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Capillary Electrophoresis and Capillary Electrophoresis–Mass Spectrometry in Catecholamine Studies

Katariina Vuorensola

Academic Dissertation

To be presented with the permission of the Faculty of Science of the University of Helsinki for public criticism in Auditorium A129 of the Kumpula Chemistry Building on November 8th, 2002, at 12 noon.

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PREFACE

This thesis is based on research begun in 1999 and completed in 2002 in the Laboratory of Analytical Chemistry of the Department of Chemistry, University of Helsinki. The experimental work was done in two laboratories: at VTT Processes, Technical Research Centre of Finland (during 1999-2001), and at Viikki Drug Discovery Technology Center, Department of Pharmacy, University of Helsinki (during 2001-2002). The project was carried out in co-operation with HUCH Laboratory Diagnostics, Helsinki University Central Hospital, Finland.

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ABSTRACT

Capillary electrophoresis (CE) methods were developed for the separation of eight structurally similar catecholamines and methoxycatecholamines of clinical importance. The hydrolysis of sulphate and glucuronide conjugates of catecholamines and methoxycatecholamines in urine samples was performed enzymatically with *Helix Pomatia*. The urinary matrix compounds were removed from the samples by solid phase extraction (SPE) based on a copolymer resin of N-divinylpyrrolidone–divinylbenzene.

Dynamic and permanent coatings of fused-silica capillary wall were studied with catecholamines and methoxycatecholamines. Dynamic coating reagents were triethylamine (TEA), morpholine and glycine, and permanent coating was done with ν-metacryloxypropyltrimethoxysilane (MAPT). The performance of the coatings was evaluated in terms of resolution, plate numbers and electrophoretic mobilities of the analytes. Among the dynamic coating reagents, TEA was most effective in increasing separation efficiency and resolution. The best permanent coating was obtained with 30-50% MAPT solution. Dynamic coating was applied in urine sample analysis to increase the resolution between catecholamines, methoxycatecholamines and matrix compounds.

The coupling of CE to an electrospray ionization mass spectrometer (ESI-MS) with a coaxial sheath liquid construction was studied in terms of separation conditions in CE, CE–ESI-MS interface performance and MS parameters. The optimum sheath liquid composition was found to be methanol–water (80:20, v/v) with 0.5% acetic acid as the electrolyte. A flow rate of 6 σl/min provided maximum performance in terms of analyte signal intensity, peak-width at base and resolution. With the carefully optimized conditions, a sensitive method was obtained, which was applied to the determination of dopamine and methoxycatecholamines in urine samples.

Determinations of dopamine and methoxycatecholamines in urine samples with enzymatic hydrolysis, polymer based SPE and CE–UV and CE–ESI-MS analysis were compared with determinations including acid hydrolysis, cation exchange (CEX) extraction and liquid chromatographic (LC) separation with electrochemical (EC) detection. The enzymatic hydrolysis was found to be less effective in deconjugation than was acid hydrolysis. However, SPE purification could not be directly applied to acid hydrolysed samples because of the high ionic strength of the samples, leading to low recovery of the internal standard. Furthermore, the SPE method, which was developed for CE, could not be used with LC and, vice versa, the CEX method developed for LC could not be applied to CE. LC–EC analysis was more sensitive than CE–UV and CE–ESI-MS analyses owing to the higher injection volume and more sensitive detector for the catechol structure. Both CE–UV and CE–ESI-MS analyses were nevertheless sensitive enough for the analysis of dopamine and methoxycatecholamines in urine of healthy persons.

The effect of the electrolyte solvent in catecholamine and methoxycatecholamine analysis was studied, as water was replaced by non-aqueous solvents methanol, ethanol and 1-propanol. The effects of the different alcohols were evaluated with the UV and MS detectors in terms of electrophoretic and electroosmotic mobilities, resolution, separation efficiency, diffusion, and sensitivity. The electrophoretic mobilities of the analytes were decreased from water to 1-propanol owing to reduced dissociation, increased hydrodynamic size and increase in solvent viscosity. The electroosmotic mobility was also decreased. Best resolution between catecholamines and methoxycatecholamines in the non-aqueous solvents was obtained in ethanol-based solution. Separation efficiency was higher in all the non-aqueous solvents than in water. The solvents did not induce major changes in sensitivity with UV or ESI-MS detections. The higher the surface tension and viscosity of the solvent used in the CE electrolyte solution, the higher the ESI voltage needed to initiate the electrospray process with the sheathless nanospray coupling of CE and MS.

