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Child cerebrospinal fluid analysis by capillary electrophoresis and laser-induced fluorescence detection

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Abstract

Capillary electrophoresis with laser-induced fluorescence detection (CE–LIF) was used to analyze a 50-μl sample of cerebrospinal fluid from leukaemic children treated with high doses of methotrexate. Free amino acids and primary amines are labelled with fluorescein isothiocyanate prior to analysis. Electropherograms containing more than 50 peaks were obtained in less than 22 min. Twenty-one peaks were identified, and 19 were quantitated. Observed differences in individual amino acid levels are compared with healthy reference values. The results indicate that CE–LIF is useful as a selective, rapid and sensitive tool for the determination of free amino acids and amines in clinical biology studies.

1. Introduction

Cerebrospinal fluid is secreted in the brain and is in a steady state with the fluid surrounding brain cells. It plays a critical role in providing a constant chemical environment for neurons and glia and is the body fluid most likely to reflect a disturbance of the amino acids metabolic pathway. Free amino acids levels in cerebrospinal fluids have been studied [1–3], and their implication in several diseases such as central nervous system disorders or metabolic diseases has been shown [3,4]. Moreover, the way of collecting samples, time of lumbar puncture and position of the patient may have some influence on the level of amino acids [5].

Analyses by gas chromatography [2], high-performance liquid chromatography (HPLC) [6], ion-exchange chromatography [7], isotachophoresis [8] and capillary electrophoresis [9] have been used to identify and quantify free amino acids in the brain with conventional detection modes. Capillary electrophoresis (CE) offers some advantageous characteristics for the analysis of biological samples [10,11]: very small injection volumes (very small quantities of samples are consumed, leaving a greater sample for other analyses), high separation efficiency, and easy use of a highly sensitive detection mode such as laser-induced fluorescence detection (LIF). LIF is used more and more for CE

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analysis, as an 'on-column' detector. Because of the very low injection volumes, the amount of product to be detected is too small for UV absorbance detection. Moreover, selectivity due to labelling reactions and appropriate laser wavelength excitation allows one to record only well-defined molecules, e.g., amines and amino acids.

A large number of studies have been published on the labelling of amines and amino acids, and different reagents have been used: fluorescein isothiocyanate (FITC) [12], naphthalenedialdehyde (NDA) [13] and 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde (CBQCA) [14]. The detection limit of each labelling dye is identical, around 10^{-11}–10^{-12} *M*.

Studies on the composition of free amino acids in the brain using CE–LIF have been reported. Glutamic and aspartic acids in mouse brain microdialysis samples have been identified [12]. More recently, Bergquist et al. [14] have carried out quantitative analyses of ten CBQCA-labelled amino acids in cerebrospinal fluid from patients with psychiatric or neurological diseases.

In this paper, we present CE–LIF in a quantitative study of FITC-labelled amino acids and amines in cerebrospinal fluid samples from acute lymphoblastic leukaemic children treated with high doses of methotrexate [15]. These samples were collected between one and three days after drug administration.

2. Experimental

2.1. Instrumentation and separation conditions

A modular injector and high-voltage power supply SpectraPHORESIS 100 (TSP, Freemont, CA, USA) equipped with a modular CE–LIF detector (Zeta Technology, Toulouse-Ramonville, France) and a 488-nm wavelength laser (Model 54225A, ILT, Salt Lake City, UT, USA) were used. Data collection, processing and analysis were performed using a Boreal software package (JMBS Developpements, Grenoble, France). Data were collected at a sampling rate of 10 Hz. A 75 cm × 50 μm I.D., fused-silica capillary (Polymicro Technology, Phoenix, AZ, USA) had an effective length of 42 cm. All chemicals were purchased from Aldrich (St. Quentin Falavier, France)

The separation buffer consisted of 100 m*M* of sodium dodecyl sulfate (SDS) and 100 m*M* boric acid, adjusted to pH 9.3 by the addition of sodium hydroxide. The capillary was rinsed with 0.1 *M* NaOH for 3 min, with water for 2 min, and then with separation buffer for 3 min. Samples were injected by hydrodynamic injection for 2 s (15 nl).

The separation voltage was +20 kV, resulting in an electrophoretic current of 42 μA.

2.2. Derivatization procedure

A $2.1 \cdot 10^{-4}$ *M* solution of FITC isomer I in acetone was prepared by dissolving 2.5 mg of FITC in 30 ml of acetone. Then 2 mg of each amino acid was dissolved in 2 ml of 0.2 *M* carbonate buffer at pH 9.0. Of each amino acid solution, 1 ml was allowed to react with 1 ml of FITC solution for 2 h in the dark. At the same time, 1 ml of a $2.1 \cdot 10^{-4}$ *M* solution of the FITC in acetone was mixed with 1 ml of 0.2 *M* carbonate buffer to obtain a blank, and was kept in darkness for 2 h.

