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Neutral amino acids monitoring in phenylketonuric plasma microdialysates using micellar electrokinetic chromatography and laser-induced fluorescence detection

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Abstract

Neutral and non-polar amino acids such as phenylalanine (Phe), valine (Val), tyrosine (Tyr), threonine (Thre) and GABA are hard to resolve by capillary zone electrophoresis (CZE). Their separation is possible by adding a surfactant to the mobile phase. This method is called micellar electrokinetic chromatography (MEKC). We used MEKC with laser-induced fluorescence detection (LIFD) to separate and quantitate these amino acids in plasma microdialysates of patients with phenylketonuria (PKU). This disease is an inborn enzymatic defect with decreased conversion of Phe to Tyr that causes severe neurological damage and mental deterioration, which is diagnosed by measuring plasma Phe and Phe/Tyr ratio. The amino acids tested had linear concentration–signal relation. PKU patients had significantly higher Phe, lower Tyr, 21 times higher Phe/Tyr ratio and decreased values of Val and Thre than controls. These results show that microdialysis of biological fluids coupled with MEKC–LIFD is a convenient technique to measure neutral amino acids in clinical disorders such as PKU.

Keywords: Phenylalanine; Tyrosine; γ-Aminobutyric acid; Threonine; Valine

1. Introduction

Micellar electrokinetic capillary chromatography (MEKC) combines the advantages of capillary zone electrophoresis (CZE) a high-voltage electrophoretic technique for charged species, with solvophobicity separation for neutral species [1]. CZE performs separation of ionic species based on their different electrophoretic mobility in a high-intensity electrical field applied between the ends of a narrow bore capillary tubing. This technique has been successfully used for biological fluid analysis (plasma, urine, cerebro spinal fluid etc) [2]. In addition to its high resolution, CZE works with small sample volumes usually nano or picoliters. This advantage is convenient to analyze active molecules present in small samples (a few microliters or less) obtained by microdialysis in various tissues [3–5]. Despite the broad range of charged molecules that can be analyzed by CZE, the neutral compounds in the sample cannot be separated by electrophoresis. This limitation can be solved by adding a surfactant above its critical micellar concentration to the running buffer [1]. Surfactants are molecules that have a long hydrophobic tail and a hydrophilic head that form

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amphiphilic aggregates or micelles in aqueous solutions. The uncharged compounds are moved by electroosmotic flow within the capillary and separated according to their partition coefficient. Thus analytes with greater affinity for the micelle have slower migration velocities compared to analytes that spend most of their times in the mobile phase. The detection of the analytes can be done by UV [6], electrochemical [4] or laser-induced fluorescence detection (LIFD) [7]. The last one can detect very small amounts of analytes several orders of magnitude more than the other techniques [8,9].

Phenylketonuria (PKU) is an inborn error of the amino acid metabolism. It has a recessive autosomic character and occurs with an incidence of 1 in 16 000 newborns. If the condition is not treated early, severe mental retardation and neurological deterioration will occur. In the classical PKU there is an enzymatic deficit of phenylalanine hydroxylase, which transforms the amino acid phenylalanine (Phe) to tyrosine (Tyr). The early diagnosis in this metabolic defect allows starting a Phe free-diet to prevent the deleterious consequences of the accumulation of Phe in blood. There are several techniques that have been used to screen PKU in newborn blood such as, a bacterial enzymatic inhibition assay: the Guthrie test [10], liquid chromatography [11], and more recently tandem mass chromatography [12]. In PKU patients, Phe plasma concentration can reach very high levels, Tyr plasma levels can be reduced and the Phe/Tyr ratio is usually high. Since Phe and Tyr are neutral amino acids, they should be suitable to MEKC analysis in this clinical condition.

In the present report, we used microdialysis, a technique that allows chemical monitoring of the extracellular environment of different tissues and biological fluids [13–15], to extract some neutral amino acids from plasma of PKU patients and controls. These amino acids were separated and detected by MEKC coupled with LIFD respectively.

