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Journal of Pharmaceutical and Biomedical Analysis
15 (1997) 1257–1263

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Capillary electrophoretic analyses of β-trace protein and other low molecular weight proteins in cerebrospinal fluid from patients with central nervous system diseases[1]

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Received 21 August 1996; accepted 30 September 1996

Abstract

Ordinary capillary-zone electrophoresis (CZE), as well as CZE in a sodium dodecylsulfate-containing polymer solution (SDS-CZE) and capillary isoelectrofocusing (CIEF), was applied to the analysis of low molecular weight proteins in cerebrospinal fluid (CSF) from patients with various neuropsychiatric disorders. Under the CZE conditions employed, a peaks of β-trace protein (βTP), which is the most abundant low MW protein in CSF, was clearly detected on the electropherograms of all the samples examined, and the CSF βTP level could be tentatively determined using allylamine added at a constant concentration as the internal standard. The results revealed that βTP in CSF was non-specifically increased in organic diseases in the central nervous system (CNS), especially in ones giving severe physical damage to the brain tissues. On the other hand, SDS-CZE allowed us to determine simultaneously the CSF minor low MW proteins other than βTP, such as β_2-microglobulin, γ-trace protein, myelin basic protein, etc., while the CIEF electropherograms suggested that βTP were separated into several fractions with the different PI values. These capillary electrophoresis systems seem to be powerful as aids in the biochemical examinations of βTP and other low molecular weight proteins in CSF from patients with CNS diseases. © 1997 Elsevier Science B.V.

Keywords: Capillary electrophoresis; Cerebrospinal fluid; Low molecular weight protein; β-trace protein; Central nervous system disease

1. Introduction

Cerebrospinal fluid (CSF) has been biochemically studied in connection with neuropsychiatric diagnosis. It has been known that CSF contains some minor proteins derived from the central nervous system (CNS) tissues in addition to major

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[1] Presented at the Seventh International Symposium on Pharmaceutical and Biomedical Analysis, August, 1996, Osaka, Japan.

PII S0731-7085(96)01986-3

proteins originating from the blood plasma through blood-brain-CSF barrier [1]. Among such CSF minor proteins originated in the CNS, the most abundant component is β-trace protein (βTP) with the mean molecular weight of 26 000–27 000, which was discovered by Clausen [2]. It has also been elucidated by recent studies that βTP is a sialoglycoprotein synthesized in the CNS [3] and that its primary structure is identical with that of prostaglandin D synthease [4,5]. Some other CSF low MW proteins synthesized in the CNS, such as γ-trace protein (γTP, MW: 12 300), myelin basic protein (MBP, MW: 18 000), etc., are also characteristic to CSF [6]. It is therefore accepted that the qualitative and quantitative changes in these CSF low MW proteins can reflect pathological conditions in the CNS more accurately than those in the CSF major proteins from the blood plasma.

Capillary electrophoresis (CE) has been noticed in the field of clinical biochemistry as a rapid and simple microanalysis for various substances including proteins [7]. The authors applied this new technique to the analysis of low MW proteins of CSF, the bulk of which is originated in the CNS tissues. Ordinary capillary-zone electrophoresis (CZE) was mainly employed, although in some cases the CZE analysis in a sodium dodecyl sulfate (SDS)-containing polymer solution (SDS-CZE) and capillary isoelectrofocusing (CIEF) were also applied.

2. Materials and methods

2.1. Chemicals

All the reagents were of analytical grade. The authentic samples of α_1-acid glycoprotein (αAGP) and β_2-microglobulin (βMG) were purchased from Sigma. The standard sample of βTP was supplied by Dr. Yoshihiro Urade of Osaka Bioscience Institute.

2.2. CSF samples

A total of 100 CSF samples were taken by lumbar puncture from 87 patients with various neuropsychiatric disorders later described in Section 3. Among these, 89 CSF samples from 76 patients were examined only by CZE and another 11 were analyzed by SDS-CZE. Among these, four were further treated by CIEF.

