acids of importance in PKU and MSUD was long (about 30 min). Zahou *et al.* (2000) used phenylisothiocyanate derivatisation for the detection of amino acids in protein hydrolysate, however, they did not demonstrate if the assay could be used for the quantitation of free amino acids in blood.

Thus, in this research we aimed to develop a Micellar Electrokinetic chromatography method for the quantitation of phenylalanine, valine, leucine and isoleucine, which was relatively rapid and used simple UV detection, for the purpose of screening neonates for PKU and MSUD. We validated the assay and compared it with a HPLC method, based on the OPA derivatisation of amino acids, used routinely for the screening of neonates in a diagnostic laboratory.

## MATERIALS AND METHODS

**Reagents and chemicals:** Unless stated otherwise, the work was carried out in the Capillary Electrophoresis Lab, Razi Institute for Drug Research, Iran University for Medical Sciences, Tehran, Iran. Boric acid, Sodium Dodecyl Sulphate (SDS), Triton-X 100, 1-propanol, 2-propanol, ethanol triethylamine, were purchased from Merck. Imidazole, 2-N-morpholinoethane sulfonic acid (MES), poly ethylene glycol 6000 and L-amino acids (alanine, valine, leucine, isoleucine, phenylalanine, cycteine, aspartic acid, glutamic acid, aspargines, glutamine, glycine, ornithine, citruline, homoserine, serine, tryptophane, tyrosine, lysine, arginine, methionine, proline, histidine, threonine) were obtained from Sigma

chemical company, St Louise, USA. Acetonitrile and methanol were HPLC grade. All other reagents were analytical grade.

**Preparation of the samples:** Four milliliter of blood was collected into EDTA containing Vacutainer tubes (BD Vacutainer Systems, Belliver Industrial Estate, Plymouth, UK). Collected blood sample was immediately centrifuged at 3500 rpm, for 10 min at 4°C, in a bench top refrigerated centrifuge. The supernatant plasma was transferred into a clean tube and stored at -20°C until needed.

To deproteinise plasma sample 400 µL methanol was added to 100 µL plasma in a glass tube. The mixture was shaken for 10 sec and allowed to stand for 30 sec. The turbid mixture was centrifuged at 4000 rpm for 4 min. Two hundred and fifty microliter of supernatant was transferred to a clean tube and dried under a stream of nitrogen prior to derivatisation.

**Preparation of the standards:** Solutions of mixtures of phenylalanine (P), valine (V), leucine (L) and isoleucine (I) were prepared to contain the amount of amino acids indicated in Table 1. Two hundred fifty microliter of each mixture was dried under a stream of nitrogen prior to derivatisation.

For the purpose of peak identification, solution of each of the four amino acids alone at a concentration of 100 µM was prepared. Two hundred fifty microliter of the solution was dried under a stream of nitrogen prior to derivatisation.

**PITC derivatisation for CE analysis:** Amino acids were derivatised using the PITC method as described by Zahou *et al.* (2000). Forty microliter 99.5% ethanol:water: triethylamine (2:2:1 by vol) were added to each tube containing dried amino acids residue. After mixing the tube content were evaporated under a stream of nitrogen. Three microliter 50%v/v ethanol was added to each tube and mixed. Subsequently 7 µL 99.5% ethanol: triethylamine: PITC (7:2:1 by vol) was added. After mixing the tubes were covered with parafilm and incubated for 30 min at room temperature. The mixtures were dried under a stream of nitrogen. The derivatised amino acids in samples and standards were dissolved in 400 µL of sample buffer and mixed for 10 sec. After centrifugation at 14000 g for 2 min the samples were ready for CE analysis.

**HPLC analysis of plasma amino acids:** A validated HPLC assay for plasma amino acids used for routine diagnosis of aminoacidopatheis, in Masoud Diagnostic Laboratory, Tehran, Iran, was selected for comparison of CE results.

The HPLC assay was based on the ortho-phthaldehyde derivatisation of amino acids followed by HPLC separation with fluorometric detection as described by the document number V7228, Version 0790, Knauer Company, Hegauer Weg 38, 14163 Berlin, Germany.

### **Capillary electrophoresis**

**Preparation of the capillary:** Bare fused silica capillaries with OD of 0.360 mm, total length of 42 cm and effective length of 37 cm were used. New capillaries were first rinsed with 1 M NaOH for 20 min followed by distilled water wash for 5 min. The capillary was then equilibrated with the run buffer for 6 min before the electrophoretic run. After every run the capillary was rinsed with water for 4 min, with a mixture of 3:1 DMSO:NaOH (1M) for 6 min and water again for 4 min. The capillary was then equilibrated with the run buffer before run.

