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Journal of Chromatography B, 717 (1998) 327–339

JOURNAL OF CHROMATOGRAPHY B

Review

Analysis of compounds containing carboxyl groups in biological fluids by capillary electrophoresis

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Abstract

Capillary electrophoresis (CE) is one of the suitable separation techniques used to analyze drugs or metabolites in complicated sample matrices such as plasma, serum and urine. It sometimes requires only a simple process of sample pretreatment, deproteinization, dilution or extraction for biological fluids, otherwise no pretreatment is necessary. Various metabolic disorders concerning the compounds which possess carboxyl groups such as organic acids have been monitored by CE. Drug metabolism in the body can be monitored by the same technique. Recent publications suggest the feasibility of an automated system for diagnosis based on CE technique. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Reviews; Drug metabolism; Organic acids

Contents

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PII: S0378-4347(98)00290-4

1. Introduction

Capillary electrophoresis (CE) is a powerful technique used to separate a small amount of analytes with a high efficiency in a short time. It requires much simpler sample treatment prior to separation than high-performance liquid chromatography (HPLC), and its instrumental set-up such as an autosampler and an on-line detector is appropriate to design automatic analytical systems. The above characteristics reveal that CE might be suitable for clinical analysis, including applications for biological fluids, i.e., serum, plasma, urine, cerebrospinal fluid (CSF) and dialysate.

We can easily find a couple of reviews of clinical applications with CE in the past few years. Anderson et al. [1] described a general review in clinical chemistry by diverse analytical tools including CE. Reviews of CE in clinical chemistry were published by Lehmann et al. [2], by Jenkins and Guerin [3], by Lehmann et al. [4], and by von Heeren and Thormann [5] and by Jellum [6]. Jellum introduced examples of the clinical applications of CE to routine analysis [6]. The reviews by Holland et al. [7], by Dawson [8] and by Yang et al. [9] also provide useful information on this subject.

When biological fluids are analyzed, the metabolism of drugs or biomolecules in a living body and the diagnosis of metabolic disorders can be investigated. The small sample volume requirement, one of the characteristics of CE, reduces suffering of patients during sample collection, especially, blood samples from neonates and little children, and CSF samples.

We will introduce the recent publications on the analysis of the drugs and biomolecules containing carboxyl groups by CE, focusing on biological fluids as sample matrices in this review.

2. Principles of CE separation of analytes in biological fluids

2.1. Separation modes

Two main separation modes employed for the analyses of drugs or biomolecules containing carboxyl groups are capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC).

In CZE, the most common applied mode in CE, the analytes are separated in a simple electrolyte. Since carboxyl groups are weakly acids, their charges change dependent on pH within the range of 3 and 11 where CE experiments are mostly carried out. The contaminants in biological fluid, e.g., proteins and lipids, may easily and strongly adsorbs to the capillary inner surface. It often causes poor resolution and reproducibility. In these cases, the use of coated capillaries or sample pretreatment to remove the contaminants is recommended.

The MEKC method by using sodium dodecyl sulphate (SDS)-containing electrolytes as a pseudo-solid-phase [10] can be applied to the compounds containing carboxyl groups. The advantage of the application of MEKC to biological fluid, particularly to serum or plasma, is the complexation of protein molecules with SDS micelles. The SDS–protein complexes migrate slower than the original protein molecules due to negative charges of SDS micelles. Accordingly, the disturbance for the peak identification by the contaminant protein peaks is eliminated and reproducible results are obtained by the suppression of protein adsorption to capillary inner surface [11].

2.2. Sample preparation

While biological fluids can be sometimes analyzed with direct sample injection to a separation capillary, sample treatments prior to sample injection were carried out to eliminate the contaminants, mainly proteins. The pretreatments simplify the washing processes of capillaries between electrophoretic runs, improve the reproducibility, and prevent the peak broadening in some cases.

Table 1 summarizes the experimental conditions of CE analyses that are explained in this review. In the third column of the table, sample pretreatment processes are mentioned. When the low-molecular-mass solutes in serum or plasma are analyzed by CE, various problems arise from complicated sample matrices, involving proteins, lipids and ionic compounds. The ingredients frequently cause the insufficient reproducibility, poor resolution, peak broadening, etc. To solve the problems, deproteiniza-

Table 1
Survey of recent publications on CE analyses of –COOH compounds in biological fluids

