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One-step capillary isoelectric focusing of the proteins in cerebrospinal fluid and serum of patients with neurological disorders

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Abstract

One-step capillary isoelectric focusing (cIEF), which uses reduced but non-zero electroosmosis flow to mobilize the focused proteins, was applied to the analysis of proteins in cerebrospinal fluid (CSF) and serum of patients with various neurological disorders. Under the conditions employed, pathological changes in the CSF proteins were clearly detected on the electropherograms within 25 min, although the serum proteins did not vary significantly between samples. The present one-step cIEF system seems to be useful in routine laboratory examinations of a large number of CSF samples as an aid in neurological diagnosis. © 2002 Published by Elsevier Science B.V.

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1. Introduction

Human cerebrospinal fluid (CSF) contains proteins derived mainly from blood plasma and, partly, from central nervous system (CNS) tissues. The content (normal range: 15–40 mg/dl) and the distribution of human CSF proteins do not vary as distinctly as the low-molecular-mass (low-M_r) components do, because the blood–brain barrier (BBB) restricts penetration of blood plasma proteins (normal range: 6–8 g/dl) into the CSF. It is known, however, that due to disturbance in the BBB function and increase in secretion into the CSF of the CNS-specific proteins, CSF proteins change quantitatively and qualitatively under various pathological conditions of the CNS. From the viewpoint of clinical biochemistry, we have been studying pathological changes in proteins in CSF of patients with neurological disorders by capillary zone electrophoresis (CZE) [1,2] and sodium dodecylsulfate–capillary gel electrophoresis (SDS–CGE) [3,4]. However, in order to obtain further information from the results of CSF protein analyses, development of other separation modes is required. Although isoelectric focusing (IEF) is a powerful tool for the analysis of proteins with different isoelectric point (pI) values, it has not been utilized fully in the field of clinical biochemistry. This could possibly be caused by its experimental complexity and the difficulties encountered in the quantitation of the separated proteins. Recently, capillary IEF (cIEF) with acid mobilization was introduced by replacing, after separation, the sodium hydroxide catholyte with acetic acid in the analysis of proteins in clinical samples [5–7], including CSF [7]. However, this system (two-step cIEF), which requires more than 1 h for the analysis of one sample [5–7], did not seem to be suitable for the routine

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examination of a large number of clinical samples. The one-step cIEF method employing reduced but non-zero electroosmosis flow to mobilize the focused proteins has an advantage over the two-step system, in that the analysis time is shorter and the procedures are simpler [8]. Indeed, using one-step cIEF, we succeeded in detecting pathological changes in the charge microheterogeneity of β-trace protein (βTP) in CSF samples from patients with various CNS diseases [9]. In this study, we analyzed by cIEF the whole protein complements in CSF and serum of patients with various neurological disorders.

2. Experimental

2.1. Subjects

Forty-six pairs of lumbar CSF samples and the corresponding sera were taken from 38 patients with cerebral infarction (five pairs from four cases, 5:4), meningitis (4:2), Parkinson's disease (5:5), Altzheimer's disease and senile dementia of Altzheimer type (AD/SDAT) (7:7), amyotrophic lateral sclerosis (ALS) (2:2), multiple sclerosis (MS) (3:2), Guillain–Barré syndrome (GBS) (4:1), epilepsy (2:2), diabetic neuropathy (3:3), alcoholism (2:2), sequelae of head injury (2:1), schizophrenia (2:2), depressive illness (3:3) and neurosis (2:2). Informed consent was obtained from all subjects. Except for seven cases (in total) with psychotic disorders (schizophrenia and depressive illness) and neurosis, they all had some organic damage in the nervous system. All CSF and serum samples were stored at $-20\,°C$ leaving the rest required for routine laboratory tests in the hospital.