ABBREVIATIONS

A	adrenaline
CE	capillary electrophoresis
CEX	cation exchange
CZE	capillary zone electrophoresis
DA	dopamine
DHBA	3,4-dihydroxybenzylamine
EC	electrochemical detector
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene-bis-(oxyethylenenitrilo)tetraacetic acid
EOF	electroosmotic flow
ESI	electrospray ionization
HMBA	4-hydroxy-3-methoxybenzylamine
i.d.	internal diameter
IS	internal standard
LC	liquid chromatography
LLE	liquid-liquid extraction
LOD	limit of detection
MAPT	ν-metacryloxypropyltrimethoxysilane
MN	metanephrine
MS	mass spectrometry
3MT	3-methoxytyramine
NA	noradrenaline
NMN	normetanephrine
o.d.	outer diameter
PBA	diphenylboronic acid
PLRP-S	polymer based sorbent for SPE
PRP-1	polymer based sorbent for SPE
RSD	relative standard deviation (%)
SPE	solid phase extraction
TCA	trichloroacetic acid
TEA	triethylamine
UV	ultraviolet

SYMBOLS

Al_2O_3	alumina
ζ	degree of dissociation
C18	octadecylalkyl
D	diffusion coefficient (cm^2/s)
E	electric field (V/cm)

ξ	viscosity (Ns/m^2)
κ	dielectric constant
HCl	hydrochloric acid
$HClO_4$	perchloric acid
k	Boltzman constant, $1.380658*10^{-23}$ J/K
K	Kelvin
ς	wavelength (nm)
L_{det}	detection length (cm)
L_{tot}	total length (cm)
$[M+H]^+$	protonated molecular ion
σ_{act}	actual electrophoretic mobility (m^2/V)
σ_{app}	apparent electrophoretic mobility (m^2/V)
σ_{eff}	effective electrophoretic mobility (m^2/V)
σ_{EOF}	electroosmotic mobility (m^2/V)
m/z	mass to charge ratio
N	plate number
pH*	apparent pH
pK_a	acid constant
ζ_θ	wall potential (mV)
ζ_ς	Stern potential (mV)
r	hydrodynamic radius (nm)
T	absolute temperature, 273.15 K
ζ	zeta potential (mV)

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which will be referred to in the text by their Roman numerals (I-V):

I Vuorensola, K. and Sirén, H. Determination of urinal catecholamines with capillary electrophoresis after solid phase extraction. *J. Chromatogr. A 895* (2000) 317-327.

II Sirén, H. and Vuorensola, K. Separation of catecholamines by capillary electrophoresis using in-run modified surfaces and covalently bonded surface coatings in capillaries. *J. Microcol. Sep. 13* (2001) 126-133.

III Vuorensola, K., Kokkonen, J., Sirén, H. and Ketola, R. A. Optimization of the capillary electrophoretic–electrospray ionization mass spectrometric analysis of catecholamines. *Electrophoresis 22* (2001) 4347-4354.

IV Vuorensola, K., Sirén, H. and Karjalainen, U. Determination of dopamine and methoxycatecholamines in patient urine by liquid chromatography with electrochemical detection and by capillary electrophoresis coupled with spectrophotometry and mass spectrometry. *J. Chromatogr. B* (2002) submitted.

V Vuorensola, K., Sirén, H., Kostiainen, R. and Kotiaho, T. Analysis of catecholamines by capillary electrophoresis and capillary electrophoresis–nanospray mass spectrometry: Use of aqueous and non-aqueous solutions compared with physical parameters. *J. Chromatogr. A* (2002) in press.

1 INTRODUCTION

Catecholamines are neurotransmitters in the central and peripheral sympathetic nervous system. The diagnosis of diseases like Parkinsonism includes the determination of catecholamines and their metabolites in biological samples. A widely used analytical method for the determination of catecholamines and methoxycatecholamines in urine involves acid hydrolysis of the sulphate and glucuronide conjugates, purification based on cation exchange (CEX) and separation by liquid chromatography (LC) with electrochemical (EC) detection.

Capillary electrophoresis (CE) techniques include capillary zone electrophoresis (CZE), electrokinetic capillary chromatography, capillary isotachophoresis and capillary isoelectric focusing. In CE, the sample is introduced to a narrow fused-silica capillary filled with an electrolyte solution. A high electric field is applied across the capillary, usually with anode at the capillary inlet and cathode at the capillary outlet. Under the electric field, positively charged ions (cations) migrate towards the cathode and negatively charged ions (anions) towards the anode. The negative charge on the wall of the fused-silica capillary gives rise to a flow of the bulk solution. This flow, which is called electroosmotic flow, carries both cations and anions past an on-line installed detector towards the cathode, allowing the analysis of positively and negatively charged analytes in the same run. The separation of analytes under the electric field is based on differences in their sizes and charges. The hydrodynamic size of an analyte can be modified, for example by the choice of the electrolyte solvent, and the charge can be manipulated by adjustment of the pH of the electrolyte solution.

The active sites at the fused-silica capillary wall can be blocked to reduce the analyte–wall interactions and to modify the electroosmotic flow. The coating can be performed dynamically by introducing a coating reagent to the electrolyte solution, or permanently with covalent bond formation between the coating reagent and silanol groups at the capillary wall. The coating reagents may be charged or neutral, and reagents such as amine compounds, surfactants and various polymers have been used in coating procedures.

Usually, CE separations are performed in aqueous electrolyte solutions. Though less often used, organic solvents are of interest because of the different properties they introduce to the separation. The solvent affects the dissociation and hydrodynamic size of the analytes, the electroosmotic flow and the viscosity of the bulk solution. In addition, many organic solvents have lower conductance than water, and, for these, higher electric fields can be applied to the separation, increasing the separation efficiency.