Analytes	Sample matrix	Sample pretreatment	Separation mode electrolyte	Separation and detection conditions	Ref.
Short-chain mono-, di, tricarboxylic acid					
Oxalate, fumarate, acetate, pyruvate, lactate, glutamate, glutamine	CSF from patients with central nervous system diseases	Ultrafiltration	CZE: (A) 50 m*M* sodium tetraborate, pH 9.2, 2.5% TTAB; (B) 100 m*M* borate, pH 8.3	(A) 60 cm×75 μm I.D., 20 kV, 185 nm; (B) 50 cm×75 μm I.D., 20 kV, 200 nm	[42]
Organic acids	Human urine	SPE	CZE: 50 m*M* borate, pH 10.0, 0.5 m*M* CIA OFM Anion-BT (Waters)	100 cm×75 μm I.D., 20 kV, 25°C, 185 nm	[14]
Oxalate, citrate	Human urine from patients having kidney stones	Acidification followed by dilution	CZE: 10 m*M* chromate, 0.5 m*M* TTAB, pH 8.1	60 cm×75 μm I.D., 25 μA, indirect UV at 254 nm	[20]
Lactate, pyruvate	Human urine from children with metabolic errors	Dilution	CZE: 50 m*M* tetraborate, pH 10, 1 m*M* CIA OFM Anion-BT (Waters), 0.4 m*M* Ca^{2+}	70 cm (60 cm effective length) ×75 μm I.D., −70 μA, 196 nm	[48]
Lactate, citrate	Human seminal plasma	Ultrafiltration	CZE: 10 m*M* chromate, 1 m*M* 5,5-diethylbarbiturate, pH 8.0	40 cm (20 cm effective length) ×50 μm I.D., dynamically coated with hexadimethrine bromide between runs, 30 kV, 254 nm	[18]
2-Ketoisocaproate, citrate, malate, acetoacetate, citramalate, lactate	Human serum from paediatric patients with respiratory insufficiency, in shock, with severe head injury and with multiple myeloma	Direct injection	CZE: 10 m*M* ε-aminocaproic acid–10 m*M* mandelic acid, pH 3.8	60 cm (50 cm effective length) ×75 μm I.D. polyacrylamide-coated capillary, −24 kV, indirect UV at 220 nm	[21]
Methylmalonic acid	Human serum, plasma	Protein precipitation with methanol followed by derivatization with 1-pyrenyldiazomethane and dilution	CZE: 30 m*M* tris–citrate, pH 6.4, 50% DMF, 0.1% HPMC	47 cm (40 cm effective length) ×75 μm I.D. polyacrylamide-coated capillary, −24 kV, 20°C, LIF (ex=325 nm)	[23]
Glyceric acid, methylmalonic acid, mevalonic acid, propionic acid	Human urine from patients with metabolic diseases	Direct injection	CZE: 300 m*M* borate, pH 8.5	64.5 cm (56 cm effective length) ×50 μm I.D., 30 kV, 200 nm	[49,50]
Aromatic carboxylic acid					
Phenylpyruvate, phenyllactate, 2-hydroxyphenylacetate, phenylacetate	Urine, serum from patients with phenylketonuria	Extraction with ethylacetate	CZE: 0.1 *M* ε-aminocaproic acid, 0.1 *M* adipic acid, pH 4.3	36.3 cm (26.3 cm effective length) ×50 μm I.D., 11 kV, 260 nm	[40]
Homovanillic, vanillylmandelic, homogentisic, *p*-hydroxyphenylacetic acid	Urine from patients with neuroblastoma, alkaptonuria and galactosemia	Direct injection	CZE: 300 m*M* borate, pH 8.5	64.5 cm (56 cm effective length) ×50 μm I.D., 30 kV, 200 nm	[51]
Hippuric acid, *p*-hydroxyhippuric acid	Human uremic serum	Ultrafiltration and dilution	CZE: 10 m*M* acetate, pH 3.8 or 10 m*M* MES, pH 6.1	15 cm at pH 3.8 or 25 cm at pH 6.1×200 μm I.D. PTFE, 50 μA at pH 3.8 or 35 μA at pH 6.1, 254 nm	[35]
Hippuric acid	Human uremic serum, hemodialysate	Ultrafiltration	CZE: 150 m*M* borate, pH 9.0	64.0 cm(55.8 cm effective length) ×50 μm I.D., 22 kV, 25°C, 210 nm	[15]
Hippuric acid	Human uremic serum	Ultrafiltration	CZE: 150 m*M* borate, pH 9.0; MEKC: 20 m*M* borate, 80 m*M* SDS, pH 9.0	80.5 cm(72 cm effective length) ×50 μm I.D., 24 kV (CZE) or 22 kV (MEKC), 25°C, 210 nm	[37]

(Cont.)