2. Experimental

2.1. Subjects

Plasma samples were obtained from adult volunteers and two patients who had been referred from Pediatric Ambulatory Centers to the Human Development Center for evaluation.

2.2. Microdialysis

Details of the removable probes and the procedure have been described elsewhere [14–16]. In brief, the active area of the probe was made of 15-mm long cellulose hollow fiber, 220-μm O.D. and 13 000 molecular weight cut-off (Spectrum Medical Industries, Los Angeles, CA, USA). Probes and connectors were sterilized with ethylene oxide at least 24 h before use. The probes were perfused with sterilized 0.9% NaCl solution at 1 μl/min flow-rate. The probes were introduced into a vial that contained 500 μl of plasma, and 1-h-samples collection began after 30 min of dialysis stabilization period. In this way we obtained a plasma ultrafiltrate free of proteins. The recovery of these microdialysis probes has been tested previously for compounds of the same range of molecular weight of the amino acids tested, such as glucose and it is around 70% [15].

2.3. Derivatization of standards and samples

Maximal derivatization of neutral amino acids was done by mixing 1 ml of a 10^{-5} *M* standard of each amino acid with 1 ml of 1:1 (v/v) mixture of 6.4×10^{-3} *M* solution of FITC in acetone and 20 m*M* carbonate buffer at pH 9.4. Calibration curves for each amino acid were done by derivatizing increasing concentrations of the amino acid from 10^{-7} *M* to 10^{-5} *M* with 10^{-3} *M* of FITC. The dialysates were derivatized 1:1 (v/v) ratio with the FITC–carbonate buffer mixture. The derivatized sample volume was 30 μl. Spiking solutions were derivatized by mixing 1 ml of a 10^{-5} *M* standard solution of each amino acid with 5 μl of the FITC–carbonate buffer mixture. The derivatized samples, standards and spiking solutions were incubated overnight at room temperature.

2.4. Detection

The electrophoresis instrument consisted in a Meridialysis CZE system (Model R2D2, Mérida, Venezuela) equipped with an argon ion laser (Ion Laser Technology, Salt Lake City, UT, USA) tuned to 488 nm. A fiber optic conducted the laser beam

onto a dichroic mirror centered at 510 nm (Omega Optics, Brattelboro, VT, USA). The laser is focused on the window of a capillary (Polymicro Technologies, Phoenix, AZ, USA) by means of a 0.75 NA objective. The window was made by burning out the polyimide coating at 38 cm from the anodic end of a 50-cm long 27 μm I.D.×346 μm O.D., fused-silica capillary. The fluorescence was collected through the objective and focused on an R120-05 multialkali photomultiplier tube (PMT) (Hamamatsu, Bridgewater, NJ, USA). A high pass filter and a notch filter centered at 520 and 488 nm (Andover, Salem, NH, USA) respectively, attenuated stray radiation. The running buffer was made with 23–34 m*M* sodium borate with 120-m*M* sodium dodecyl sulfate and methanol 1%. The sample was loaded at the anodic end by applying a negative pressure (19 p.s.i. for 1 s) at the cathodic end of the capillary. The actual sample volume injected was 1 nl. Electrophoretic separation was achieved by applying 26 kV between the anode and the cathode. Between runs the capillary was rinsed with 0.1 *M* NaOH for 1 min, filtered deionized water for 1 min and then, running buffer for 3 min. The buffer reservoirs at both ends were also rinsed with fresh buffer between runs to prevent contamination and capillary clogging.

2.5. Chemicals

Fluorescein isothiocyanate (FITC), lauryl sulfate sodium salt, phenylalanine, valine, tyrosine, threonine and γ-aminobutyric acid, were purchased from Sigma (St. Louis, MO, USA). Sodium borate from Merck (Rahway, NJ, USA) and methanol from J.T. Baker (Phillipsburg, NJ, USA).