Mass spectrometry (MS) is a highly specific detection method providing structural information and thus reliable identification of analytes. In electrospray ionization (ESI), which is the most frequently applied method of ionization in CE–MS couplings, the analytes are ionized in liquid phase and transferred to gas phase. Ions fragmented from the analytes are separated by their mass and charge in the mass analyser. The fragmentation of the analyte occurs in a specific way

in MS, depending on the molecular structure of the analyte. CE can be interfaced with MS by coaxial sheath liquid, liquid junction and sheathless nanospray couplings.

In this study, a new analytical method for urinary dopamine and methoxycatecholamines was developed, based on enzymatic hydrolysis with *Helix Pomatia*, solid phase extraction (SPE) with a copolymeric sorbent of N-divinylpyrrolidone–divinylbenzene and separation by CE with UV detection. In addition, a CE–MS method with coaxial sheath liquid coupling was developed, allowing sensitive detection of these compounds in patient urine samples. Both dynamic and permanent coatings of the fused-silica capillary were observed to increase the resolution between catecholamines and methoxycatecholamines, and dynamic coating with diisopropylamine was applied to urine sample analysis. CE–UV and CE–MS methods were compared with an existing LC–EC method for the determination of dopamine and methoxycatecholamines in urine. In addition, the effects of different hydrolysis and purification methods on the analysis were studied. Non-aqueous separation conditions in methanol, ethanol and 1-propanol were applied to catecholamine and methoxycatecholamine analysis, and the results were compared with those obtained in aqueous conditions. The analyses were evaluated in terms of electrophoretic mobility, separation efficiency, diffusion and sensitivity. In addition, the effect of organic solvent on the ionization efficiency of the analytes was studied by CE–MS where coupling was achieved with a sheathless nanospray interface.

2 AIMS OF THE STUDY

The aims of the present study were

- to develop a new analytical method for urinary dopamine and methoxycatecholamines based on enzymatic hydrolysis of the conjugates with *Helix Pomatia*, purification by solid phase extraction with a new copolymer of N-divinylpyrrolidone–divinylbenzene and analysis by capillary electrophoresis with UV detection,

- to study the effect of dynamic and permanent coatings of the fused-silica capillary wall on the efficiency and resolution of the separation of catecholamines and methoxycatecholamines by capillary electrophoresis,

- to study the determination of dopamine and methoxycatecholamines by capillary electrophoresis–mass spectrometry using coaxial sheath liquid coupling, and to apply the method to urine samples,

- to compare the concentrations of dopamine and methoxycatecholamines in patient urine samples obtained by the methods developed in this study and by a commonly used liquid chromatographic–electrochemical method including acid hydrolysis and cation exchange purification,

- to develop a non-aqueous separation of catecholamines and methoxycatecholamines using capillary electrophoresis with UV and sheathless nanospray–mass spectrometric detection, and to compare the results, in terms of resolution, efficiency and sensitivity, with those obtained in aqueous separation conditions.

3 CATECHOLAMINES

This chapter describes the biosynthesis of catecholamines and methoxycatecholamines and explains the diagnostic importance of catecholamine and methoxycatecholamine determinations. The methods used in catecholamine and methoxycatecholamine determinations are then reviewed, with attention to the hydrolysis techniques used for the deconjugation of glucuronide and sulphate conjugates, purification and extraction methods for urine sample pretreatment, and conditions for the analysis.

3.1 Background

The catecholamines, dopamine (DA), noradrenaline (NA) and adrenaline (A), are compounds containing a 1,2-dihydroxybenzene nucleus with a side chain containing an amine group. The biosynthesis of catecholamines starts from amino acid tyrosine, which is hydroxylated by tyrosine hydroxylase to form DOPA [1]. By a decarboxylating reaction, DA is formed from DOPA (Figure 1). The introduction of a hydroxyl group to the benzylic carbon of DA yields NA, and finally the methylation of the amine group turns NA into A. The methylation of the 3-hydroxy group of DA, NA and A by catechol O-methyltransferase yields the methoxycatecholamines 3-methoxytyramine (3MT), normetanephrine (NMN) and metanephrine (MN), respectively. In addition to these basic metabolites, the metabolism of catecholamines further yields neutral metabolites like alcohols and aldehydes as well as acidic metabolites [1].