Table 1. Continued

Analytes	Sample matrix	Sample pretreatment	Separation mode electrolyte	Separation and detection conditions	Ref.
Hippuric acid, *o*-, *m*-, *p*-methylhippuric acid	Human urine from normal and toluene-exposed patients	Protein precipitation by acetone or ultrafiltration, or only dilution	MEKC: 20 m*M* phosphate, pH 8.0, 100 m*M* DTAB, 4 *M* urea	67 cm (47 cm effective length) ×50 μm I.D., −22 kV, 30°C, 224 nm	[38]
Hippuric acid, tryptophan	Human urine	Direct injection	MEKC: 30 m*M* sodium tetraborate, pH 10, 75 m*M* SDS, 10 m*M* β-CD	44 cm (37 cm effective length) ×75 μm I.D., 20 kV, 15°C, 195 nm	[39]
Salicylate	Human serum and urine	Direct, dilution (urine), ultrafiltration (serum)	MEKC: 75 m*M* SDS, borate–phosphate, pH 9.1, CZE: 33 m*M* phosphate, pH 8.3	90 cm (70 cm effective length) ×75 μm I.D., 20 kV, 195 to 320 nm	[12]
Salicylate	Human serum	Direct injection following various washing	MEKC: 60 m*M* borate pH 10.0, 200 m*M* SDS	42.5 cm (34.5 cm effective length) ×50 μm I.D., 10 kV, 25°C, 200 nm	[33]
Naproxen, salicylate	Human serum and urine	Direct injection or dilution for urine	MEKC: borate–phosphate, pH 9.2, 75 m*M* SDS	70 cm (50 cm effective length) ×75 μm I.D., 20 kV, absorbance at 220 nm, fluorescence (ex=220 nm, em=340 nm)	[30]
Naproxen	Human serum	Direct injection, ultrafiltration, extraction	MEKC: borate–phosphate, pH 9.2, 75–200 m*M* SDS	65–75 cm effective length×75 μm I.D., 25 kV, 35°C, 215 nm or 240 nm	[17]
4-Fluorobenzoyl propionic acid, 4-fluorophenyl acetic acid	Guinea pig hepatic microsomal incubation, human urine from a patient treated with haloperidol	SPE	CZE: 50 m*M* ammonium acetate, 10% methanol, 1% glacial acetic acid, pH 4.1	65 cm×50 μm I.D., 30 kV, 25°C, 214 nm	[16]
Heterocyclic N-containing carboxylic acid					
Orotic acid, pyroglutamate, adenylosuccinate	Human urine from patients with metabolic deseases	Direct injection	CZE: 300 m*M* borate, pH 8.5	64.5 cm (56 cm effective length) ×50 μm I.D., 30 kV, 200 nm	[49,50]
Orotic acid	Human urine	SPE followed by centrifuge with a cut-off filter	CZE: 100 m*M* phosphate, pH 3.0	64.5 cm×50 μm I.D. polyvinyl alcohol coated capillary, 20 kV, 35°C, 278 nm	[52]
Cefpiramide	Human plasma	Direct injection	MEKC: phosphate, I=0.05, pH 8, 10 m*M* SDS	75 cm (55 cm effective length) ×50 μm I.D., 15 kV, 37°C, 280 nm	[11]
Cefixime	Human urine	Direct injection	CZE: 50 m*M* phosphate, pH 6.8	75 cm (53 cm effective length) ×50 μm I.D., 20 kV, 295 nm	[55]
Cefotaxime	Human plasma	Direct injection (MEKC) and protein precipitation with acetonitrile (CZE)	MEKC: phosphate, pH 8.0, 165 m*M* SDS, CZE: borate, pH 9.2	57 cm (50 cm effective length) ×75 μm I.D., 15 kV, 25°C, 254 nm	[32]
Flunixin	Equine serum and urine	NaOH hydrolysis, SPE	MEKC: 50 m*M* borate, 2% ethanol, pH 9.2, 75 m*M* SDS	57 cm (50 cm effective length) ×75 μm I.D., 20 kV, 23°C, 286 nm	[13]
Vitamins					
Nicotinic acid and its metabolites	Human plasma	Protein precipitation by acetone	CZE: 10 m*M* borate, pH 9.36	57 cm (51 cm effective length) ×50 μm I.D., 25 kV, 25°C, 254 nm	[36]
Nicotinic acid and its metabolites	Rat urine	Dilution	MEKC: 50 m*M* borate, pH 9.0, 0.15 *M* SDS	72.5 cm (60 cm effective length) ×75 μm I.D., 214 nm, 37°C, 15 kV	[54]
Amino acids					
Phenylalanine	Human serum or plasma	Protein precipitation with ethanol	CZE: 0.025 *M* borate, pH 10	65 cm (45 cm effective length) ×50 μm I.D., 20 kV, 214 nm	[34]
Cysteine, homocysteine, penicillamine, glutathione, adenylsuccinate, taurine	Human serum and urine, heart muscle from human and rat	Protein precipitation with PCA (serum, heart muscle), or filtration (urine). Derivatization with MB or with FMOC	CZE: 50 m*M* phosphate, pH 7.5 or pH 2.5	40 cm×75 or 100 μm I.D., 25 or 20 kV, fluorescence (ex=375 nm or 265 nm, em=480 nm or 305 nm, UV at 214 nm	[25]