**Capillary electrophoresis procedure:** For all experiments a Spectrophoresis 1000 capillary electrophoresis system, Spectra Physics USA, was used. The system was equipped with a UV/Vis detector, set at 254 nm and an air cooling system. The detector output was recorded using Chromperfect Spirit 5 software, Justice Laboratory Systems, USA. All buffers were degassed by sonication for 10 min. Samples and standards were dissolved in sample buffer and centrifuged at 14000 g for 5 min to remove any particulate matter, before injection.

**Optimization of capillary electrophoresis conditions:** The electrophoretic separation buffer was optimized with regards to composition, concentration and pH. The run buffer contained 6 mM sodium borate, 45 mM imidazole and 208 mM SDS. The pH of the running buffer was adjusted to 9.0 by titrating with MES. The buffer was filtered through a 0.45 µm filter prior to use.

Effects of capillary diameter were examined on the efficiency of separation. Capillaries with internal diameters (ID) of 75, 50 and 25 µm were tested. The capillary with 25 µm ID yielded the best separation efficiency.

Effects of separation voltage and separation temperature on the efficiency of separation were examined. The best resolution was obtained with a separation voltage of 18 kV and a separation temperature of 17°C.

Effects of sample buffer composition were also examined. A sample buffer containing 6 mM sodium borate, 45 mM imidazole, 69 mM SDS and 0.5% w/v polyethylene glycol 6000, adjusted to pH 9.0 by adding MES was found to produce the sharpest peaks with the most reproducible migration times. The buffer was

centrifuged at 14000 g for 10 min, in order to remove particulate matter. Injection of the sample was carried out for 7 sec, hydrodynamically.

With the optimized buffer system and temperature and voltage conditions, inter-day and intra-day RSD for the migration times of the four amino acids were determined (Table 1).

**Validation of the assay:** In order to validate the assay, a calibration curve of amino acid concentration against peak area was constructed. The amino acid concentrations were chosen such that the calibration curve covered concentrations lower, in the middle of and higher than the range found in normal serum (Table 1). Inter-day and intra-day variations of peak areas for the four amino acids examined were determined (Table 1).

The recovery of the assay system was determined by comparing the peak areas of the 4 amino acids in a plasma sample before and after spiking with 500 µM valine, 500 µM leucine, 300 µM isoleucine and 400 µM phenylalanine.

In order to determine Limit of Detection (LOD) and Limit of Quantitation (LOQ) of the assay, a calibration curve of amino acid concentration against peak height was constructed. For LOD calculations a signal to noise (S/N) ratio of 3 was chosen (Table 2) and the minimum detectable concentration was calculated from the slope of the peak height calibration curve. LOQ was calculated for each amino acid using a S/N ratio of 20.

In order to assess the overall assay performance, amino acid concentrations in plasma samples were determined using both the developed CE system and a fluorometric HPLC system. For each amino acid, the curve of the CE concentration compared with the HPLC concentration was constructed.

## **RESULTS AND DISCUSSION**

Under optimized conditions described in the previous section, all four amino acids appear to have been separated within the first 11 min (Fig. 1). In HPLC these four amino acids tend to elute towards the end of a 45-50 min run. Most other workers, have developed CE methods for the quantitation of amino acids which have long run times (more than 30 min) for the four amino acids important in PKU and MSUD (Ummadi and Weimer, 2002; Lu and Cheng, 2002; Boulat *et al.*, 2000). Some of the CE methods developed for the rapid separation of amino acids, were unable to resolve leucine and isoleucine (Komarova *et al.*, 2004; Shen *et al.*, 2002). Therefore, the CE method developed in this study is faster for screening

Table 1: Linearity, reproducibility and accuracy of quantitative analysis of PTC-labeled amino acids by CE

Amino acid	Rang ( $\mu\text{M L}^{-1}$ )	Calibration equation <sup>a</sup>	R <sup>b</sup>	Migration time (min)		Peak area		Recovery (%) (n = 2) <sup>d</sup>
				Intra-day RSD (%) (n = 10)	Inter-day RSD (%) (n = 6) <sup>c</sup>	Inter-day RSD (%) (n = 14)	Inter-day RSD (%) (n = 3) <sup>c</sup>	
Valine	85-375	$Y = 399.6x + 3563$	0.9976	1.15	2.08	4.99	8.28	159.2
Leucine	45-325	$Y = 722x + 3848.8$	0.9956	1.07	3.49	4.30	8.73	116.8
Isoleucine	10-175	$Y = 611.4x + 3107$	0.9969	1.01	2.40	4.55	11.29	117.5
Phenylalanine	75-200	$Y = 1175x - 20853$	0.9199	1.34	3.04	6.53	10.64	109.4
Average RSD (%)				1.145	2.75	5.09	9.74	