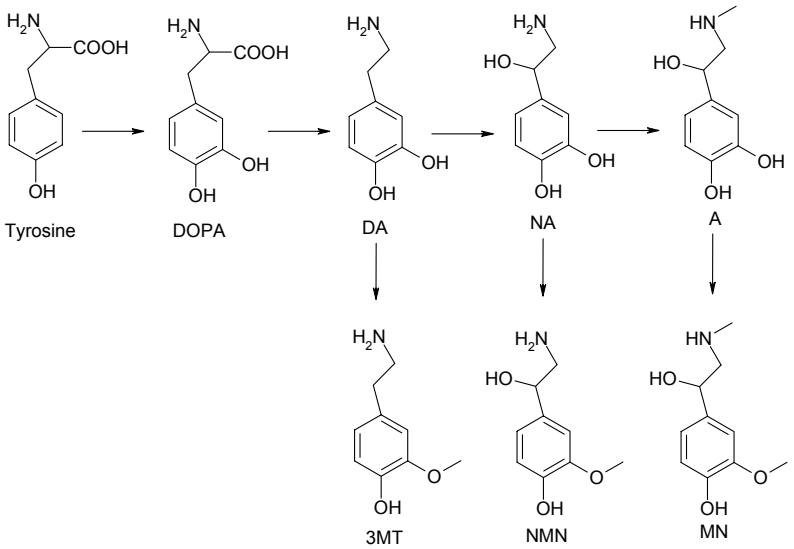


Figure 1. Biosynthesis of catecholamines and methoxycatecholamines.

Conjugation to glucuronic acid and sulphuric acid also takes place during the metabolism of catecholamines and methoxycatecholamines [2]. NMN and MN are mainly excreted as sulphate conjugates and are routinely measured after hydrolysis [3]. The extent of the sulphate conjugation of DA, NA, A, 3MT, NMN and MN in human urine has been reported to be 42-77%, 43-78%, 12-69%, 14-97%, 49-87% and 41-80%, respectively [4-6]; glucuronide conjugates form only a few per cent of the total [7].

DA and NA are neurotransmitters in the central and peripheral sympathetic nervous systems, respectively, and A is an adrenomedullary hormone [1, 2]. Anomalies in the excretion of catecholamines and their metabolites are used in the diagnosis of pheochromocytoma, a catecholamine secreting tumor; common disorders in cardiology, such as essential hypertension; psychiatric disorders such as depression and schizophrenia; and neurological disoders such as Parkinsonism [1]. The determination of total NMN and MN is of particular importance in the diagnosis of pheochromocytoma [1, 3, 8], and DA is sometimes included [9].

3.2 Determination of urinary catecholamines and methoxycatecholamines

In general, there are three steps in the determination of catecholamines and methoxycatecholamines in urine samples: deconjugation of the catecholamines and methoxycatecholamines by hydrolysis, extraction from the urine matrix, and separation and detection.

3.2.1 Hydrolysis of glucuronide and sulphate conjugates

The catecholamines and methoxycatecholamines in living organisms occur conjugated to glucuronic and sulphuric acids. Conjugation enhances the polarity of the molecule, which facilitates excretion. Usually, the conjugates in samples are hydrolysed and the free amines are then analysed. Hydrolysis can be performed enzymatically with η-glucuronidase and arylsulphatase, or chemically with acid or base. Base hydrolysis is rarely applied to catecholamine deconjugation, as the free catecholamines are easily oxidized in alkaline conditions [10]. Acid is efficient in hydrolysing sulphate ester bonds in the conjugates [10] but is not suitable for deconjugation of ether bound glucuronides [11]. Enzymatic hydrolysis is a more specific method than acid hydrolysis, and preparations of η-glucuronidase [11, 12] arylsulphatase [6] and mixtures with both activities [13] are commercially available.

Table 1 outlines the conditions of acid hydrolysis used for the deconjugation of urinary catecholamines and methoxycatecholamines. In addition, some conditions applied for plasma samples are included. Most often the hydrolysis is performed with perchloric or hydrochloric acid at low pH in a boiling water bath for 20-30 minutes. Antioxidants like sodium metabisulphite, ascorbate, EDTA, dithiotreitol and glutathione have been added to the incubation mixture to prevent the free amines from degradation. Without the addition of

Table 1. Conditions for the acid hydrolysis of catecholamine and methoxycatecholamine conjugates.

Matrix	Analytes	Acid	pH, additives	Incubation ∀C/min	Notes	Ref.
Urine	DA, NA	0.1M $HClO_4$	pH 1.5, sodium metabisulphite	100/60	poor recovery	13
Urine	DA, NA	0.1M $HClO_4$	sodium metabisulphite, dithiotreitol	lyophiliz./180	higher results than with enz. hydrol.	13
Urine	DA, NA, A	0.4N $HClO_4$	EDTA, sodium metabisulphite	100/120	hydrol. has no effect on stds, maximal recovery after 120 min, hydrol. complete	40
Urine	DA, NA, A, NMN, MN	3M HCl	pH 1.0, EDTA, sodium ascorbate	100/7		4
Urine	DA, NA, A, 3MT, NMN, MN and acidic compounds	1.0M $HClO_4$	EDTA, glutathione	100/20	in the absence of EDTA and glutathione, 7-25% of CA were decomposed	5
Urine	3MT, NMN, MN	-	pH 0.8-1.0	90-100/30		17
Urine	3MT, NMN, MN	-	pH 0.5-1.0	100/20	25% loss of MN during hydrol.	15
Urine	3MT, NMN, MN		pH 1.0	100/30		22
Urine	3MT, NMN, MN	1M HCl		100/30	IS (HMBA) added after hydrol.	20
Urine	3MT, NMN, MN	6N HCl	pH 1.0	100/20		19
Urine	NMN, MN	-	pH 1.0-1.5	100/15	heating beyond 20 min depres results	14
Urine	NMN, MN	HCl (conc.)	pH 1.0	100/30		21
Urine	NMN, MN	0.6M HCl	1:1 (v/v) with sample	100/30		41
Plasma	DA, NA, A	5N $HClO_4$		98/10	hydrol. complete in 10 min; heating for >10 min results in severe loss of stds	11
Plasma	DA, NA, A	3M $HClO_4$	dithiothreitol, sodium metabisulphite	100/15	quantitative recovery from sulphates	50
Plasma	DA, NA, A	0.6M $HClO_4$	EGTA	95/10-50	103, 97 and 96% of DA, NA and A were recovered, hydrolysis complete in 30 minutes	51