2.2. Chemicals

All reagents were of analytical grade. The standard samples of albumin (Alb), transferrin (Tf), immunoglobulin G (IgG), celuroplasmin (Cp), α_1-acid glycoprotein (AGP) and β_2-microglobulin (βMG), as well as reagents for cIEF, such as hydroxypropylmethylcellulose (HPMC), N,N,N',N'-tetramethylenediamine (TEMED) and Pharmalyte 3-10, were purchased from Sigma (St Louis, MO, USA). Cp was used as a standard protein with a

known pI value of 4.4. An immunoaffinity column with monoclonal antibody against βTP [10] was supplied by Dr Yoshihiro Urade of Osaka Bioscience Institute. The standard proteins with pI values of 9.6 (cytochrome c), 8.8, 8.6 and 8.2 (lentil lectins), 7.3 (myoglobulin), 6.6 (carbonic anhydrase I), 5.9 (carbonic anhydrase II) and 5.1 (β-lactoglobulin) were obtained from Beckman (Fullerton, CA, USA).

2.3. Pretreatment of samples

The CSF samples were quantitatively fractionated into two portions by centrifugal ultrafiltration using membranes with M_r cut-off values of 10 000 and 50 000. The major protein fraction (50 μl each from 2.2 ml of intact CSF) contained components with M_r values above 50 000, while the low-M_r protein fraction (50 μl each from 2.0 ml of the ultrafiltrate thus obtained) contained components with M_r values between 10 000 and 50 000, as described previously [3,4]. Prior to analysis, both CSF concentrates were diluted with 2 ml of deionized water and re-centrifuged for desalting and re-concentration to 50 μl. The serum samples were diluted 40 times with deionized water.

2.4. One-step cIEF

One-step cIEF was carried out with a Beckman P/ACE 2000 unit (Beckman) equipped with an eCAP neutral capillary (20 cm×50 μm) operated in the reversed polarity mode. The anolyte (10 mM phosphoric acid) and catholyte (20 mM sodium hydroxide) vials were placed at the outlet and inlet, respectively. The capillary was rinsed first with the anolyte, then filled with the mixture (1:1, v/v) of a sample to be injected (desalted CSF concentrates and diluted sera) and the ampholyte solution consisting of deionized water, 1% (w/v) HPMC, TEMED and Pharmalyte 3-10 in the ratio of 9:80:3:20 (v/v). The final 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. Injection of the sample mixtures by pressure required 2 min. IEF was achieved at 125 V/cm; the detector was operated at 280 nm. The pI values of peaks on the electropherograms were determined from the

electrophoresis of the nine standard samples with known p*I* values between 4.4 and 9.6 (see Section 2.2). The electropherogram of the standard proteins was essentially the same as that obtained by earlier workers [8] except for the presence of a peak for Cp with a migration time of ca. 16.8 min (p*I* 4.4). The analytical conditions were essentially the same as those employed in Ref. [8] and in our previous study on the charge microheterogeneity of βTP in human CSF [9].

2.5. Identification of proteins

Identification of Alb, Tf, IgG, AGP and βMG on the electropherograms was performed by analyzing samples mixed with authentic proteins. Among CSF-specific proteins of which authentic samples were not available, βTP was identified by disappearance of the corresponding peaks after passing the samples through an immunoaffinity column with the monoclonal antibody (see Section 2.2), while γ-trace protein (γTP) and asialoTf were identified by comparing their p*I* values with those in the literature [11], respectively. The reproducibility of electropherograms was checked by duplicate injections of a diluted serum on three different days, although CSF could not be treated due to its small amount.

2.6. Others

βTP in CSF was determined by enzyme-linked immunosorbent assay (ELISA) as reported previously [12]. The total protein (T.P.) content in CSF was measured by routine laboratory tests in the hospital.