Table 1. Continued

Analytes	Sample matrix	Sample pretreatment	Separation mode electrolyte	Separation and detection conditions	Ref.
Hydroxyproline, proline, sarcosine	Human serum and urine	Protein precipitation with acetonitrile (serum) or hydrolysis (urine), followed by derivatization with FMOC or OPA/FMOC	MEKC: 20 m*M* borate, pH 9.2, 25 m*M* SDS	60 cm×50 μm I.D., 22 kV, LIF (ex=248 nm)	[29]
Lysine, threonine, histidine	Human urine	Dilution with NaOH solution	CZE: 0.1 *M* NaOH	80 cm×25 μm I.D., 20 kV, electrochemical detection	[31]
Amino acids	CSF from leukaemic children	Derivatization with FITC, dilution only	MEKC: 100 m*M* SDS, 100 m*M* borate, pH 9.3	75 cm (42 cm effective length) ×50 μm I.D., 20 kV, LIF (ex=488 nm)	[26]
Amino acids	CSF from patients with Alzheimer's disease and children with neurological disorders	Derivatization with CBQCA	MEKC: 50 m*M* borate, pH 9.0, 30 m*M* SDS, 20% DMSO	57 cm (50 cm effective length) ×75 μm I.D., 20 kV, 25°C, LIF (ex=488 nm)	[44]
Glutamate, aspartate, GABA, glycine	Rat brain homogenate	Derivatization with NDA	CZE: 20 m*M* sodium borate, pH 9.0	115 cm (95 cm effective length) ×50 μm I.D., 30 kV, 25°C, 420 nm	[45]
Glutamine	CSF from patients with central nervous system diseases	Ultrafiltration	CZE: 50 m*M* sodium tetraborate, 1 *M* 3-(trimethylammonio)propyl-sulfonate	60 cm×75 μm I.D., 15 kV, 185 nm	[43]
Aspartate, glutamate	Human seminal plasma	Ultrafiltration	CZE: 10 m*M* chromate, 1 m*M* 5,5-diethylbarbiturate, pH 8.0	40 cm (20 cm effective length) ×50 μm I.D., dynamically coated with hexadimethrine bromide between runs, −30 kV, 254 nm	[18]
Selenoamino acids	Human milk	Defatted, protein precipitation, and size-exclusion chromatography	CZE: borate pH 8.5, phophate pH 6.0 and pH 2.5, acetate pH 5.5 and pH 1.9	24 or 50 cm×25 or 50 μm I.D., bare and coated capillary, ±10 kV, 20°C, 200 nm	[53]
Glutamate	Rat brain by microdialysis	Derivatization with FITC	CZE: 20 m*M* carbonate, pH 9.5	30 cm (20 cm effective length) ×12 μm I.D., 21 kV, LIF (ex=488 nm)	[27]
Glutamate, aspartate	Rat brain by microdialysis	On-line derivatization with NDA/CN	CZE: 100 m*M* Tris, pH 8.65	30 cm (14 cm effective length) ×25 μm I.D., −25 to −30 kV, LIF (ex=442 nm)	[28]
Tryptophan, tyrosine, indole derivatives, catecholamine metabolites	Human serum and urine	Direct injection or dilution for urine	MEKC: borate–phosphate, pH 9.2, 75 m*M* SDS	70 cm (50 cm effective length) ×75 μm I.D., 20 kV, absorbance at 220 nm, fluorescence (ex=220 nm, em=340 nm)	[30]
Peptides					
Imidodipeptides (X-Pro, X-hydroxyproline)	Human urine from patients with prolidase deficiency	Treated with activated charcoal and filtered	CZE: 100 m*M* phosphate, pH 2.5	24 cm×25 μm I.D. coated capillary, 7 kV, 20°C, 200 nm	[41]
Vasoactive intestinal peptide (VIP)	Rat brain (cerebral cortex)	SPE followed by RP-HPLC	CZE: 20 m*M* sodium citrate, pH 2.5; MEKC: 10 m*M* sodium phosphate, pH 7.0, 20 m*M* SDS	72 cm×50 μm I.D., 25 kV, 30°C (CZE) or 35°C (MEKC), 200 nm	[46]
Neurotensin	Rat duodenum and adrenal glands	SPE followed by RP-HPLC	CZE: 20 m*M* sodium citrate buffer, pH 2.5, 4.5 or 5.5	72 cm×50 μm I.D., 25 kV, 30°C, 200 nm	[47]

tion is often held utilized for serum and plasma. Serum or plasma is deproteinized by precipitation or ultrafiltration. Perchloric acid (PCA), acetone, acetonitrile or alcohols are generally used as a protein-precipitating agents. Ultrafiltration is a simple deproteinization by the centrifugation. In cases with high concentrations of analytes, sample solutions can be injected directly or only with a simple pretreat-

ment such as dilution or ultrafiltration [12]. However, when the concentrations of solutes of interest are relatively low, below μM levels in UV detection, solute enrichment by extraction is often required [13]. Organic acids are extracted from the sample solution by adding of water-immiscible organic solvent such as ethyl acetate or by solid-phase extraction (SPE) procedures, adsorbing and eluting through a solid-phase C_{18} column.

For urine, sample solution is directly injected more often than serum or plasma, because urine has a sample matrix containing a smaller amount of protein. Ultrafiltration and dilution with buffer are familiar pretreatment methods for urine. When the solute is at the concentration below the limit of detection or when an endogenous substance interferes with the peak of interest, the extraction has been completed.

2.3. Detection and sensitivity

On-line UV absorption is commonly employed as a detection method with CE. Since carboxyl groups absorb the light in the wavelength range below 250 nm, direct UV detection can be commonly achieved. Higher sensitivity is obtained by measuring below 200 nm [14]. When the analyte of interest shows the characteristic absorption spectrum, direct UV detection with photodiode array provides the information for identification [15–17]. A disadvantage of the direct UV is its relatively lower sensitivity. Indirect UV detection is generally more sensitive than direct and universal detection for all kinds of analytes [18–21]. Chromate [18,20] and ε-aminocaproic acid [21] as chromophoric electrolytes have been successfully used to detect organic acids in biological fluids. Electroosmotic flow (EOF) is reversed with tetraalkylammonium salt [20] or hexadimethrine bromide [18] to shorten the analysis time. A neutrally coated capillary is used to suppress EOF instead of an EOF reversing agent [21].

UV detection has the following disadvantages for CE analyses of biological fluids. Firstly, many interfering peaks can be observed due to the complicated sample matrix. Secondly, the detection sensitivity is relatively low (10^{-6}–10^{-4} M) [22], arising from a short light path of a capillary cell. The two problems, poor selectivity and low sensitivity, may be improved when the fluorescence detection is applied to CE. Derivatization with a fluorescent dye to a certain functional group of the analyte of interest can visualize only the analyte and much reduce interference from the contaminant. Since few chemical modifications for carboxyl groups in aqueous solution have been reported, CE analysis with fluorescence detection has been rarely used for the products labeled through their carboxyl groups with fluorescent probes. One of the successful approaches is the determination of methylmalonic acid in human serum labeled with 1-pyrenyldiazomethane [23]. Stable and highly fluorescent 1-pyrenylmethyl monoesters were formed from methylmalonic acid and other short-chain dicarboxylic acids. If the analytes contain other functional groups, i.e., amino groups, the derivatization can be readily applied. Issaq and Chan [24] reported several derivatization reactions by attaching to reactive amino groups. Monobromobimane (MB) reacts with thiol groups forming fluorescent derivatives [25]. This modification reaction has been applied for sulfur-containing amino acids.