A 50-μl sample of each cerebrospinal fluid was mixed with 50 μl of $2.1 \cdot 10^{-4}$ *M* FITC solution and left to react for 2 h in darkness. Each solution obtained was diluted either 1000 times or 100 times prior to injection.

2.3. Identification and quantitation

Peaks were identified by spiking cerebrospinal fluid samples with known quantities of standard solutions of amino acids. Amino acids were quantified using linear calibration curves based on peak height. Each calibration curve contained data points at a minimum of six different concentrations, and each curve spanned the range of concentrations found in the cerebrospinal fluid sample diluted 100 or 1000 times. Linearity was assessed using standard least-squares analysis of the logarithm of peak height versus logarithm of concentration plots. Co-injection of known quantities of fluorescein thiocarbamyl (FTC)

glutamic acid, aspartic acid, ornithine, glycine and taurine showed that experimental errors on quantitation (without internal standard) remain below 1.2%.

2.4. Cerebrospinal fluid

Cerebrospinal fluid (CSF) came from children with acute lymphoblastic leukemia (6–14 years old), who were treated with high amounts of methotrexate. This is a cytotoxic drug used to eliminate leukaemic cells [15]. Table 1 shows the details of the patients.

A high dose of methotrexate (HDMTx) was given in a 24-h infusion (5 g/m^2) with 500 mg/m^2 given in 1 h and 4500 mg/m^2 given in the 23 subsequent hours. During the HDMTx infusion, alkaline hydration (3 l/m^2) was administered in order to maintain urinary pH above 7. Adjuvant drugs: 6-mercaptopurine (6MP; 25 mg/m^2) was given orally every morning after fasting; cytosine arabinoside (CA; 1 g/m^2) was given during 1-h infusion after the beginning of the methotrexate infusion.

Samples were collected to quantitate methotrexate in cerebrospinal fluid, and we carried out quantitative studies of amines and amino acids on unused samples. Lumbar punctures were performed in the L3-4 or L4-5 interspace in the morning with the patient in a recumbent position. Samples were collected between one and three days after drug administration. The first 3 ml of cerebrospinal fluid was collected in plastic tubes and gently mixed to avoid a gradient effect. This amount of each sample was used for different analyses, i.e., methotrexate quantitation, and only 50 μl of these samples was used to perform CE–LIF experiments. The albumin ratio [cerebrospinal fluid albumin (in mg/l) divided by serum albumin (g/l)] was the measure of blood barrier function, in order to exclude samples from patients with pathologically increased levels of serum in their cerebrospinal fluid [16].

Table 1
Characteristics of the patients

Patients	J	M	C
Age (years)	11	14	6
Sex	M	F	F
Diagnostic	ALL	ALL	ALL
Plasmatic proteins concentration	63	65	67
CSF proteins concentration	0.38	0.41	0.3
Associated drugs	6MP	6MP and CA	6MP

ALL = Acute lymphoblastic leukemia, 6MP = 6-mercaptopurine, CA = cytosine arabinoside.

3. Results and discussion

Fig. 1 shows the electropherogram of the standard FTC-amino acids and amines levels under experimental conditions. FTC-amines and -amino acids have a quantity of 15 amol ($1 \cdot 10^{-9}$ *M* solutions injected).

The fluorescent intensity measured for all amino acids and amines was linearly correlated with the sample concentration injected over a range of 10^{-8}–$5 \cdot 10^{-10}$ *M*. The linearity was determined from repeated injection at six different concentrations of each amine and amino