3. Results and discussion

Typical electropherograms of the CSF major protein fraction, the CSF low-M_r protein fraction and the diluted serum are shown in Fig. 1a–c, respectively. One analysis time was ca. 25 min, which was less than half of that required by two-step cIEF (more than 1 h [5–7]). As shown in Fig. 1a,c, many peaks were detected in the electropherograms of the CSF major protein fraction (Fig. 1a) and the diluted serum samples (Fig. 1c). They resembled each other. Indeed, Alb, Tf and IgG were identified in both of
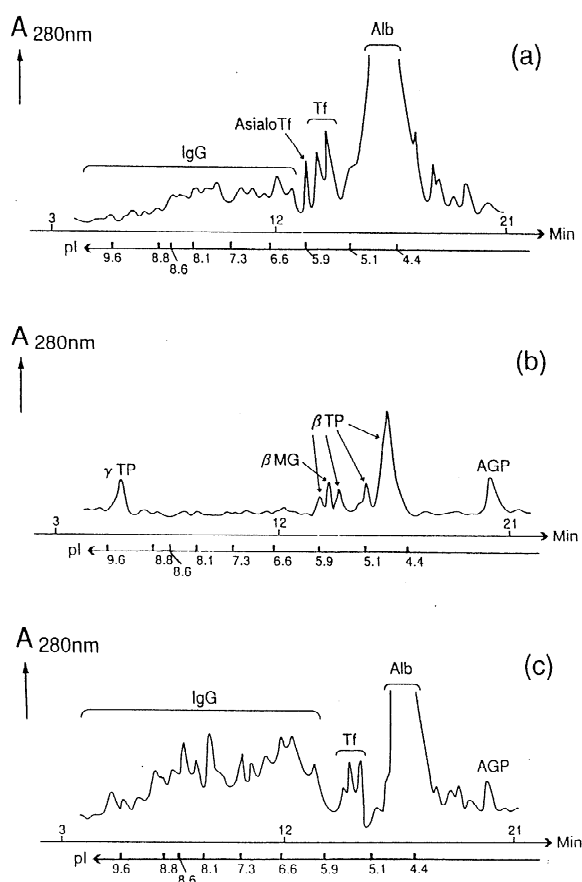

Fig. 1. Typical electropherograms of the CSF major protein fraction (a), the CSF low-M_r protein fraction (b) and the diluted serum (c); Alb, albumin; Tf, transferrin; IgG, immunoglobulin G; AGP, α_1-acid glycoprotein; βTP, β-trace protein; βMG, β_2-microglobulin.

these electropherograms (Fig. 1a,c). However, the sizes of the peaks of components with p*I* values higher than 6.0 were relatively lower in CSF (Fig. 1a) than in serum (Fig. 1c). They were mainly IgG. A peak of CSF-specific asialoTf (p*I* 5.8–6.0) was also observed only in the CSF electropherograms (Fig. 1a). A peak of Cp (p*I* 4.4) was not detected in these electropherograms, because it overlapped with the huge peak of Alb (Fig. 1a,c). Peaks of γTP (M_r 12 300, p*I* 9.3) and four isoforms of βTP (M_r 27 000 as the mean value, p*I* 4.7–4.8, 5.1–5.2, 5.6–5.7 and 5.9–6.0), as well as those of AGP (M_r 44 100, p*I* 2.8) and βMG (M_r 11 700, p*I* 5.7–5.8), were found in the electropherograms of the CSF low-M_r protein

fraction (Fig. 1b). In the electropherograms of diluted serum, AGP was also identified, although the peak of βMG was not clearly detected, because, presumably, its amount is small and overlapped with a cluster of peaks near Tf (Fig. 1c). The electropherogram patterns of serum proteins exhibited virtually no variation between samples, as well as between different injections (see Section 2.5), while those of CSF proteins varied in some samples, as follows.