Fluorescently labeled analytes can be detected with a highly sensitive laser-induced fluorescence (LIF) detection mode. An argon ion laser (λ_{ex}=488 nm) was used as a light source for amino acid derivatives labeled with fluorescein isothiocyanate (FITC) [26,27]. 1-Pyrenyldiazomethane-labeled methylmalonic acid [23] and naphthalene-2,3-dicarboxyaldehyde (NDA)-labeled amino acids [28] were detected with a He–Cd laser, which excited fluorophores at 325 nm and 442 nm, respectively. A compact Kr–F excimer laser (λ_{ex}=248 nm) is suitable to detect the products of secondary amino acids labeled with *o*-phthalaldehyde (OPA)/9-fluorenylmethyl chloroformate (FMOC) [29]. The limits of LIF detection for biological fluids are reported to be much less than 10^{-6} mol/l. The non-laser fluorescence detector was used for MB-labeled derivatives [25].

Caslavska et al. [30] designed a CE system with simultaneous fluorescence and absorbance detection, modified a conventional tunable UV–Vis absorbance detector. Examples were shown to monitor indole derivatives (tryptophan, 5-hydroxytryptophan, tyrosine, 3-indoxyl sulfate and 5-hydroxyindole-3-

acetic acid) and catecholamine metabolites (homovanillic acid and vanillylmandelic acid) in urine, as well as some drugs, naproxen, quinidine, salicylate and their metabolites in urine or serum, by using the direct sample injection. Native fluorescence detection showed increased selectivity and for certain compounds increased sensitivity [30].

Electrochemical detection has a high sensitivity usually down to the femtomole level over a wide variety of analytes. CE could profile important metabolites including amino acids, creatine and uric acid in urine, with electrochemical detection [31].

Mass spectroscopy (MS) is a developing detection method for CE. Although MS is remarkably powerful to identify the substance, the reports concerning CE–MS for biological fluids can be seldom found. Tomlinson et al. [16] introduced in vivo study of drug metabolism using CE–MS.

2.4. Reproducibility and quantification

Reproducibilities of migration times with biological samples are often poor with direct sample injection, resulting from the complicated matrix. Among numerous interfering substances, proteins tend to adsorb to capillary surface. Consequently, the EOF velocity changes and reproducibility becomes worse. The other feasible effect of proteins to the separation of ionic small molecules is the interaction between proteins and small molecules. Deproteinization solves the problems in both cases. High ionic strength of intact serum or plasma occasionally induces the abnormal peak shape with direct sample injection [17,32], worsening the limit of detection. The dilution of the sample solution with water reduces this phenomenon due to the concentration effect of lowering the conductivity [32].

Direct sample injection, however, is convenient and favorable for clinical use, especially to design an automatic clinical-purpose instrument. Some investigators reported reasonable reproducibility with direct sample injection of biological fluids in MEKC mode by devising the washing process inside the capillary between runs. Schmutz and Thormann [17] examined the performance and the reproducibility of 50 μg/ml naproxen in serum with direct sample injection. The results were obtained that relative standard deviations (R.S.D.s) for migration times and peak areas were below 1% and 3.5%, respectively, even under conditions where the migration times were shorter than 2 min. The R.S.D. values of migration times of cefotaxime and deacetylcefotaxime in human plasma were determined to be below 1% and relative migration times to internal standard showed the improvement of deviations as below 0.4% by Penalvo et al. [32]. In both of the publications, the capillary was washed with 0.1 *M* NaOH and then SDS-containing running buffer prior to sample injection between each run for MEKC [17,32]. In CZE, a rinsing process with 0.1 *M* NaOH for 2 min followed by a 5 min buffer wash brought satisfactory R.S.D.s of the migration times, typically better than 1% [32]. Kunkel et al. [33] made an effort to achieve reproducible and reliable quantification of drugs including salicylate, even with direct sample injection of human plasma. Intra- and inter-day precision values of relative peak areas of about 1–2% R.S.D. ($n=20$) and 2–3% R.S.D. ($n>80$), respectively, were obtained using SDS-containing borate buffer at pH 10 and acetonitrile (50:50, v/v) as a between-run rinsing solution.

For deproteinized samples, good satisfactory reproducibility and quantitative precision were shown in the SDS-free electrolytes, CZE mode. The CZE analysis of phenylalanine reveals the intra-day R.S.D. of the migration time to be 0.41% [34]. In this series of measurements, sufficient accuracy and precision were obtained with intra-day and inter-day variation lower than 4% and 7%, respectively. The R.S.D. values of migration times and peak areas were 0.7% and 6%, respectively, for hippuric acid and *p*-hydroxyhippuric acid in ultrafiltrated serum samples, with the similar accuracy and precision of the quantification to HPLC gradient-elution method [35]. For human plasma deproteinized upon the addition of acetone, the precision of nicotinic acid in the CZE is less than 10% of R.S.D. values in the concentration range between 2 and 12 μg/ml [36].

For diluted urine, the R.S.D. values of quantitative precision were reported to be 1.2–5.6% for oxalate and citrate by Holmes [20]. The recovery of added oxalate ranged from 94% to 101%. The author remarked that acidification of urine sample prior to dilution improves the broadening of the peak and the quantitative precision of oxalate.