- not reported, CA:catecholamines

Table 2. Conditions for the enzymatic hydrolysis of catecholamine and methoxycatecholamine conjugates.

Matrix	Analytes	Enzyme source	Enzyme activity /ml sample	Buffers, additives, pH	Incubation ∀C/h	Notes	Ref.
Urine	DA, NA	glusulase	1850 U of arylsulphatase-glucuronidase	sodium metabisulphite, pH 5.6	37/2, 6, 18	lower recovery than in acid lyophilization	13
Urine	3MT, NMN, MN	*Aerobacter aerogenes*	1 U of sulphatase	Tris HCl, EGTA, pH 7.5	37/1	50-75% recovery of added stds, complete hydrol.	6
Urine	NMN, MN	glusulase	glucuronidase and sulphatase	pH 5.5 with acetic acid	37/24	urine desalting prior hydrol., same results as with acid hydrol., recovery of NMN, MN from urine 55-116%	14
Plasma	DA, NA, A	*Aerobacter aerogenes*	0.5-4000 mU of arylsulphatase	Tris HCl, Tyrode buffer, pH 7.4	37/0.25-1	maximum hydrol. with 33mU/ml, 0.75 min	16
Plasma	DA, NA, A	*Aerobacter aerogenes*	200 mU of arylsulphatase	glutathione, EGTA, pH 7.8	-/1	combined enz. hydrol. yields more total NA and A than acid hydrol.	11
Plasma	DA, NA, A	*E. coli*	η-glucuronidase	glutathione, EGTA, pH 7.8	-/-		11
Plasma	DA, NA, A, DOPA	*Aerobacter aerogenes*	250 mU arylsulphatase	glutathione, EGTA	37/1	complete hydrol. in <1h	52
Plasma	DA, NA, A	ketodase (bovine liver)	2000 U η-glucuronidase	ascorbic acid, pH 4.5-5.0	37/16-18	recovery of stds >90%, complete hydrol.	12
Plasma	DA, NA, A		20 σl sulphatase/ml	Tris HCl, pH 7.2	-/1	complete hydrol.	12

- not reported

preservatives, losses in recoveries of the free amines may occur, especially with prolonged heating [14, 15].

Enzyme hydrolysis is more gentle than acid hydrolysis. However, the enzyme activity depends on the electrolyte content of the medium, and therefore the degree of hydrolysis may vary from one sample to another [13]. For example, sulphatase is highly inhibited by the inorganic salts of urine, but desalting by electrodialysis has been reported to effectively abolish the inhibition [14]. In addition, the optimal pH may vary for enzymes obtained from different sources [11, 16]. Table 2 presents the conditions for enzymatic hydrolysis of catecholamines and methoxycatecholamines. As can be seen, sulphatase from *Aerobacter aerogenes* in buffered medium at +37 °C yields complete hydrolysis of the sulphate conjugates within 1-18 hours incubation time.

Acid hydrolysis is fast but non-specific compared to enzymatic hydrolysis. Moreover, the stability of the analytes needs to be considered in the harsh conditions of low pH and high temperature. Enzymatic hydrolysis, in turn, needs more careful optimization of the conditions for the deconjugation to be successful. Since the major part of the catecholamines and methoxycatecholamines in human urine are sulphate conjugated, hydrolysis with acid or arylsulphatase is most often chosen.

3.2.2 Extraction of catecholamines and methoxycatecholamines

Cation exchange (CEX) is one of the most common purification methods for urinary catecholamines and methoxycatecholamines [4, 5, 15, 17-24]. A widely used procedure involves the addition of EDTA to the urine sample, the adjustment of pH to 6.0-6.5 and application of the sample to CEX sorbent. The sorbent is usually washed with water, occasionally also with acid or base [17]. Ammonium hydroxide (4 M) can be used for the elution of methoxycatecholamines [19, 21, 22], but acidic solutions, such as formic acid (4 M) and boric acid (4%), are more effective if catecholamines and methoxycatecholamines are to be eluted from the same sample [4, 5, 17, 18, 21]. The pH of the sample and washing solutions in CEX has been reported to have an effect on the recoveries of catecholamines and methoxycatecholamines [5, 17]. Specificity in extraction has been obtained through optimized pH adjustment [17] and the addition of ammonium pentaborate to urine samples [20], leading to the complexation of catecholamines with the pentaborate. The complex is not retained in the CEX sorbent, allowing the specific elution of methoxycatecholamines from the sample [20]. Recoveries for catecholamines and methoxycatecholamines in CEX purification have been reported to be 73-95% [5, 15, 22].