<sup>a</sup>y: Peak area, x: Analyte concentration ( $\mu\text{M}$ ); <sup>b</sup>Correlation coefficient: Calculated from three replicates at five concentration levels; <sup>c</sup>This number was determined between-days over 2 months; <sup>d</sup>100  $\mu\text{L}$  samples spiked with valine 500, leucine 500, Isoleucine 300 and phenylalanine 400  $\mu\text{M}$

Table 2: Limit of detection and limit of quantitation of PTC-derivatized amino acids by CE

Amino acid	Rang ( $\mu\text{M L}^{-1}$ )	Calibration equation <sup>a</sup>	R <sup>a</sup>	LOQ ( $\mu\text{M}$ ) s/n = 20		LOD ( $\mu\text{M}$ ) s/n = 3	
				CE	HPLC	CE	HPLC
Valine	85-375	$h = 0.004x - 0.017$	0.9811	0.157	0.39	0.023	0.059
Leucine	45-325	$h = 132.2x + 1568$	0.9967	0.096	0.42	0.016	0.062
Isoleucine	10-175	$h = 0.004x - 0.010$	0.9955	0.107	0.52	0.014	0.077
Phenylalanine	75-200	$h = 0.0034x - 0.012$	0.9613	0.065	0.56	0.009	0.084

h: Peak height, x: Analyte concentration of CE ( $\mu\text{M}$ ); a Correlation coefficient obtained from three replicates at five concentration levels of CE

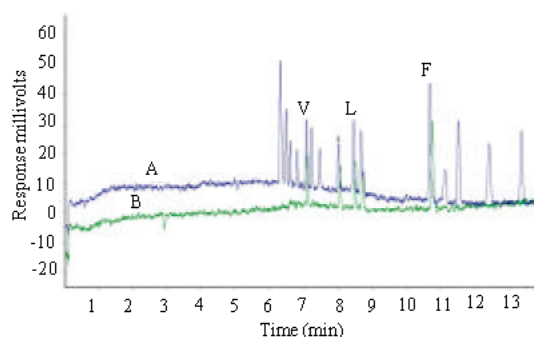


Fig. 1: Electrophrogram of mixtures of 23 amino acids (A) and 4 amino acids (B) standards. The PTC-amino acids were separated by CE under optimized conditions and calibration curve was constructed by plotting peak areas against amino acid concentrations

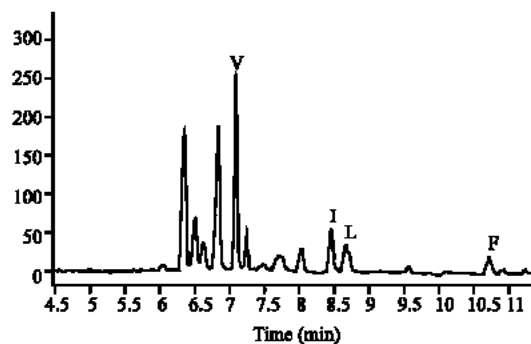


Fig. 2: Electrophrogram of healthy serum, MEKC condition same as Fig. 1. The PTC-amino acids were separated by CE under optimized conditions and calibration curve was constructed by plotting peak areas against amino acid concentrations

for abnormal concentrations of these four amino acids in serum samples (Fig. 2). There was a linear relationship between the concentration and peak area (Table 1) over the concentration range tested. For each amino acid the concentration range was chosen to include standards which were both more dilute than the minimum of normal plasma concentration in man and more concentrated (Fig. 3). For each amino acid LOD was calculated based on a S/N ratio of 3 (Table 2). The LOD values were two orders of magnitude lower than the minimum normal levels in human plasma. For the calculation of LOQ, a S/N ratio of 20 was chosen which represents an error of 5% in measuring peak area (Table 2). Under such conditions, LOQ values were at least ten times lower than the

minimum plasma concentration in normal individuals. This suggests that the method can measure concentrations of these 4 amino acids relevant to clinical conditions.