3. CE for diagnosis of metabolic disorders

3.1. Metabolic disorders in kidney function

Uremia represents the re-adsorption of many nitrogenous compounds into the tissues and blood, normally excreted in the urine. When uremic serum or hemodialysate is analyzed, some organic metabolite levels are elevated, including organic acids, creatine, urea, phenols, etc. [15]. Several organic acids containing carboxyl groups such as hippuric acid are found in uremic sera.

Schoots et al. [35] reported CE separation and quantification with UV detection for hippuric acid, *p*-hydroxyhippuric acid and uric acid in the blood serum of chronic renal-failure patients [35]. Prior to CE analysis, the samples were deproteinized by ultrafiltration and diluted. The solutes of interest could be analyzed in CZE mode at pH 6. While accuracy and precision of this method were comparable with HPLC, the analysis time by CE was much shorter than that by HPLC. Petucci et al. [15] achieved similar experiments but at pH 9, for serum samples from healthy and uremic individuals. The electrophoresis at pH 9 enabled to extend a variety of target molecules in a short time less than 16 min. More than 19 low-molecular-mass anionic metabolites could be identified, including hippuric acid, kynurenic acid, indole-3-acetic acid, 4-hydroxyphenylacetic acid, nicotinic acid, 2-hydroxyhippuric acid, tyrosine and tryptophan. A few of the metabolite levels, e.g., hippuric acid were significantly elevated in uremic patients. Separation in MEKC mode with SDS-containing electrolyte was published by Tran et al. [37].

The conditions of CE separation in MEKC mode of hippuric and *o*-, *m*-, *p*-methylhippuric acid have been optimized by changing pH, the species and concentration of micelles, and adding urea in electrolyte [38]. A precise analysis with acceptable sensitivity (detection limit of 1.5~2.5 μg/ml) and reproducibility could be done for human urine following occupational exposure to toluene and xylene. The UV-absorbing constituents in human normal urine including hippuric acid and tryptophan were analyzed by CE in MEKC mode which provided better resolution and selectivity than CZE mode [39]. Several separation factors were optimized, i.e., pH, applied voltage, SDS concentration, temperature, β-cyclodextrin (β-CD) concentration and organic modifier concentration. Under the optimum conditions, 70 peaks were observed within 12 min.

Analyses of urinary oxalate and citrate are expected for clinical diagnosis of patients with calcium oxalate nephrolithiasis. However, the CE separation of oxalate is difficult, because high concentrations of other small anions interfere with the detection of oxalate. The acidification to pH below 2.5 followed by dilution before CE analysis provided sufficient electrophoretic determination of oxalate and citrate in the electrolyte consisting of chromate and tetradecylammonium bromide at pH 8.1 with indirect UV detection [20]. This procedure could be applied to urine samples from 108 stone-forming patients [20].

3.2. Analysis of carboxyl-containing compounds in metabolism of amino acids

Phenylketonuria is an inborn genetic disease with metabolic disorder of phenylalanine. In urine of the patient of phenylketonuria, pathological metabolites of phenylalanine have been found at higher concentrations than in healthy individuals. Dolník [40] realized the separation of the metabolites, phenylpyruvate, 2-hydroxyphenylacetate, phenyllactate and phenylacetate, in extracted urine and serum samples with the CZE buffer at pH 4.3. The significant difference is recognized in the levels of these metabolites between phenylketonuria and healthy individuals. Tagliaro et al. [34] suggested the feasibility of an easy pediatric diagnosis of phenylketonuria to monitor the content of phenylalanine in serum or plasma. Borate buffer at pH 10 was used for the separation of phenylalanine both from tryptophan and tyrosine.

A quick and simple tool could be offered by CE analysis of sulphur-containing amino acids in urine and in red blood cells labeled with MB by fluorescence detection to diagnose the human diseases of homocystinuria, cystinuria and glutathione synthetase deficiency. This method was proved to be applicable to determine the content of taurine, an aminosulphonic acid, in biopsies of heart muscle by Jellum et al. [25].

Chan et al. [29] explained that the combination of

MEKC and LIF enables the rapid and sensitive detection of hydroxyproline in serum and hydrolyzed urine. Hydroxyproline in biological fluids was derivatized with FMOC. Zanaboni et al. [41] used CE to monitor imidodipeptides containing C-terminal prolyl or hydroxyprolyl residues excreted into the urine of patients with prolidase-deficiency. The pattern of the dipeptide excretion was heterogenous for the patients with different clinical severities.

3.3. Metabolite levels in CSF and its related organs

Hiraoka et al. [42] investigated the levels of organic acids in CSF. The contents of lactate and pyruvate and the ratios of lactate to pyruvate were elevated in patients with cerebral infarction and bacterial meningitis. They also examined the unusual contents of amino acids and proteins in CSF from patients with a variety of central nervous system (total number of patients is 74) by CE [43]. Although a minor protein peak with the relative molecular mass (M_r) range of 10 000–30 000, assumed to be a β-trace protein, was recognized in all CSF samples, an increase of glutamine was found in hepatic encephalitis in the M_r range below 10 000.

Free amino acid levels in CSF reflect the conditions of patients with central nervous system disorders or metabolic diseases, including chronic schizophrenia, multiple sclerosis, Parkinson's syndrome and Alzheimer's diseases. Nouadje et al. [26] applied CE–LIF in a quantitative analysis of amino acids labeled with FITC in CSF from acute lymphoblastic leukaemic children treated with high dose of methotrexate. While 21 peaks were identified, 19 of them could be determined quantitatively. Bergquist et al. [44] attempted a sensitive and rapid analysis of amino acids labeled by 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde (CBQCA) in CSF using LIF–CE. Differences in amino acid levels were clearly observed among controls, patients with Alzheimer's diseases, and children with different neurological disorders. The amino acid neurotransmitters, glutamate, aspartate, γ-aminobutyric acid (GABA) and glycine, and several other amino acids in rat brain homogenate were separated and detected by CE with UV–Vis detection at 420 nm, labeled with NDA by Weber et al. [45]. A large number of theoretical plates around 400 000 and a low limit of detection in the 3–10 μ*M* range were obtained.