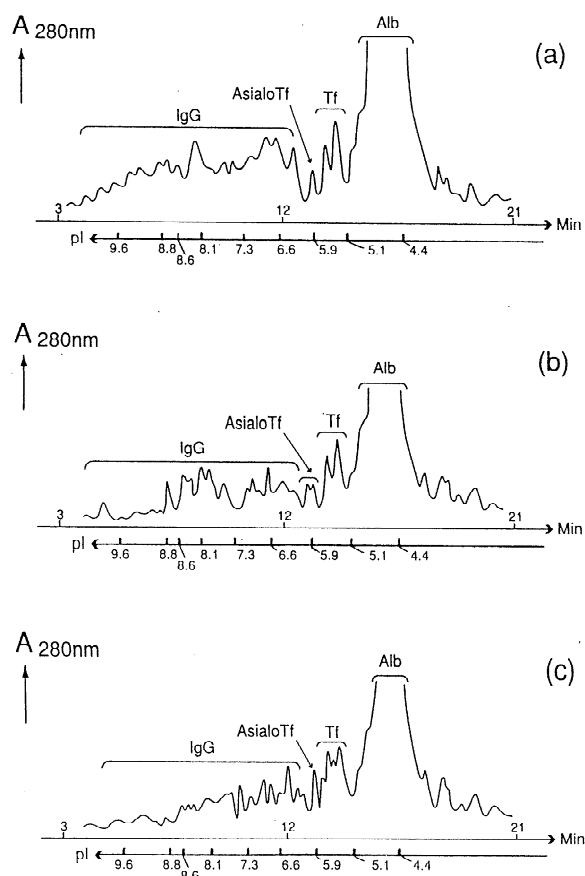As shown in Fig. 2, enhancement and/or appearance of peaks in the electropherograms of the CSF

Fig. 2. Electropherograms of the CSF major protein fraction in an ALS patient (a), an MS case (b) and a cerebral infarction subject (c). Abbreviations are as in Fig. 1. In (a) and (b), IgG peaks are uniformly (a) and non-uniformly (b) higher than those in Fig. 1a. In (b), the asialoTf peak found as a single peak in Fig. 1a is divided into two fractions. In (c), aberrant peaks are observed in the Tf region and in the acidic part of the IgG region.

major protein fraction were found mainly in the regions of IgG (pI above 6.0), CSF-specific asialoTf (pI 5.8–6.0) and Tf (pI 5.2–5.8). Often, such changes in the electropherogram patterns of the CSF major proteins occurred in an overlapped manner. Enhancement of peaks in the IgG region was usually accompanied by elevation of the CSF T.P. level. CSF T.P. was elevated beyond the upper normal limit of 40 mg/dl in 17 samples from 14 patients (in total) with various organic disorders in the nervous system, such as cerebral infarction (3 samples: 3 cases), meningitis (3:2), Parkinson's disease (1:1), AD/ SDAT (2:2), ALS (1:1), MS (1:1), GBS (3:1), epilepsy (1:1), diabetic neuropathy (1:1) and seculae of head injury (1:1). The highest value of the T.P. content in CSF examined was 180 mg/dl in one of four samples from a GBS case. In the electropherograms of these CSF samples with elevated T.P. levels, IgG peaks were uniformly (Fig. 2a) or non-uniformly (Fig. 2b) enhanced. The uniform increase in CSF IgG was detected in 12 CSF samples from 10 patients (in total) with cerebral infarction (2 samples: 2 cases), Parkinson's disease (1:1), AD/SDAT (2:2), ALS (1:1), GBS (3:1), epilepsy (1:1), diabetic neuropathy (1:1) and sequelae of head injury (1:1). This phenomenon may be due to the increased entry of blood plasma macromolecular proteins, including IgG, through the dysfunctional BBB. On the other hand, non-uniform increase in IgG was found in the other five CSF samples from four patients (in total) with cerebral infarction (1:1), meningitis (3:2) and MS (1:1). This may be caused at least partly by pathological acceleration of IgG production within the CNS. In four patients with cerebral infarction, meningitis, MS and GBS (from whom CSF samples were taken 2–4 times during the hospital treatments) with both uniform and non-uniform increases in CSF IgG and elevation of CSF T.P. contents, the elevated levels disappeared or were reduced in association with their clinical improvements as responses to therapy. As shown in Fig. 2b, occurrence of doublet peaks in the CSF-specific asialoTf region was observed in eight CSF samples from six patients (in total) with meningitis (2 samples: 1 case), AD/ SDAT (1:1), MS (2:1), alcoholism (1:1), schizophrenia (1:1) and depression (1:1). This variation in CSF asialoTf was originally reported in 1978 by Stibler who analyzed the human CSF proteins by thin-layer