2.6. Statistical analysis

Student *t* test for comparison of control and PKU groups was used. Significance level was set at $P \leq 0.05$.

3. Results and discussion

To our knowledge, this is the first report using the combination of microdialysis and MECK–LIFD to measure neutral amino acids in PKU patients.

It was possible to separate five neutral amino acids in plasma dialysates using sodium borate, lauryl sulfate and methanol mixture in the mobile phase or running buffer. With 23-m*M* sodium borate: Phe, Val, Thre and GABA were well separated and identified (Fig. 1). The total electropherogram run time was between 5 and 10 min. The migration time of the neutral amino acids tested was between 7 and 8 min. The identification was done by spiking of the samples with a 10^{-5} *M* standard solution of each amino acid tested. The spiking solutions were added to the samples one at the time.

For a better separation of Tyr, it was necessary to increase the borate concentration to 34 m*M* in the buffer. This prolonged the migration time between 8 and 9 min but separated Tyr. In Fig. 2 the separation of Tyr and its identification using spiking with 10^{-5} *M* Tyr standard solution, is shown.

A calibration curve with 3 to 100 μ*M* concentrations for each of these five amino acids was achieved. The regression lines and the equations for each amino acid tested showed an excellent relationship between the signal (peak height) and the amino acid concentration. For Phe $y=8.533x-4.006$, $r=0.999$; Tyr $y=9.982x-6.865$, $r=0.999$; Val $y=13.225x-3.620$, $r=0.998$; Thre $y=5.975x-1.555$, $r=0.998$; GABA $y=15.331x-51.131$ $r=0.996$.

Thus, we were able to identify and measure five neutral amino acids in plasma dialysates in PKU patients and controls using MEKC coupled with LIFD. One of the patients with PKU had been previously diagnosed by means of HPLC and the other one was a presumptive PKU case. The plasma dialysates from controls and PKU patients were diluted 1:80 in 20 m*M* carbonate buffer and run twice with 23 and 34 m*M* sodium borate buffer. Large Phe peaks were found in PKU patients compared with the controls (Fig. 3). This made it necessary to dilute PKU patient samples to 1:360 in order to measure Phe (Fig. 4). We found in these patients high amounts of Phe, and a very high Phe/Tyr molar ratio, 21 times more compared to controls, confirming in both cases the existence of PKU. Additional information on other neutral amino acids that are not usually measured in PKU was obtained with the present technique as well. It was possible to separate and measure Val, Thre and GABA (Table 1). Levels of Val and Thre were reduced and GABA did not change in PKU patients compared with the controls. This aspect of the biochemical profile of