Soucheleau and Denoroy [46] determined a neuropeptide in rat cerebral cortex by CE with UV detection. Vasoactive intestinal peptide, a 28-amino acid polypeptide was identified both by CZE at different pH values and by MEKC. A SPE of tissue homogenate followed by reversed-phase (RP) HPLC was performed prior to CE separation. A similar method was applied to detect neurotensin, a 13-amino acid polypeptide, in rat duodenum and adrenal glands [47].

3.4. Monitoring of other disorders of organic acid metabolism

Lactate, pyruvate and other organic acids in urine could be determined by Willetts et al. [48]. Only by the dilution of urine from children with metabolic disorders prior to sample injection, abnormal levels in organic acids can be monitored.

Organic acids in serum of children could be analyzed with indirect UV detection. Serum samples were injected into a coated capillary without any deproteinization processes and analytes of interest were migrated and detected within 12 min [21]. Profiles in organic acids, i.e., lactate, citrate, malate and acetoacetate, were different among paediatric patients with duodenal ulcer, respiratory insufficiency, severe head injury and multiple trauma.

Jellum et al. [49] analyzed organic acids such as glyceric acid, methylmalonic acid, mevalonic acid and orotic acid in urine samples from patients with glyceric aciduria, methylmalonic aciduria, mevalonic aciduria and lysinuric protein intolerance, respectively. Abnormal metabolites were observed, especially organic acids, in urine samples from patients by the same group, i.e., orotic acid for hyperornithinemia–hyperammonemia–homocitrullinuria (HHH) syndrome, pyroglutamate for pyroglutamic aciduria, adenylosuccinate for adenylosuccinase deficiency, and propionate for propionic acidemia [50]. They also successfully detected the abnormally elevated levels of vanillylmandelic and homovanillic acids, homogentisic acid and *p*-hydroxyphenyllactate in urine from patients with neuroblastoma, alkaptonuria and galactosemia, respectively [51]. From the results with direct sample injection and photodiode array

detection, they proposed a routine screening system for diagnosis of metabolic disorders.

The concentration of methylmalonic acid in serum or in urine is a marker for cobalamin deficiency. Methylmalonic acid in deproteinized human plasma or serum was fluorescently labeled with 1-pyrenyldiazomethane and analyzed by CE–LIF [23]. A discontinuous buffer system enabled sample stacking in the capillary. The assay showed linearity in the methylmalonic acid concentration range of 0.12–200 μmol/l. The quantitative results obtained by CE reasonably agreed with those by the two established GC–MS methods.

Orotic acid is an intermediate in the biosynthesis of pyrimidines and its level elevates in several metabolic diseases. Franke and Nuttall [52] designed the quantitative assay of orotic acid in urine with sample pretreatment of SPE followed by cut-off filter centrifuge. The quantitative range was estimated to be 5–500 μmol/l. Jellum and co-workers [49,50] reported the experimental results without sample pretreatment as already mentioned.

3.5. Other miscellaneous purposes

Shirao et al. [14] described CE separation of organic acids in human urine from healthy individuals. Oxalate, formate, malonate, fumarate, succinate, α-ketoglutarate, citrate, acetate, pyruvate, lactate, isovalerate and hippuric acid were separated with the electrolyte at pH 10.0 in the presence of cationic surfactant as an EOF modifier.

Oefner [18] proposed a buffered electrolyte for indirect UV detection of anionic compounds in biological fluids to improve reproducibility of migration time. Organic anions in human seminal plasma including citrate, carbonate, lactate, aspartate and glutamate were determined in a minute.

Selenium is an essential trace element for humans. Newborns show very low serum selenium levels. Estimation of selenoamino acids in human milk is important to evaluate the bioavailability of selenium. The identification of selenoamino acids poses a problem caused by shifts of migration times, because ionic compositions differ among the samples. The problem was overcome by the procedures that two CZE separation methods for each analyte were applied and migration times were normalized by the addition of analyte standards to the samples [53].

4. Metabolism of drugs containing carboxyl groups

After the administration of a drug, it is adsorbed initially and distributed throughout the body. The drug is transformed into its derivatives by the catalysis of the enzyme in human body. Since various metabolites have different pharmacological and toxicological effects each other, the monitoring of metabolite levels in biological fluids is clinically important for diagnosis and treatment. The CE technique is expected to be a valuable tool for such a kind of monitoring, because it may provide a rapid and automatic analytical system with a simple pretreatment of sample solution.

Nicotinic acid is widely used for therapy of hyperlipaemia. Zarzycki et al. [36] investigated separation conditions of nicotinic acid from nicotinamide, nicotinamide *N*-oxide, *N′*-methylnicotinamide, 6-hydroxynicotinic acid and nicotinuric acid by CE. This method in CZE mode could be applied to deproteinized plasma samples. Nicotinic acid, nicotinamide and their six possible metabolites were separated in MEKC mode by Iwaki et al. [54]. They studied pharmacokinetics in rat urine after oral and intravenous administration of nicotinic acid and nicotinamide.