2.3. Preparation of the samples to be examined

This was carried out by the combination of centrifuging ultrafiltration using membranes with the different cut-off values of MW. In the case of 89 samples examined by CZE, a constant volume each (2.5 ml) of intact CSF was centrifuged at first in Centricon-30 miniconcentrators (Amicon Japan) at 2000 × g for 30 min at 4°C, until the complete concentration to the dead stop volume of 50 μl was achieved. Then, 2 ml portions each of the first step ultrafiltrates were further treated with Centricon-10 (Amicon Japan) in a similar manner. The ultra-filtrating membranes of these tools allow only solutes with the MW below 30 000 and 10 000 to pass through into the ultrafiltrates, respectively. Therefore, it was expected that the final concentrates (50 μl) in the second step ultrafiltration contained low MW proteins with the MW between 10 000 and 30 000 at their highest concentrations. This is the low MW protein fraction. In order to detect components characteristics to this fraction, the first step concentrates in which major CSF proteins with the MW more than 30 000 (e.g. albumin, transferrin, immunoglubulins, etc.) were concentrated (the major protein fraction) and the second step ultrafiltrates containing only CSF components with the MW below 10 000 (the small substance fraction) were also examined by CZE. Another 11 CSF samples were treated in the first step ultrafiltration with Centricon-50 having the ultrafiltrating membrane with the cut-off MW value of 50 000, and only the second step concentrates in which CSF components with the MW between 10 000 and 50 000 were concentrated at their highest concentrations were analyzed by SDS-CZE (and CIEF).

2.4. Capillary electrophoresis

2.4.1. CZE

CZE was carried out by the use of a Beckman P/ACE 2000 unit equipped with a fused silica

capillary (75 μm × 50 cm) and an ultraviolet (UV) absorbance detector operated at 280 nm. Aliquots (20 μl each) of the samples to be examined were placed in sample vials, and a constant volume each was withdrawn and injected into the capillary by pressure of 0.5 psi. The injection time was 10 s in examination of the major protein fraction and 40 s in that of the low MW protein fraction and the small substance fraction, respectively. In the case of the low MW protein fraction, prior to the injection, the samples to be examined were mixed with 5% aqueous allylamine as the internal standard for determination of βTP, at a ratio of 9:1 (18 and 2 μl, respectively). Separation was achieved at 20 kV using 50 mM sodium tetraborate (pH 9.2) with or without 1 M 3-(trimethylammonio)propylsulfonate (Z_1-methyl) (Nihon Waters) as an electrolyte. Z_1-methyl was added only in the analysis of the major protein fraction. These conditions were generally same as those employed in our preliminary research on the CZE analysis of CSF protein and amino acids except for the apparatus used and the detection wavelength [8].

2.4.2. SDS-CZE

SDS-CZE employing a Beckman P/ACE 2000 unit and eCAP™ SDS 14-200 kit was performed in a fused silica capillary (100 μm × 47 cm) operated in the reversed polarity mode. Twenty-five μl each of the samples to be analyzed was mixed with 50 μl each of 120 mM trishydroxyaminomethane buffer (pH 6.6)–1% SDS (the sample buffer of this kit), 5 μl of 0.1% Orange G (OG) (the front marker), 2.5 μl of 2-mercaptoethanol and 20 μl of dionized water. The mixtures thus prepared were heated at 95°C for 5 min. After cooling, a constant volume each of the mixtures was injected by pressure (0.5 psi) for 60 s into the capillary filled with a SDS-containing polymer solution (the gel buffer solution of this kit whose contents have not been published by Beckman except that it is a polymer solution containing SDS). Separation was achieved at 14.1 kV, and the detector was operated at 214 nm. The correlation between the migration time (Mt) and MW was evaluated by coanalysis of marker proteins (Beckman) and the authentic samples with the known MW of 92 500 (phosphorylase b), 66 200 (bovine serum albumin), 45 000 (ovalbumin), 31 000 (carbonic anhydrase) and β_2-microglobulin (MW: 11 700). These conditions are the same as those employed in our previous study on oxidative degradation of human serum albumin by the action of L-ascorbic acid and Cu^{++} [9].