Acid washed alumina (Al_2O_3) has been used for the purification of urinary catecholamines [23, 25]. In this procedure, the slightly alkaline urine sample is mixed with acidic alumina and centrifuged. The resulting supernatant is the unadsorbed sample, containing also the methoxycatecholamines. Alumina is washed with water and the catecholamines are eluted with acidic solution. The problem with alumina extraction is that it yields varying recoveries and

reproducibilities for catecholamines due to the nonreproducible chemical activation of the Al_2O_3 with hydrochloric acid [26]. EDTA has been added to urine samples to avoid degradation of the catecholamines in the oxidative alkaline conditions needed for their retention on alumina [27].

In liquid-liquid extraction (LLE) of urinary catecholamines, the catecholamines are complexed with diphenylboronic acid (PBA). The diphenylboronate anion is formed in alkaline solution, and compounds, such as catecholamines, containing a vicinal cis-diol group, covalently bind to the diphenylboronate with the concomitant release of water. The polar complexes are exctracted as ion pairs to a non-polar organic solvent, like hexane, with an extraction recovery of 71% [28]. The complexes are degraded in acidic solution to release the free catecholamines. The PBA pretreatment is specific to compounds containing the catechol group, and it has been reported to provide a more rapid purification for catecholamines than extraction with alumina [26]. Tetraphenylboronate has been applied for specific complexation of methoxycatecholamines before diethylether extraction [6]. Neither catecholamines nor their acidic metabolites form complexes with tetraphenylboronate, and are not then extracted into diethylether. Recovery of 50-75% has been reported for methoxycatecholamines with the LLE method and complexation with tetraphenylboronate.

Solid phase extraction (SPE) with silica-based C18 sorbents differing in hydrophobicity, and with polymer-based PLRP-S and PRP-1 sorbents, also had been tested for urinary catecholamine purification [29]. As in LLE, the catecholamines are complexed with diphenylboronate at alkaline pH before extraction. Good recoveries for the catecholamines were obtained with the polymer-based resins, but the performance of silica-based packings was inadequate. In addition, the C18 sorbents from different suppliers have been shown to differ in their extraction efficiencies for catecholamines [30]. Methoxycatecholamines have been purified with C18-based SPE without complexation [31]. Extraction was performed after adjustment of the sample pH to 8. Better recoveries have been reported with SPE than with CEX or LLE with ethylacetate. In the on-line SPE purification with LC, the filtered urine samples were directly injected into LC, and column switching was utilized to exclude interfering and contaminating matrix compounds from the analytical C18-column [32, 33]. In addition to C18-precolumns [33], precolumns based on size exclusion [32] and restricted access affinity [34] have been studied.

Different methods of extraction have been combined to increase the efficiency of the purification. The combination of SPE on C18, silica and alumina, exploiting nonpolar, polar and adsorption interactions, respectively, resulted in total recovery of 40-50% of catecholamines [35]. Also, a combination of CEX and alumina purifications has been reported [36], and a combination of cation and anion exchange extractions [15]. A combination of CEX extraction and PBA complexation yielded recoveries above 90% for the catecholamines [37]. The strong CEX retained cationic species including catecholamines and their basic metabolites, and the more selective and efficient PBA in its ionized form selectively retained the catecholamines, while other polar and non-polar molecules were washed away. Both sorbents

can withstand methanol, so the nonpolar components could be removed without affecting the recovery of catecholamines. LLE with ethylacetate–acetone has been employed as an additional purification after CEX [21, 22, 38]. For chromatographic analysis of catecholamines with UV detection, urinary pigments have been extracted with toluene–isoamyl alcohol before CEX purification [22]. Wu and Gornet [39] compared four sorbents for the purification of urinary catecholamines: namely, acid washed alumina, CEX, CEX–alumina and PBA complexation. Chromatographic interferences were observed for the alumina and CEX extractions, while minimal interference due to the matrix was observed with CEX–alumina method and PBA complexation.

3.2.3 Analytical methods based on chromatography

Separation by liquid chromatography (LC) with electrochemical (EC) detection is a popular method for the determination of urinary catecholamines and methoxycatecholamines [4, 6, 15, 17, 19, 20, 21, 24, 29, 35-37, 40, 41]. Both amperometric [24, 26, 39] and coulometric [20, 23, 36, 41] detections are employed. Separations are performed with reversed phase C18 columns with ion-pair reagents in the mobile phase. 1-Heptanesulphonic acid [4, 6, 24, 29, 35-37] and 1-octanesulphonic acid [15, 17, 19, 20, 21, 40] are widely used as the ion-pair reagent. The mobile phase for an isocratic run usually consists of phosphoric acid and EDTA, with methanol or acetonitrile as the organic modifier [4, 19, 24, 29, 36, 37, 40]. In addition to ion-pair chromatography, ion chromatographic separation with EC detection has been reported [39, 42]. The use of an ion chromatographic column removes the need for the ion-pair reagent used with reversed phase columns, allowing lower back pressures and shorter equilibration times of the column [39]. Detection limits for catecholamines and methoxycatecholamines with EC detection have been reported to be 1-250 nmol/l [20, 40]. 3,4-Dihydroxybenzylamine (DHBA) and 4-hydroxy-3-methoxybenzylamine (HMBA) have traditionally been employed as internal standards for catecholamines and methoxycatecholamines, respectively [15, 17].