IEF [13] as the doublet tau-fraction. Successful detection of this phenomenon by the present cIEF system seemed to be due to the removal of such low-M_r proteins as the highest pI isoform of βTP (pI 6.0, M_r 27 000) and βMG (pI 5.8, M_r 11 700) (see Section 2.3). The doublet asialoTf peaks appeared independently of changes in the CSF T.P. and IgG levels, non-specifically to the properties of diseases. No difference in its presence/absence was revealed between samples taken at different days from the same subjects. Therefore, it seems to have a poor diagnostic value, and may be a genetic factor as described by Siden and Kjellin [14]. Some aberrant peaks appeared in the Tf region (pI 5.2–5.8) in the electropherograms of five CSF samples from three cerebral infarction patients and two meningitis subjects. This was also not usually accompanied by elevation of CSF T.P. and IgG levels. Furthermore, in two cerebral infarction patients with normal protein levels of 23 and 36 mg/dl, as shown in Fig. 2c, a cluster of aberrant peaks was detected also in an acidic part of IgG (pI 6.0–7.3), possibly due to the absence of enhanced IgG peaks in this region. In a cerebral infarction patient and two meningitis cases, from whom CSF samples were taken twice at different days, disappearance of aberrant peaks in the Tf region agreed with their clinical improvements. In the first samples from these patients (T.P. 54–80 mg/dl), occurrence of aberrant peaks in the most acidic part of the IgG region was not confirmed due to uniform enhancement of the IgG peaks. In the second samples taken after 2 and 4 weeks (T.P. 27–40 mg/dl), the aberrant IgG peaks were absent. These data may reflect the occurrence of such pathological changes in the CNS as partial deasilation of CSF Tf (and a part of IgG).

Typical pathological changes in the electropherograms of the CSF low-M_r protein fraction are shown in Fig. 3. As shown in Fig. 1c and Fig. 3a–c, βTP is the most abundant among the components in this fraction. The total βTP content in intact CSF samples was measured by ELISA (see Section 2.6). The mean value (±SD) of the βTP concentration in the CSF samples examined was 43.4±28.5 μg/ml (n= 46). We have revealed changes in its total level [12] and microheterogeneities in M_r [4] and charge [9] in association with diseases. Essentially the same results have been obtained here (data not shown).
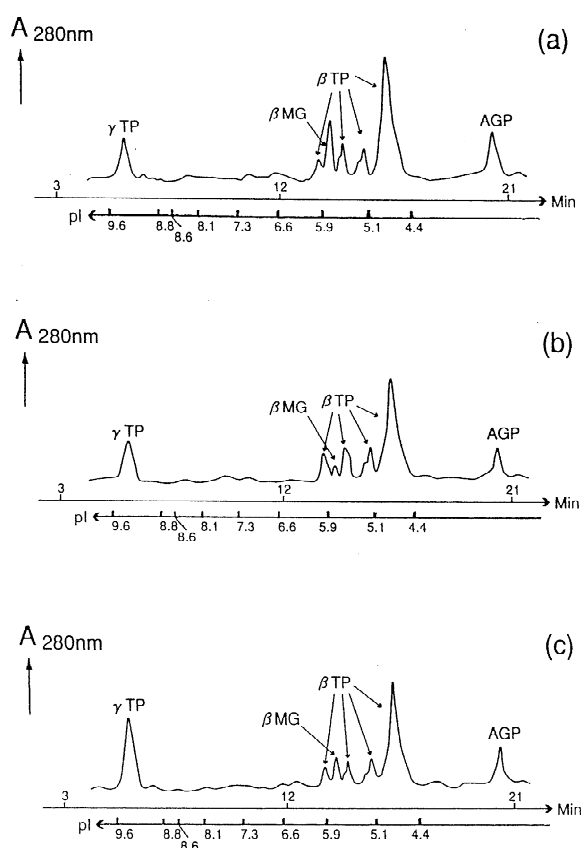

Fig. 3. Electropherograms of the CSF low-M_r protein fraction in a meningitis patient with elevated AGP and βMG levels (a), a SDAT case with a reduced βMG level (b) and a cerebral infarction subject with an increased γTP content (c) Abbreviations as in Fig. 1.