2.4.3. CIEF

One-step CIEF was done using a Beckman 5000 unit equipped with a eCAP™ Neutral Capillaray (50 μm × 20 cm) operated in reversed polarity mode. The anolyte (10 mM phosphoric acid) and the catholyte (20 mM sodium hydroxide) were placed at the outlet and the inlet, respectively. The capillary is rinsed at first with the anolyte, and then filled with the mixture (1:1) of samples to be examined and the ampholyte solution consisting of purified water, 1% hydroxypropylmethylcellulose (HPMC), *N,N,N′,N′*-tetramethylethylene-diamine (TEMED) and Pharmalyte 3-10 (Sigma) at a ratio of 9:80:3:20. Injection of the sample mixtures required 2 min. HPMC was added to provide viscosity and reduce the electroosmosis flow. The concentration of TEMED as a gradient extender was adjusted so that when an electric field was applied the ampholyte pH gradient forms in the 7 cm between the detector and the capillary outlet. The isoelectrofocusing was achieved at 4.67 kV, and the detector was operated at 280 nm. The correlation between the Mt and isoelectric point (PI) values were obtained by coanalysis of marker proteins (Beckman) with the known PI values of 9.6 (cytochrome C), 8.8, 8.6, and 8.2 (lentil lectin), 5.9 (carbon anydrase II) and 5.1 (β-lactogloblin). Identification of proteins were performed by the mixed analysis with the authentic samples, and all the CE data were treated with a computer software of System Gold (Beckman).

3. Results and discussion

The CZE analysis of three fractions of CSF: Typical electropherograms of the major protein fraction, the low MW protein fraction and the

small substance fraction were shown in Fig. 1. As shown in Fig. 1a, the patterns of fractionated major proteins resembled to the densitograms of stained cellulose acetate strips and agarose-gel films except that the order of migration was reversed. On all the electropherograms, seven peaks of the major proteins (γ-globulin, β_2-globulin, β_1-globulin, α_2-globulin, α_1-globulin, albulin and prealbumin) in addition to an artifact peak of NaN_3 (an ultrafiltrating membrane component) and neutral signal were observed on the electropherograms (Fig. 1a). One analysis required only 10 min. These electropherograms resembled to those obtained in our preliminary study on the CZE analysis of CSF proteins and amino acids except for the absence of the peak of glutamine (Gln) with no UV absorbance, since in that work employing a Waters Quanta-4000 unit equipped with the detector operated at 185 nm, the peak of Gln was detected within the region of α_1-globulin [8]. On the other hand, as shown in Fig. 1b and

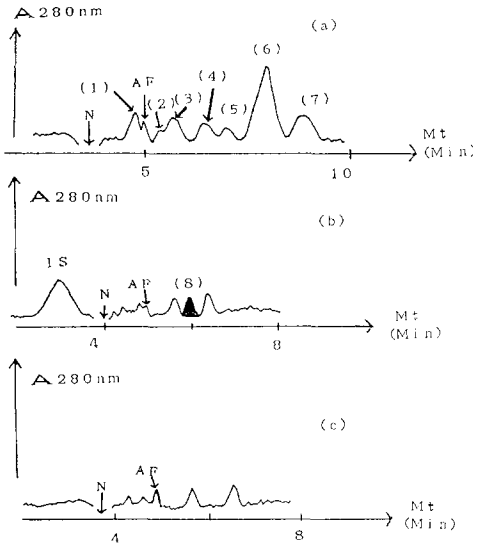

Fig. 1. Typical CZE Electropherograms of the Major Protein Fraction (a), the Low MW Protein Fraction (b) and the Small Substance Fraction (c); N: neural signal, AF: an artifact peak of NaN_3 (a component of the ultrafiltrating membranes), IS: 0.5% allylamine as the internal standard, (1) γ-globulin, (2) β_2-globulin, (3) β_1-globulin, (4) α_2-globulin, (5) α_1-globulin, (6) albumin, (7) prealbumin, (8) β-trace protein, Mt: migration time (min).