Nakagawa et al. [11] reported that cefpiramide, a cephalosporin antibiotic, in human plasma was determined by using SDS-containing buffer in MEKC mode. Though direct sample injection without any sample pretreatment was applied, reproducible results were obtained by the addition of internal standard and the suitable choice of cleaning solution. The CE separation of another cephalosporin antibiotic, cefixime and its metabolites was examined by Honda et al. [55]. Better separation was observed in CZE mode rather than in MEKC. Spiked cefixime in human intact urine samples could be determined in phosphate buffer at pH 6.8 without any additives.

Cefotaxime and its metabolite, deacetylcefotaxime, in human plasma could be well determined by the addition of theobromine as an internal standard [32]. The separation electrolyte consists of phosphate buffer at pH 8.0 containing 165 m*M* SDS. Despite the distortion or/and broadening of the peak was observed with direct sample injection of plasma, the dilution with water at the ratio of 3:1 or 3:2 (plasma–water, v/v) prior to sample injection im-

proved the peak shape and gave the detection limit of 1 mg/l.

Salicylate, widely used as a keratolytic, antiseptic or antifungal drug, in biological fluids was analyzed by certain research groups. Thormann et al. [12] compared three CE methods, CZE, MEKC and capillary isotachophoresis in order to examine the suitability of these techniques for the rapid screening and confirmation of drugs in serum and urine. Salicylate could be identified with all of the three methods by comparing the absorbance spectrum of the peak with that of a model compound. Simultaneous monitoring of UV absorption and fluorescence was applied to detect salicylate and its metabolites in human urine and serum from a patient with salicylate intoxication [30]. Valuable information was provided for the evaluation of intoxications, with the sample dilution before injection for urine and with direct sample injection for serum.

Naproxen is an anti-inflammatory, analgesic and anti-pyretic drug. Schmutz and Thormann [17] presented a high-speed MEKC separations with direct sample injection of serum containing 50 μg/ml of naproxen. By shortening the run time from 15 min to 3.5 min, no loss in reproducibility was observed.

Metabolism of haloperidol, a neuroleptic drug, was studied by using CE with photodiode array detector and MS to identify its metabolites. Identification and structure elucidation of the metabolites were shown both in guinea pig hepatic microsomal incubation and in urine from a patient treated with haloperidol [16].

Flunixin, 3-pyridine-carboxylic acid 2-[(2-methyl-3-trifluoromethyl) phenyl] amine, is an anti-inflammatory drug and widely used in horses as a therapeutic medicine or as a doping agent. Flunixin in equine urine and serum was quantitated by CE [13]. With SPE prior to CE separation, low limits of detection were achieved, which were 3.4 ng/ml and 16.9 ng/ml for serum and urine, respectively.

5. Combination of microdialysis and CE

Microdialysis is a useful technique for in vivo monitoring of metabolites in biological fluids collected from certain organs or tissues, e.g., brain, of free moving animals [28]. For microdialysis, a microdialysis probe consisting of a semi-permeable membrane and tubing is inserted to the tissue of interest and the perfusate is pumped through the probe. The metabolites can be followed to analyze collected dialysate. CE is suitable to combine with microdialysis for metabolite analysis, because it requires a small sample volume with a short analysis time and high separation efficiency. Microdialysis is often applied to brain study. Glutamate is known to be released and taken up by specialized neurons and is thought to play an important role in learning and memory. The problem to detect neurotransmitters such as glutamate and aspartate is insufficient sensitivity of UV detection, above 1 μmol/l. Derivatization with a fluorescent reagent and LIF detection solve this problem. Hernandez et al. [27] showed the CE–LIF detection of glutamate in brain dialysate labeled with FITC. The analysis takes 3 min or less. An acute injection of haloperidol decreased glutamate in the dialysate.

Zhou et al. [28] designed continuous in vivo monitoring of aspartate and glutamate in rat brain, an on-line coupling of microdialysis sampling with CE–LIF analysis. Target molecules were on-line derivatized with NDA. Microperfusion rate, derivatization parameters and separation conditions were optimized. Near-real-time analysis of glutamate and aspartate with a temporal resolution of less than 2 min was performed. Detection limits for glutamate and aspartate were 0.1 μmol/l. For an anesthetized rat, it was observed by this technique that concentrations of glutamate and aspartate in dialysate increased after stimulation of brain with high concentration of K^+.

6. Conclusions

When the low-molecular-mass solutes, i.e., organic acids and amino acids, in biological fluids are analyzed by CE, high contents of interfering admixtures such as proteins, lipids and small ionic compounds often cause the problems of insufficient reproducibility, poor resolution and peak broadening. However, the problems have been overcome with a simple process of sample pretreatment or even without any pretreatment, by the optimization of the separation conditions and rinsing processes. If the

detection sensitivity is not enough, sample extraction or derivatization with fluorescent chromophores can be an effective and alternative approach.

The CE separations of the compounds containing carboxyl groups in serum, plasma, urine and cerebrospinal fluid provide useful information for clinical diagnosis. Organic acids, amino acids and their derivatives show the abnormal levels in biological fluids from patients with several metabolic diseases including neurological disorders, organic acidurias, phenylketonuria, uremia and cobalamin deficiency.

The monitoring of a drug and its metabolites in a body after administration is an important process for treatment of patients. CE is suitable for the purpose, since it is performed in a short analysis time with a small sample volume.

The combination of microdialysis and CE has been recently attempted. An instrumental design involving on-line derivatization enables near-real-time analysis of metabolites in a living body.

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