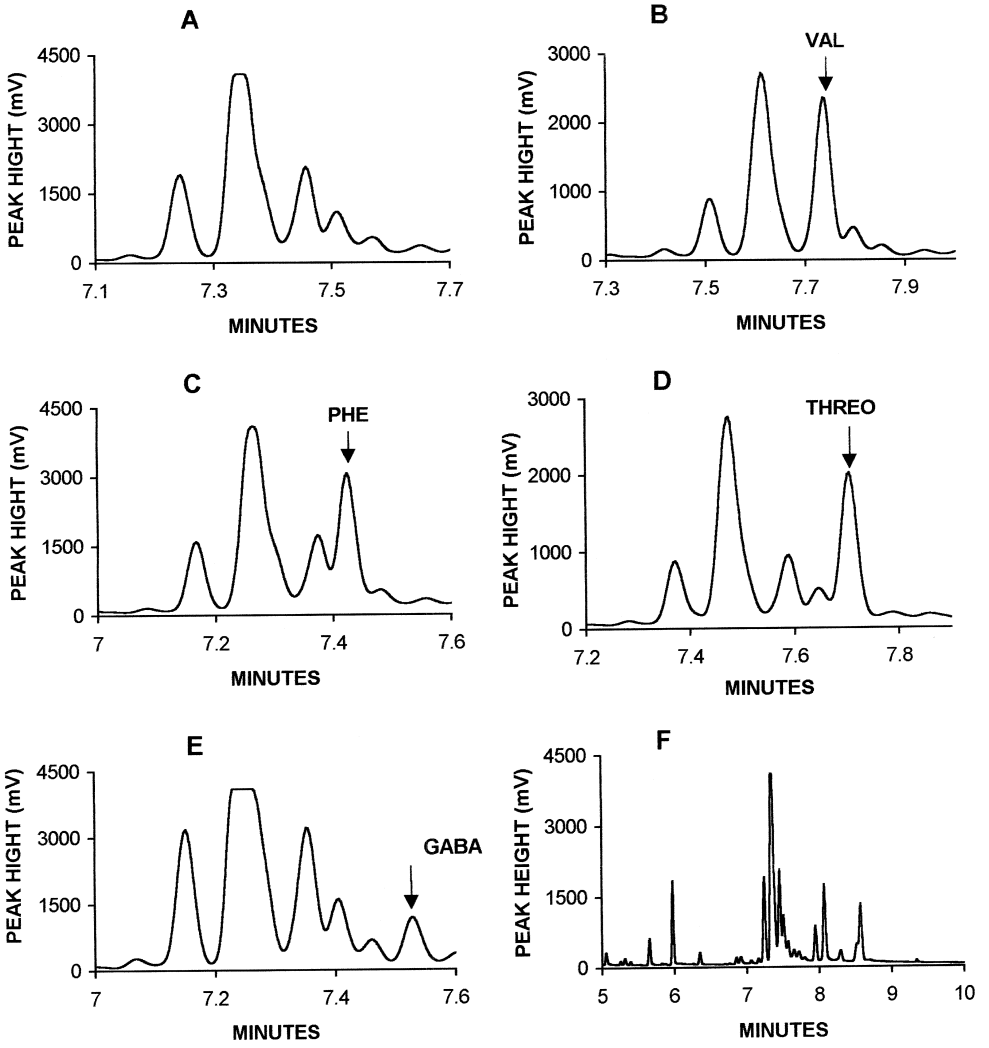

Fig. 1. Electropherograms obtained from a control plasma dialysate. At F is shown the complete run 5 to 10 min. The electropherograms A to E correspond to an amplification of the section located between 7 and 8 min. (A) Sample without spiking; (B) spiking with 10^{-5} *M* Val standard solution; (C) spiking with 10^{-5} *M* Phe standard solution; (D) spiking with 10^{-5} *M* Thre standard solution; (E) spiking with 10^{-5} *M* GABA standard solution.

PKU patients has not been previously reported as far as we know and needs more study to be explained.

CZE is a highly efficient technique with high resolution that has been frequently used to separate negatively charged neuroactive amino acids such as glutamate and aspartate [5,8,19,20]. But there are other active amino acids that CZE cannot resolve because they are neutral. The present work demonstrates that it is possible to separate them by MEKC. To do this we increased the SDS concentration above limits previously reported by others [21,22]. By increasing the surfactant concentration, the resolution power of the mobile phase increased. In addition, we used a narrow capillary of 26-μm I.D., smaller than that reported in previous MEKC studies [1]. Narrow bore capillaries have a reduced cross section area that increases the resistance and decreases the intensity of current, leading to an increase of the resolution power. Narrow bore capillaries also have larger surface to volume ratio that