Table 1
Results of the within-run reproducibility test for determination by CZE of the CSF βTP level

Run	Values of the βTP level[a]
1	0.52
2	0.56
3	0.53
4	0.50
Mean value	0.53
S.D.[b]	0.03
R.S.D.[c]	5.67%

[a]Peak area ratios against the internal standard.
[b]Standard deviation.
[c]Relative Standard Deviation.

1c, some peaks other than NaN_3 and neutral signal were commonly observed on both of the electropherograms of the low MW protein fraction and the small substance fraction. They were presumably low MW components of CSF, such as aromatic amino acids, ascorbic acid, uric acid, etc. However, several peaks were also observed only on the electropherograms of the low MW protein fraction, and one of them with the Mt of ca. 6 min. was the most abundant (Fig. 1b). This peak was detected in all the 89 samples examined by CZE, and was identified as βTP. Its level in these CSF samples could be tentatively determined using 0.5% (the final concentration) allylamine as the internal standard (see Section 2). Precision of the determination of βTP by this system was confirmed by the within-run reproducibility tests (Table 1). One measurement took only 8 min.

3.1. Determination of βTP in CSF from patients with various neuropsychiatric disorders

Typical electropherograms indicating the raised and moderate levels of CSF βTP are shown in Fig. 2. The peak area ratios of βTP against the internal standard were calculated in individual samples. Its mean value and the standard deviation (S.D.), as well as its distribution range, in various disease and disease groups are summarized in Table 2. As summarized in Table 2, the mean $\pm$ S.D. value of the βTP level determined by this method in CSF so far examined was 0.29 $\pm$

0.27 ($n = 89$, range: 0.04–0.88). A clinically interesting finding was a fact that the CSF βTP levels in patients with organic diseases in the CNS, such as cerebrovascular diseases, inflammation of brain and/or meninges, degenerative disorders, multiple sclerosis and epilepsy (mean ± S.D.: 0.38 ± 0.23, $n = 54$, range: 0.05–0.88) was significantly higher ($P < 0.05$) than that of ones with other neuropsychriatric disorders giving no organic damage to the CNS, such as psychotic diseases, neurosis and peripheral neuropathy (mean ± S.D.: 0.15 ± 0.06, $n = 25$, range: 0.04–0.23). The most elevated value of CSF βTP obtained by this method (0.88) was found in a multiple sclerosis patient. Furthermore, in patients with cerebrovascular diseases, the value in cerebral infarction tended to be higher than that in cerebroarteriosclerotic Parkinsonism and/or dementia, and among epileptic patients, one suffered from uncontrolled seizure tended to exhibit the higher CSF βTP level than others whose seizure had been suppressed by the action of anti-epileptic drugs (Table 2). These data indicated that CSF βTP is increased nonspecifically in patients with organic diseases of the CNS, especially in ones which give severe physical damage to the brain tissues (e.g. cerebral infarction, multiple sclerosis, etc). It was also noteworthy that such increase of βTP in CSF was virtually independent of elevation of the total CSF protein contents. However, in patients from whom CSF samples were taken several times during the hospital treatments, the correlation between changes in the CSF βTP level and the clinical improvements were unclear. Therefore, it was considered that the elevated level of CSF βTP, as well as that of neuron-specific enolase [10] and MBP [11], can be used as an indicator of

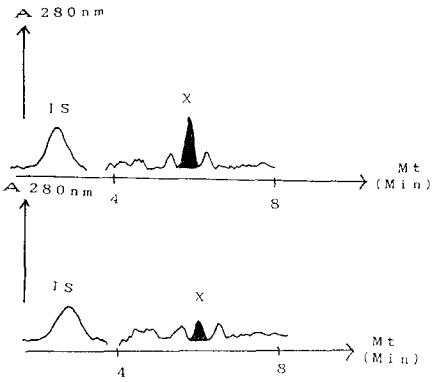

Fig. 2. The CZE Electropherograms of the Low MW Protein Fractions in CSF with the Raised Level (a) and the Moderate Level (b) of TP Levels; IS: the internal standard, X: β-trace protein, Mt: migration time (min).