In addition to EC, fluorescence detection is suitable for the analysis of urinary catecholamines and methoxycatecholamines. Where sensitivity was important, hexacyanoferrate treatment followed by trihydroxyindole derivatization of catecholamines was employed [32, 33]. Also, postcolumn reaction with 1,2-diphenylethylenediamine [5], 1,2-diethylenediamine [23] or 9-fluorenylmethyloxycarbonyl chloride [43] has been applied to achieve limits of detection ranging from 0.3 to 13 nmol/l [5, 33]. The use of UV detection at 280 nm with LC separation of methoxycatecholamines requires the removal of urinary pigments from the sample, but advantages of UV detection over EC detection are faster establishment of baseline and insensitivity to changes in flow rate, temperature and mobile phase composition [22]. Detection limits with UV are reported to be 6-10 σmol/l [22].

Urinary catecholamines have been determined by LC–MS with ionspray ionization using deuterated analytes as internal standards [28]. Atmospheric pressure chemical ionization has been utilized in the identification of methoxycatecholamines [18] and their 9-fluorenylmethyloxycarbonyl derivatives [43]. Hexafluorobutyric acid has been observed to be

an ESI-MS compatible ion pair reagent in the LC–MS analysis of NA [44]. Gas chromatography–mass spectrometry has been applied to catecholamine and methoxycatecholamine determination after derivatization with pentafluoropropionic anhydride [31, 45] or trifluoroacetic anhydride [9] to increase their volatility. Chemical ionization was utilized with deuterated catecholamines and methoxycatecholamines as the internal standards.

The separation of urinary catecholamines and methoxycatecholamines has recently been investigated by CE with UV [46], EC [47] and luminescence [48] detection. The dual-electrode amperometric detection was of high specificity resulting after injection of filtered and diluted urine samples directly into CE in a highly sensitive analysis with detection limits of 0.1-0.4 nmol/l [47]. Methods other than chromatographic ones include also spectrofluorometry after oxidation with ferricyanide [14] or iodine [14, 25]. An enzyme immunoassay determination for urinary methoxycatecholamines has also been reported [49].

4 CAPILLARY ZONE ELECTROPHORESIS

The basic theory behind capillary zone electrophoresis is now briefly presented. The dynamic and permanent fused-silica capillary coating procedures employed to enhance separation efficiency are discussed, and the three coupling methods – coaxial sheath liquid, liquid junction and nanospray – for interfacing capillary electrophoresis with mass spectrometry are described, in the context of factors influencing the electrospray ionization process. Finally, the benefits of non-aqueous solvents as separation media in capillary electrophoresis are presented.

4.1 Theory of capillary zone electrophoresis

In capillary zone electrophoresis, separation is based on an ion's electrophoretic mobility, which is a function of the charge-to-size ratio of the ion [53]. The effective mobility, σ_{eff}, of a monovalent weak acid or base is determined by

$$\sigma_{eff} = \sigma_{act}\ \zeta \qquad (1)$$

where σ_{act} is the actual mobility of the fully charged acid or base, and ζ is the degree of dissociation, given for monovalent acids by

$$\zeta = 1 / (1 + 10^{\,pKa - pH}) \qquad (2)$$

and for monovalent bases by [54]

$$\zeta = 1 / (1 + 10^{\,pH - pKa}). \qquad (3)$$

pK_a is the acid constant and pH is the pH of the electrolyte solution. The charge and, thus, the electrophoretic mobility of an ion are affected by the pH of the electrolyte solution [55]. The apparent mobility, σ_{app}, of an ion is a function of both the ion's effective mobility and the electroosmotic flow, σ_{EOF} [53]:

$$\sigma_{app} = \sigma_{eff} + \sigma_{EOF}. \qquad (4)$$

The effective mobility of an analyte is zero if the analyte is completely undissociated, and the mobility is maximum if the analyte is fully ionized. A spherical analyte ion moving under the influence of an electric field engages in ion-dipole and intermolecular hydrogen bonding interactions with the solvent molecules, resulting in dielectric friction [56]. The analyte also has electrostatic interactions with the counter-ions of the electrolyte [57]. The higher the ionic strength of the electrolyte solution, the more the counter-ions will shield the analyte ions, thereby reducing their effective charge [56]. These retarding viscous effects are moderated with a solvent of high dielectric constant since this reduces the Coulombic interactions, leading to reduced ion association [56].