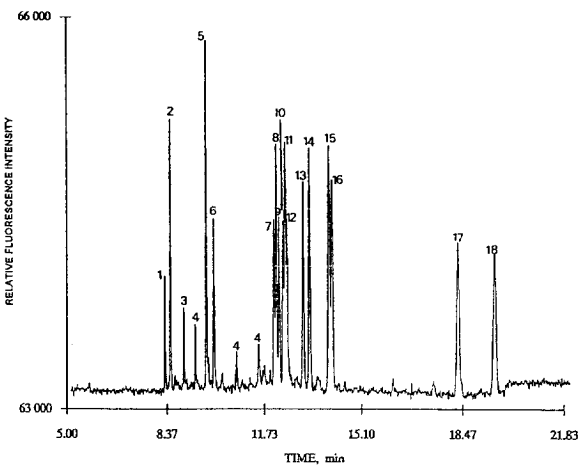

Fig. 1. Electropherogram of 17 FTC-standards (10^{-9} *M*). Electrophoretic conditions: buffer, boric acid 100 m*M*, SDS 100 m*M*, pH = 9.3. Analysis: +20 kV, 42 μA, hydrodynamic injection 2 s. Peaks: 1 = Lys, 2 = Arg, 3 = ornithine, 4 = blank, 5 = ammonia, 6 = tyramine, 7 = Leu, 8 = Gln, 9 = Tyr, 10 = Val, 11 = Thr, 12 = Phe, 13 = Ser, 14 = Ala, 15 = taurine, 16 = Gly, 17 = Glu, 18 = Asp.

acid. The following equation was used to evaluate the linearity of the method:

$$\log Y = \log A + r \log C$$

where $\log C$ is the log of concentration, $\log A$ is a constant, $\log Y$ refers to the log of relative fluorescence intensity, and r is the slope of the curve. The regression equations of these curves and their correlation coefficients are shown in Table 2. The linearity was achieved without an internal standard, which probably would have increased the correlation coefficients.

By running five replicates of the standard (10^{-9} M), each FTC-amino acid or -amine showed high reproducibility in terms of peak heights or elution times, giving standard deviations of standard products between 1.7 and 4.2%. The detection limit for each amino acid was less than 1.3 amol ($2 \cdot 10^{-10}$ M). The separation of amino acids was completed within 22 min. Fig. 2 shows the electropherograms of cerebrospinal fluid from leukaemic children treated with methotrexate.

Table 3 shows the mean CSF levels of free amino acids (taken from the literature [1]) in agreement with data obtained previously by ion-exchange chromatography, HPLC and GC [5–7]. Table 4 indicates the results we have found for three children.

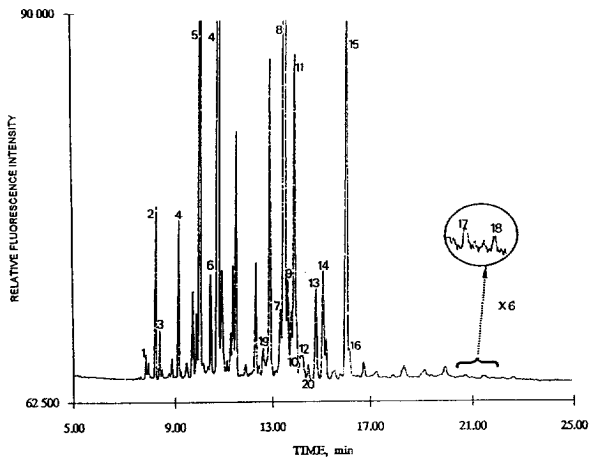

Fig. 2. Separation of FTC-amino acids and -amines from the acute lymphoblastic leukemic patient J. Conditions and peaks are identical to those in Fig. 1, with 19 = citruline and 20 = Asn.

Table 2
Logarithm of amino acid or amine concentration (x) versus fluorescence intensity (y) ($n = 6$)

Amino acid or amine	Regression equation	r^2
Lys	$y = 10.955 + 0.96496x$	0.999
Arg	$y = 11.012 + 0.92160x$	0.993
Ornithine	$y = 10.454 + 0.90610x$	1.000
Tyramine	$y = 11.420 + 0.98768x$	0.983
Leu	$y = 11.303 + 0.99481x$	0.996
Gln	$y = 12.022 + 1.0509x$	0.995
Tyr	$y = 11.501 + 0.99318x$	1.000
Val	$y = 11.488 + 1.0240x$	0.999
Thr	$y = 11.443 + 0.9960x$	0.996
Phe	$y = 11.673 + 1.0267x$	0.994
Ser	$y = 11.277 + 1.0127x$	0.995
Ala	$y = 11.705 + 1.0595x$	0.994
Taurine	$y = 11.667 + 1.0150x$	0.998
Ammonia	$y = 11.022 + 0.9216x$	0.984
Gly	$y = 11.571 + 1.0470x$	0.992
Asp	$y = 12.515 + 1.1557x$	0.995
Glu	$y = 11.571 + 1.0670x$	0.985

Table 3
Levels of free amino acids and amines in cerebrospinal fluids from [1]

Amino acid or amine	CSF, mean values ($\mu M/l$)	Range ($\mu M/l$)
Ammonia[a]	30.9	14.2–40.5
Ala[b]	30.3	18–44
Arg[b]	20.9	7–30
Asp[b]	2.6	0.3–6
Gln[b]	535	355–885
Glu[b]	9.8	1–48
Gly[b]	6.9	3–11
Leu[b]	13.8	7–23
Lys[b]	25.5	15–37
Ornithine[b]	4.9	3–7
Phe[b]	9.8	5–15
Ser[b]	28.5	20–41
Taurine[b]	7.2	4–11
Thr[b]	32.8	18–45
Tyramine[c]	<0.1	<0.1
Tyr[b]	8.7	5–17
Val[b]	18.5	7–31

[a] Ref. [24].
[b] Ref. [1].
[c] Ref. [21].