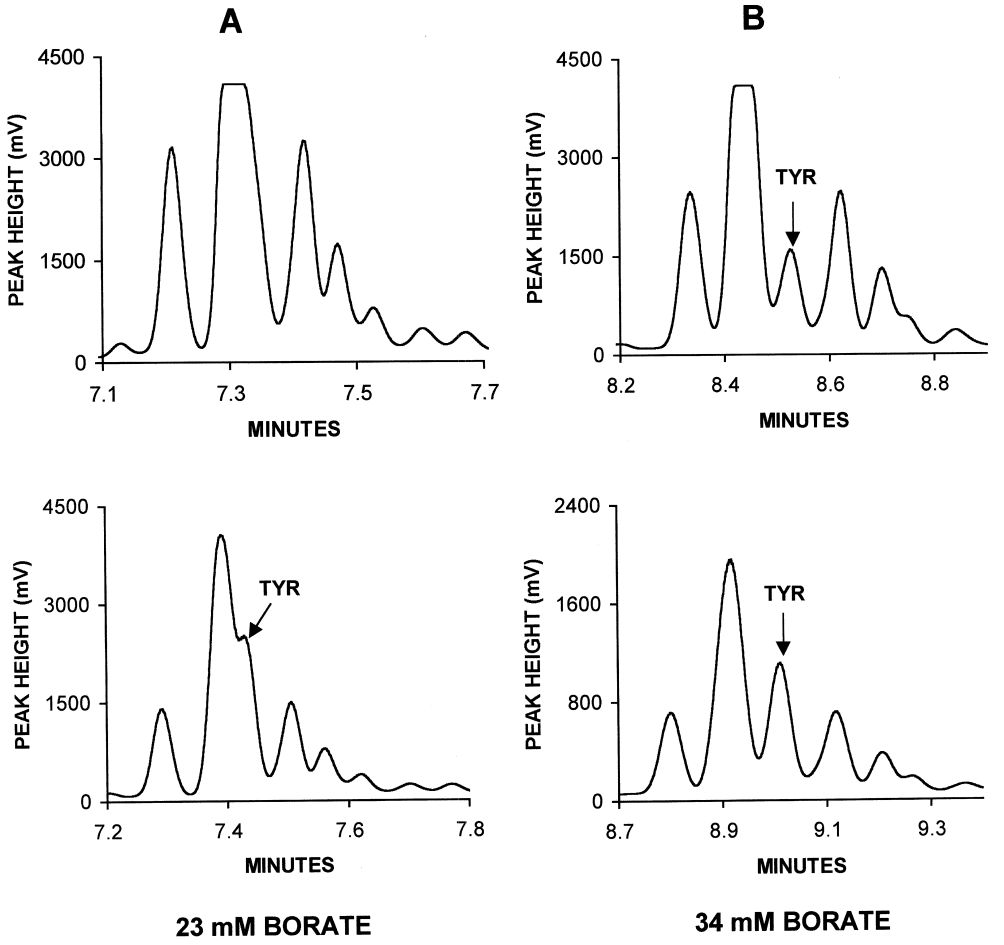

Fig. 2. Comparison of separation of Tyr with 23 m*M* and 34 m*M* sodium borate in a control plasma dialysate. (A) 23 m*M* borate running buffer; (B) 34 m*M* borate running buffer. Top: Sample without spiking. Bottom: Spiking with 10^{-5} *M* Tyr standard solution. The Tyr peak is not resolved at 23 m*M* borate buffer. However, Tyr is well resolved at 34 m*M* borate buffer.

causes better heat dissipation. Therefore, there is less Joule heating, less band broadening and even better resolution.

CZE uses only a very small fraction of the sample (volume for injection), therefore, it is possible to carry out repeated analysis of the same sample. In the present work, the injected volume was approximately 1 nl, what is a very small volume compared with the injection volumes needed in other techniques, for instance, HPLC. In PKU screening and in successive evaluations of PKU patients [23] the use of small sample volumes represents an additional advantage. It makes the present technique highly convenient for newborn patients in which repeated analyses are necessary. With a drop of blood microdialysis and MECK–LIFD are feasible. Microdialysates are an ultrafiltrate sample free of proteins ready for the chemical analysis. The additional benefits of using microdialysis to extract the samples and the advantages of the detection of the analytes with LIFD have already been established in previous papers [3,5,7,9].