Therefore, we describe here the pathological changes in the CSF levels of AGP, βMG and γTP. The levels of AGP, βMG and γTP in the CSFs examined were tentatively determined by calculation using βTP levels obtained by ELISA and the peak area ratios against the sum of the βTP peaks. The mean concentration of these low-M_r proteins (in patients from whom two or more samples were collected, the first data were taken) in CSFs were 5.1±2.2 μg/ml (n=38, range: 1.6–10.1 μg/ml) for AGP, 3.2±1.9 μg/ml (n=38, range: 0.4–8.3 μg/ml) for βMG and 4.3±1.8 μg/ml (n=38, range: 1.2–9.7 μg/ml) for γTP, respectively. In CSFs with elevated T.P. content (47–180 mg/dl) taken from 14 patients with various organic disorders in the nervous system, the mean

values of the AGP and βMG levels were 5.8±2.8 μg/ml (*n*=14, range: 2.2–10.1 μg/ml) and 3.7±2.5 μg/ml (*n*=14, range: 0.4–8.3 μg/ml), respectively. These values tended to be greater than those in CSF with T.P. levels (15–40 mg/dl) from the other 24 patients with various organic and non-organic diseases (AGP: 4.7±2.0 μg/ml, *n*=24, range: 1.6–7.7 μg/ml, βMG: 2.9±1.8 μg/ml, *n*=14, range: 0.9–5.7 μg/ml), although the difference was not significant. Both AGP and βMG are common components in CSF and blood plasma, and elevation of the CSF T.P. level is caused mainly by dysfunction of BBB. Therefore, at least a part of the increased AGP and βMG in their CSF seemed to be derived from the blood plasma. However, the highest levels of AGP and βMG in CSF so far examined were observed not in a GBS patient with the most elevated CSF T.P. content of 180 mg/dl, but in a meningitis case with the moderately elevated CSF T.P. content of 80 mg/dl (Fig. 3a). On the other hand, in CSFs of two cerebral infarction patients with normal protein levels of 23 and 36 mg/dl (as described above, aberrant peaks appeared both in the Tf and IgG

regions in the electropherograms of the major protein fraction of their CSF), the AGP levels were as high as 9.2 μg/ml (the βMG level was 5.6 μg/ml) and 7.9 μg/ml (βMG: 4.3 μg/ml), while in two AD/SDAT subjects with slightly elevated CSF T.P. levels of 49 and 56 mg/dl, CSF βMG levels were as low as 0.4 μg/ml (AGP: 6.6 μg/ml) (Fig. 3b) and 0.8 μg/ml AGP: 5.7 μg/ml). Indeed, no significant correlation was present between the CSF levels of AGP and βMG. It is postulated from these data that at least a part of AGP and βMG in CSF is generated in the CNS and that changes in their CSF levels occasionally reflect their pathologically accelerated or reduced production in the CNS. The mean value of the CSF γTP level in patients with organic damage in the nervous system (see Section 2.1) was 4.9±2.1 μg/ml (*n*=31, range: 1.7–9.7 μg/ml), which tended to be higher than that in the other seven patients with psychotic disorders and neurosis (3.9±1.7 μg/ml, *n*=7, range: 1.2–7.5 μg/ml). The difference was not significant, but the highest and the second highest CSF γTP levels of 9.7 μg/ml (Fig. 3c) and 9.1 μg/ml were found in two patients with

Table 1
Changes in the proteins detected by one-step cIEF in CSF of patients with neurological disorders (numbers of samples: numbers of patients)