Table 2
The CSF βTP level in various diseases and disease groups

Diseases or disease groups	The βTP level determined by CZE[a]	
	Mean ± S.D.	Range
Organic diseases in the CNS		
Cerebrovascular diseases		
Cerebral infarction ($n = 11$)		0.12–0.76
Cerebroarteriosclerosis ($n = 7$)		0.09–0.37
Total ($n = 18$)	0.35 ± 0.22	0.09–0.76
Inflammation in Brain and/or meninges		
Meningitis ($n = 6$)		0.11–0.39
Meningoencephalitis ($n = 3$)		0.15–0.54
Total ($n = 9$)	0.33 ± 0.14	0.11–0.54
Degenerative disorders in the CNS		
Parkinson's disease ($n = 4$)		0.14–0.49
Altzheimer's disease ($n = 3$)		0.18–0.38
Others ($n = 5$)		0.12–0.41
Total ($n = 12$)		0.12–0.49
Epilepsy		
Controlled ($n = 5$)		0.05–0.25
Uncontrolled ($n = 5$)		0.08–0.44
Total ($n = 10$)	0.27 ± 0.18	0.05–0.44
Total ($n = 54$)	0.38 ± 0.23	0.05–0.88
Neuropsychiatric disorders giving no organic damage to the CNS		
Psychotic disorders		
Shizophrenia ($n = 5$)		0.06–0.21
Depressive illness ($n = 3$)		0.10–0.18
Others ($n = 2$)		0.09–0.15
Total ($n = 10$)	0.15 ± 0.04	0.06–0.21
Neurosis		
Total ($n = 13$)	0.15 ± 0.06	0.05–0.23
Peripheral Neuropathy		
Total ($n = 12$)	0.14 ± 0.05	0.04–0.22
Total ($n = 35$)	0.15 ± 0.06	0.04–0.23
Total ($n = 89$)	0.29 ± 0.27	0.04–0.88

[a]The peak area ratio against the internal standard.

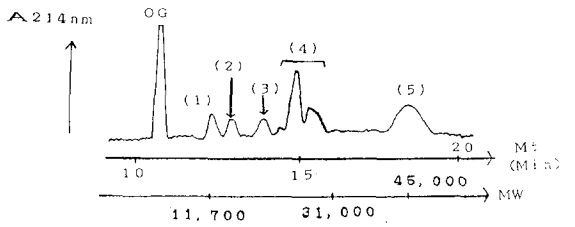

Fig. 3. A typical SDS-CZE Electropherogram of CSF MW Proteins with the MW of 10 000–50 000; OG: Orange G as the front marker, (1) β_2-microglobulin (MW: 11 700), (2) γ-trace protein (MW: 12 300), (3) myelin basic protein (MW: 18 000), (4) β-trace protein (MW: 22 000–29 000), (5) α_1-acid glycoprotein (MW: 46 000) and others, Mt: migration time (min).

the occurrence of acute demyliation or severe physical damage in the brain tissues. Indeed, earlier workers reported that CSF βTP was increased in such kinds of diseases including brain tumor [12–14]. Peaks of low MW proteins other than βTP were barely detected on the CZE electropherograms of a part of samples examined, although its determination could not be performed due to the small amount.