The present results in PKU patients: Phe significantly higher, Tyr moderately reduced and a very high Phe/Tyr molar ratio are consistent with what have been reported with other extensively used techniques for screening and for diagnoses confirmation, such as the bacterial inhibition assay or Guthrie

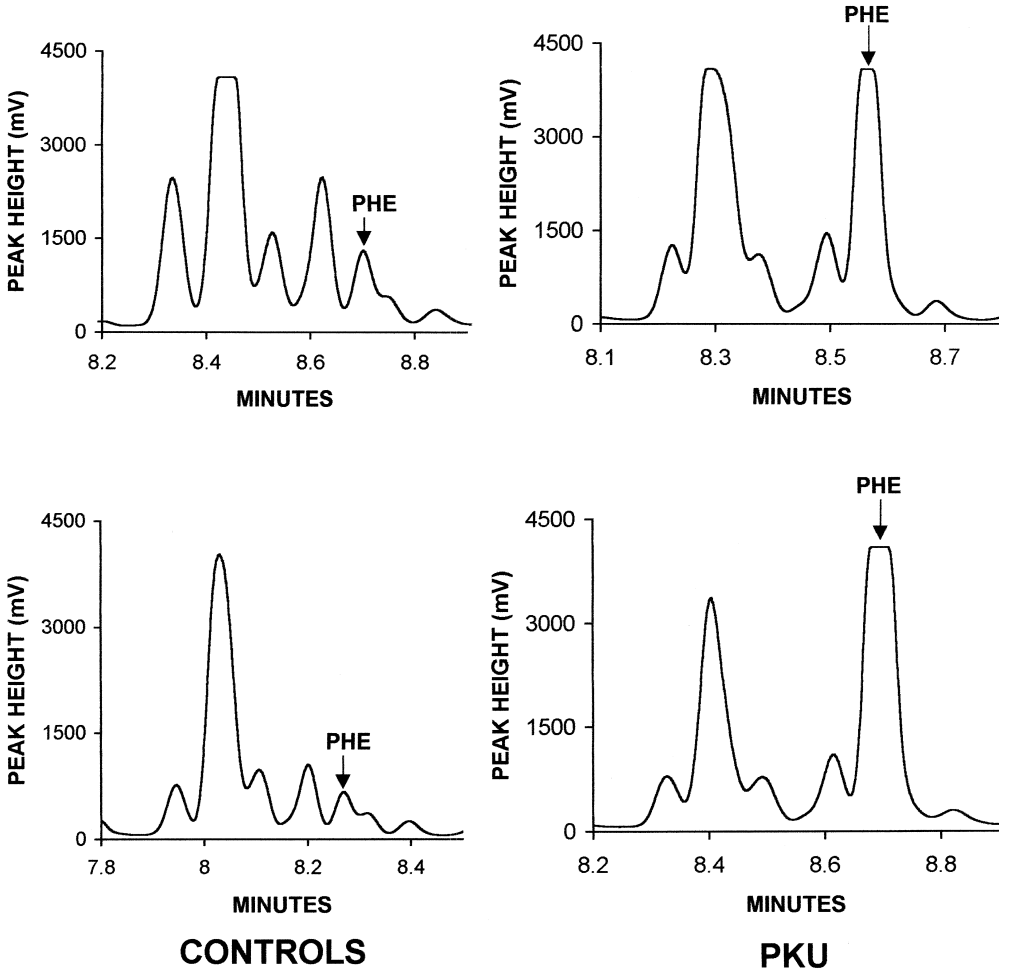

Fig. 3. Electropherograms of plasma dialysates. Running buffer 34 m*M* sodium borate. Left: Two control samples. Right: Two PKU samples.

test [10], fluorometric assay [17], ion-exchange chromatography [11] enzymatic assay [18] and more recently tandem mass spectrometry [12].