As migration time is the only criteria for determining peak identity in CE, it was important to see how much variation this parameter showed (Table 1). The intra-day RSD for migration time was less than 1.5%. Also, inter-day RSD for migration time was between 2 to 3%. The small variations in migration time suggests that the CE separation system allowed for correct peak identification.

Intra-day and inter-day variation in peak area were also determined (Table 1). Intra-day RSD was around 5% while inter-day RSD was about 10%. Both these parameters compare favorably with results obtained by other workers using CE (Lu and Cheng, 2002; Brit and

Table 3: Linearity and reproducibility of quantitative analysis of OPA-labeled amino acids by HPLC

Amino acid	Range ( $\mu\text{M L}^{-1}$ )	Calibration equation	Area ratio	
			Intra-day RSD (%) (n = 9)	Inter-day RSD (%) (n = 9)
Valine	50-200	$y = 0.004x - 0.017$	7.77	11.13
Leucine	50-200	$y = 132.2x + 1568$	4.30	14.40
Isoleucine	50-200	$y = 0.004x - 0.01$	4.55	7.73
Phenylalanine	50-200	$y = 0.0034x - 0.012$	6.53	14.10

y: Area ratio; x: Analyte concentration ( $\mu\text{M}$ )

Table 4: Comparison of quantitative analysis of four amino acid by CE and HPLC in normal, pku, msud persons

Amino acid	Correlation coefficient		
	PKU (n = 9)	Normal (n = 7)	MSUD (n = 13)
Valine	-	0.0800	0.7786
Isoleucine	-	0.7712	0.9867
Leucine	-	0.7039	0.9919
Phenylalanine	0.6828	0.7318	-

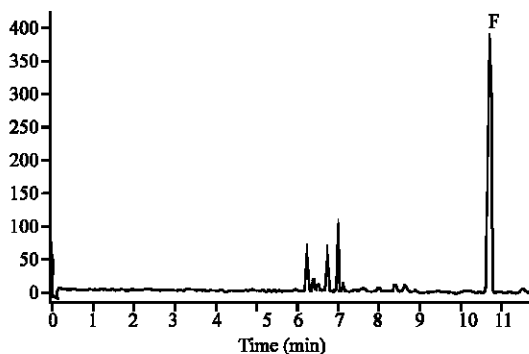


Fig. 3: Electrophrogram of patient suffering from phenylketonuria, MEKC condition same as Fig. 1. The PTC-amino acids were separated by CE under optimized conditions and calibration curve was constructed by plotting peak areas against amino acid concentrations

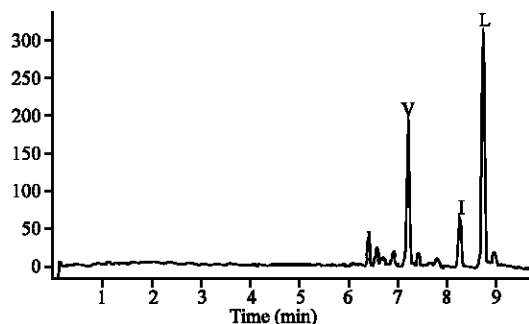


Fig. 4: Electrophrogram of patient suffering from maple syrup urinemia, MEKC condition same as Fig. 1. The PTC-amino acids were separated by CE under optimized conditions and calibration curve was constructed by plotting peak areas against amino acid concentrations

Terabe, 2003). Some of these inter and intra day variations are because of the inaccuracies in the injection volume, sample preparation and derivatisation. Inclusion of an internal standard should decrease these RSD values.

Recovery of the four amino acids was studied by spiking a known amount of each amino acid into a plasma sample. Valine showed a recovery of about 160%. This unusually high recovery could be due to co-migration of some other component with valine in the CE run.

Plasma from normal individuals and those with diagnosed MSUD and PKU was studied with the CE assay developed and the results were compared with HPLC (Table 3). CE results for phenylalanine, leucine and isoleucine showed good correlation with those obtained with HPLC. The correlation for leucine and isoleucine was greater in MSUD plasma than normal plasma (Fig. 4). Valine levels of normal plasma measured by CE did not correlate with those obtained with HPLC (Table 4). However, in MSUD plasma, valine too showed a good correlation. This also suggests that there is co-migration of some component with valine in the electrophoretic run.

## CONCLUSIONS

In conclusion, the developed CE assay appears to be suitable for measuring phenylalanine, leucine and isoleucine in normal and abnormal human plasma. The CE assay appears to produce an over-estimation of valine in normal plasma samples, however, in plasma samples with elevated valine levels, the measurement correlate well with HPLC.

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