3.2. SDS-CZE

As shown in Fig. 3, several peaks in addition to the peak of OG as the front marker were detected on the electropherograms. Among them, a complex of three or four overlapped peaks corresponding the MW of MW: 23 000–29 500 was the most abundant, and identified as βTP. The highest peak in these βTP subfractions showed the MW of 26 000. Other peaks corresponding to the MW of 11 500, 12 500 and 18 000 were clearly observed on the SDS-CZE electropherograms (Fig. 3). They were assigned as βMG, γTP and MBP, respectively, by the mixed analysis with the authentic sample (in the case of βMG) or by the fact that their MW calculated from the Mt agreed well with the reterature values (in the cases of γTP and MBP). A broad peak (MW: 42 000–47 000) also appeared on the electropherograms (Fig. 3). It was confirmed that αAGP (MW: 46 000) was contained in this peak by the mixed analysis with the authentic sample. As αAGP is a main component of the serum α_1-globulin fraction, the bulk of CSF αAGP may be derived from the blood plasma. Determination based on the peak area ratios against OG as the internal standard could be carried out even in the cases of minor components other than βTP (Table 3). It has been known that βMG is increased in CSF of patients with brain tumor [15]. As described above, increase of MBP in CSF has been accepted as a marker of acute demyliation and/or severe physical damage in the brain tissues [11], although the clinical significance of CSF γTP has not been established. The present SDS-CZE system allowed us to determine simultaneously these CSF minor low MW proteins within 20 min for one sample.

3.3. CIEF

A typical electropherogram was shown in Fig. 4. αAGP with the PI value of 2.8 and MBP with that of 10.5 were not detected by this system for the analysis of proteins with the PI values of

Table 3
The CSF low MW protein levels determined by SDS-CZE[a]

Low MW proteins	MW[b] ($n = 11$)	Mean + S.D. ($n = 11$) (%)	Range (%)
β_2-microglobulin	11 500	0.8 ± 0.3	0.4–1.2
γ-trace protein	12 500	0.6 ± 0.2	0.3–0.9
Myelin basic protein	18 000	0.5 ± 0.2	0.2–0.8
β-trace protein	23 000–29 500[c]	10.4 ± 3.3	5.6–16.2
α_1-acid glycoprotein	42 000–47 000[d]	1.8 ± 0.6	0.9–2.7

[a]Peak areas ratios against Orange G as the internal standard.
[b]The values obtained by the present SDS-CZE system (see text).
[c]A complex of 3 or 4 overlapped peaks (see Fig. 3).
[d]A broad peak containing α_1-acid glycoprotein (MW: 46 000) as one of its component.

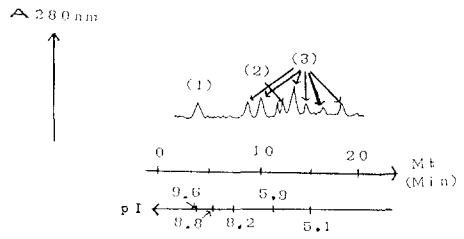

Fig. 4. A Typical CIEF Electropherogram of CSF Low MW Proteins with the MW of 10 000–50 000; (1) γ-trace protein, (2) β_2-microglobulin, (3) subfractions of β-trace protein, Mt: migration time (min).

3–10. βMG (PI: 5.6) and γTP (PI: 9.7) were detected as single peaks, while βTP was separated into several fractions with the PI values of 4–7.5 (Fig. 4). The PI value of the highest peak in the βTP subfractions was 5.4. One analysis time was 20 min.

The results of SDS-CZE and CIEF analyses suggested the presence of heterogeneity in the MW and PI values of βTP molecules. These data agreed well with the recent reports based on the analysis of CSF proteins by two-dimensional electrophoresis [16]. It is speculated that changes in the carbohydrate structure of βTP, which is a sialoglycoprotein, can reflect pathological conditions in the CNS and that such changes give those in the patterns of CSF βTP subfractions with the different MW and PI values [3]. It is therefore expected that such changes can be detected by the combination of SDS-CZE and CIEF without complicated procedures. Including ordinary CZE, which was suitable for detecting the elevating βTP level in a large number of CSF samples, these CE systems are powerful as aids in the biochemical examination of CSF from CNS disease patients.

Acknowledgements

We express our great thanks to Dr. Yoshihiro Urade of Osaka Bioscience Institute who supplied us the authentic sample of βTP (purified prostaglandin D synthease).

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