The Guthrie test is an excellent screening technique but is semiquantitative and only measures Phe. Ion exchange chromatography is quantitative, measures Phe and Tyr but is more expensive has less resolution power and is not very useful for screening. Tandem mass spectrometry has excellent resolution power, is good to measure several amino acids at once, but too expensive for low budget hospitals. Conversely, CE is simple, rapid, less expensive and with a high resolution power. CE requires very small samples. It gives the possibility of processing the same sample several times because of the very small sample injection volume. It is also possible to have capillary array in order to process many samples simultaneously. This feature would make this technique suitable to a screening test [24].

In summary, the results showed the feasibility of the analysis of neutral amino acids with MEKC and LIFD in small volume biological samples. This report proves that microdialysis and MECK–LIFD are an excellent alternative method to detect and follow up PKU patients and any other disorders where neutral amino acids may be involved.

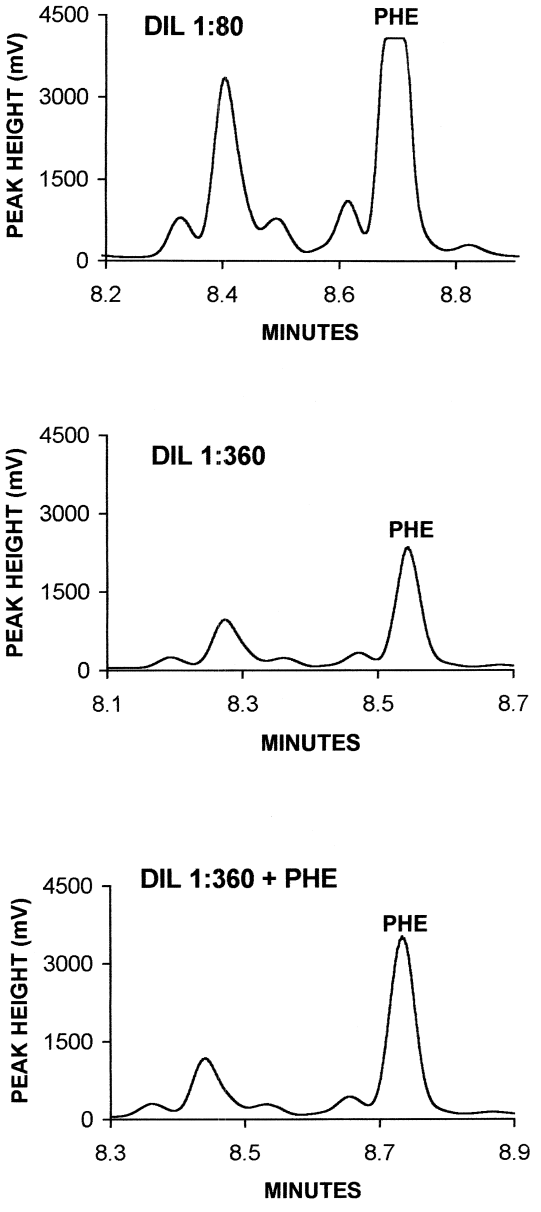

Fig. 4. Electropherograms of a PKU plasma dialysate. Running buffer 34 m*M* sodium borate. Top: 1:80 dilution. Middle: 1:360 dilution that makes possible to measure the Phe peak. Bottom: 1:360 dilution with 10^{-5} *M* Phe standard solution spiking.

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Table 1
Amino acids plasma dialysate levels measured by MEKC and LIFD

	PKU Mean±SE μM (n=2)	Controls Mean±SE μM (n=3)	t Test
Phe	398.00±11.77	41.73±01.89	$P<0.01$
Tyr	19.70±2.22	42.47±1.50	$P=0.05$
Val	31.35±1.66	62.90±1.61	$P<0.05$
Threo	15.65±2.20	37.73±1.31	$P<0.05$
GABA	7.45±1.78	8.80±0.58	NS
Phe/Tyr	19.95±01.43	0.97±0.17	$P<0.001$

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