Table 4
Levels of free amino acids and amines in cerebrospinal fluids from 1000 × dilution of the three leukemic children derivatized samples

Amino acid or amine	CSF levels, (μM) mean, S.D. ($n = 3$)		
	Patient J	Patient M	Patient C
Ammonia	140.0 ± 23.3	131.1 ± 25.2	142.3 ± 22.0
Ala	45.1 ± 0.2	45.9 ± 0.2	40.0 ± 0.2
Asn	DNQ	DNQ	DNQ
Asp[a]	0.54 ± 0.09	0.85 ± 0.08	0.73 ± 0.09
Arg	34.2 ± 0.1	19.7 ± 2	18.3 ± 2
Citruline	DNQ	DNQ	DNQ
Gln	371.2 ± 15.6	347.3 ± 16.4	350.0 ± 14.2
Glu[a]	0.43 ± 0.07	1.05 ± 0.10	1.23 ± 0.15
Gly	13.5 ± 0.2	14.8 ± 0.1	13.1 ± 0.2
Leu	23.0 ± 0.2	21.3 ± 0.2	UD
Lys	14.8 ± 0.1	8.9 ± 0.1	6.2 ± 0.1
Ornithine	25.9 ± 0.1	UD	3.5 ± 0.1
Phe	6.7 ± 0.1	4.7 ± 0.1	5.7 ± 0.1
Ser	45.0 ± 0.6	37.1 ± 0.5	49.7 ± 0.3
Taurine	78.3 ± 1.2	106 ± 3.5	73 ± 2.5
Thr	85.1 ± 1.3	59.3 ± 1.5	91.2 ± 1.9
Tyr	19.8 ± 0.3	56.3 ± 0.5	34.3 ± 0.2
Tyramine	23.0 ± 1.4	5.6 ± 0.2	3.4 ± 0.1
Val	23.8 ± 0.7	22.4 ± 0.7	35.4 ± 0.9

[a] Quantitated with 100 × dilution.
UD = undetermined, DNQ = detected but unquantitated.

In this study, most amines and amino acids are approximately within the range of normal values, but the levels of ammonia, taurine, threonine and tyrosine were increased in each leukaemic child. Ornithine was increased in patient J, tyrosine in patients M and C. Lysine was decreased in patients M and C. Little information is available on amines and amino acids in human fluids of leukaemic patients [17,18], and to our knowledge no attempts has been made to study the role of cerebrospinal fluid amino acids in this disorder. The abnormal concentrations of ammonia and taurine in the brain are often correlated to liver disorders [19], which may be induced by high doses of methotrexate [20]. Indeed, the plasma transaminase levels of these children increased in the days following chemotherapy. Tyramine has not been quantitated before in cerebrospinal fluid, but usually cathecolamines are in the 0.1 μM range [21]. The relatively high concentration of tyramine could be correlated to the high level of tyrosine because the usual biological source of tyramine is the decarboxylation of tyrosine [22]. High levels of threonine, taurine and tyrosine were described in the plasma of acute leukaemic patients [23]. No conclusions can be drawn for ornithine and lysine since the levels do not evolve in the same way for each patient. Although ammonia levels are very high, the mean level of amino acids in the cerebrospinal fluid remains constant in our patients (mean of 760 ± 50 μM), in accordance with normal mean concentrations (755 μM); no aminoaciduria is observed, as was reported for patients treated with other chemotherapy [23,24].

4. Conclusion

MEKC with laser-induced fluorescence has been used to analyze amino acids and some amines in cerebrospinal fluids in a single run. This technique allows us to use a small amount of CSF. Precolumn derivatization with FITC does not require product purification prior to analysis, and allows the detection of very small quantities of amines (in the attomole range). Studies on cerebrospinal fluids of acute lymphoblastic leukaemic children treated with high amounts of methotrexate have been made, and differences between normal children and those with leukemia have been shown, indicating that differences in CSF composition of amino acids in this kind of disease and its medical treatment can be observed by CE–LIF.

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