Disease	Types of changes detected							
	I	II	III	IV	V	VI	VII	Total
Cerebral infarction	2:2	1:1		3:3	2:2	1:1		5:4
Meningitis		3:2	2:1	2:2				4:2
Parkinson's disease	1:1							5:5
AD/SDAT	2:2		1:1				2:2	7:7
ALS	1:1							2:2
MS		1:1	2:1			1:1		3:2
GBS	3:1							4:1
Epilepsy	1:1							2:2
Diabetic neuropathy	1:1							3:3
Alcoholism			1:1					2:2
Sequelae of head injury	1:1							2:1
Schizophrenia			1:1					2:2
Depressive illness			1:1					3:3

Abbreviations, see text; I, uniform increase in IgG; II, non-uniform increase in IgG; III, occurrence of doublet asialoTf peaks; IV, appearance of aberrant Tf peaks; V, increase in aberrant IgG; VI, increase in γTP; VII, decrease in βMG. In addition to the data summarized in this table, AGP and βMG from the blood plasma tended to increase in CSF of patients with various diseases causing disturbance in the BBB function (see text).

A. Hiraoka et al. / J. Chromatogr. A 961 (2002) 147–153

153

cerebral infarction and MS which are diseases associated with severe organic damage in the brain tissues, respectively. Therefore, increased γTP in their CSF may be derived from the damaged brain tissues.

The above-mentioned findings detected by the analysis of CSF proteins employing the present one-step cIEF system are summarized in Table 1.

These results indicate that some changes in the human CSF proteins reflect pathological conditions in the CNS in association with neurological disorders, although the serum proteins have poor diagnostic value in such diseases. It is also suggested that one-step cIEF is a powerful technique for detecting pathological changes in proteins in these CSF samples. As described above, one-step cIEF requires far shorter analysis times (ca. 25 min) than the conventional two-step cIEF (more than 1 h [5–7]), and the procedures are also simpler, although both methods can be fully automated. Therefore, one-step cIEF may be suitable for the routine laboratory examination of a large number of CSF samples as an aid in neurological diagnosis.

References

[1] A. Hiraoka, I. Miura, M. Hattori, I. Tominaga, S. Machida, Biol. Pharm. Bull. 16 (1993) 949.
[2] A. Hiraoka, T. Arato, I. Tominaga, A. Anjyo, J. Pharm. Biomed. Anal. 15 (1997) 1257.
[3] A. Hiraoka, T. Arato, I. Tominaga, N. Eguchi, H. Oda, Y. Urade, J. Chromatogr. B 697 (1997) 141.
[4] A. Hiraoka, T. Arato, I. Tominaga, N. Eguchi, H. Oda, Y. Urade, J. Chromatogr. A 802 (1998) 143.
[5] T. Manabe, H. Miyamoto, A. Iwasaki, Electrophoresis 18 (1997) 92.
[6] T. Manabe, A. Iwasaki, H. Miyamoto, Electrophoresis 18 (1997) 1159.
[7] T. Manabe, H. Miyamoto, K. Inoue, M. Nakatsu, M. Arai, Electrophoresis 20 (1999) 3677.
[8] J.R. Mazzeo, I.S. Krull, J. Chromatogr. 606 (1992) 291.
[9] A. Hiraoka, K. Seiki, H. Oda, E. Naomi, Y. Urade, I. Tominaga, K. Hori, Electrophoresis 22 (2000) 3433.
[10] H. Oda, N. Eguchi, Y. Urade, O. Hayaishi, Proc. Jpn. Acad. 72 (1996) 108.
[11] A. Chramph, M.J. Dunn, B.J. Radola (Eds.), Advances in Electrophoresis, Vol. 5, VCH, Weinheim, 1991, p. 241.
[12] A. Hiraoka, K. Seiki, H. Oda, N. Eguchi, Y. Urade, I. Tominaga, T. Arato, J. Anal. Bio-Sci. 23 (2000) 110.
[13] H. Stibler, J. Neurol. Sci. 36 (1978) 273.
[14] A. Siden, G. Kjellin, J. Neurol. Sci. 39 (1978) 131.