If the separation of charges is possible, an electric double layer is created at the interface between solid and liquid phases [58]. The inner wall of fused-silica capillary is negatively charged due to the presence of weakly acidic silanol groups. Positive ions in solution gather near the capillary surface to balance this negative charge, giving rise to an electric double layer. The electric double layer comprises a diffuse layer and a compact ion-binding region, or the Stern layer [59, 60]. The excess cations that are firmly held in the region of the double layer close to the capillary surface (the Stern layer) are believed to be less hydrated than those in the diffuse region [61]. The plane where the diffuse layer begins is called the outer Helmholtz plane, and the edge for the compact region of bound cations is called the inner Helmholtz plane [59].

The potential at the fused-silica capillary wall is proportional to the charge density resulting from the dissociation of the silanol groups. The potential decreases linearly from …θ (the wall potential) to …ς (the Stern potential) in the Stern layer, and decays exponentially from …ς to zero in the diffuse layer. The potential at the surface between the Stern layer and the diffuse layer is the zeta potential, ′ [60-62]. Upon the application of an electric field across the fused-silica capillary, the cations in the diffuse layer migrate towards the cathode, dragging their hydration spheres with them. Since the water molecules associated with the cations are in direct contact with the bulk solution, all the electrolyte solution moves towards the cathode. This produces a plug-like flow called electroosmotic flow (EOF), which has a flat velocity distribution across the capillary diameter [53, 61]. The magnitude and direction of the EOF are affected by the zeta potential, and can be described by the Smoluchowski equation [53]

$$\sigma_{EOF} = - \kappa' / \xi \qquad (5)$$

where κ is the dielectric constant of the solution, ′ the zeta potential and ξ the viscosity of the solution.

The zeta potential is determined by dissociation of the silanol groups at the fused-silica capillary wall, the charge density in the Stern layer and the thickness of the diffuse layer [61]. Each of these parameters depends on several variables, such as pH, specific adsorption of ions in the Stern layer and ionic strength of the electrolyte solution. Also the dielectric constant, viscosity and nature of the solvent have an effect on the zeta potential [61, 63]. The pH of the solution has a major effect on the zeta potential [59]. An increase in solution pH directly influences the charge density on the capillary wall [55, 60, 61] due to increasing deprotonation of the surface silanol groups. Under an electric field, the thickness of the diffuse layer, to which the zeta potential is directly proportional, is indirectly proportional to the square root of the ionic strength of the electrolyte solution [58, 61]. The EOF is thus decreased with increasing ionic strength of the electrolyte solution. For binary electrolytes in aqueous solution, the double layer thickness ranges from 3 to 300 nm for electrolyte concentrations of 10^{-6} to 10^{-2} M [62]. Typical zeta potentials in CE in aqueous media are in the range of 1–100 mV [64].

The relationship between the electrophoretic efficiency and the characteristic properties of the solvent and the analyte can be expressed by the plate number, N, for an analyte as

$$N = [(\sigma_{eff} + \sigma_{eof}) L_{det} E] / 2 D \qquad (6)$$

where L_{det} is the effective separation length from point of injection to point of detection, E the applied electric field and D the diffusion coefficient of the analyte [56]. This equation assumes that the diffusion is the only source of zone broadening, even though, in practice, many factors affect it. Elevated electric fields result in high efficiency as a consequence of the short time the analyte spends in the capillary [61].

As just noted, many factors affect zone broadening in CE, including diffusion, Joule heat, electrophoretic dispersion, wall adsorption, injection plug length and detection width (Table 3) [65, 66]. Diffusion is the dominant zone broadening mechanism when the electrophoretic mobilities of the analytes are low [67]. The ionic mobility describes the transport of ions along a gradient of electric field, whereas the diffusion coefficient represents the transport of particles along a gradient of chemical potential [68]. The diffusion coefficient of a spherical analyte is related to the viscosity of the solution according to the Stokes-Einstein relation [56, 69]

$$D = kT / 6 \phi \xi r \qquad (7)$$

where k is the Boltzmann constant, T the absolute temperature and r the analyte's hydrodynamic (Stokes) radius.

Table 3. Factors affecting separation efficiency (plate numbers, N) in CE and means to reduce their contribution.

Factor	Means to decrease the contribution
Diffusion	increasing applied potential, increasing viscosity, decreasing temperature, increasing hydrodynamic size of the analyte
Joule heat	active cooling, lowering the applied potential, increasing area-to-volume ratio of capillary, increasing viscosity of the background electrolyte, reducing the ionic strength of the solution
Electrophoretic dispersion	lowering analyte/electrolyte concentration ratio
Analyte–wall interactions	optimizing the electrolyte pH or ionic strength, dynamic or permanent capillary coating
Injection plug length	decreasing the injection plug length
Detection width	decreasing detection width

The heat generated by the passage of electrical current, known as Joule heat, can result in temperature and density gradients and subsequent convection, with increased zone broadening [62, 65]. The temperature in the centre of the capillary will then be higher than that at the edges

producing a parabolic flow profile within the capillary [70]. Joule heating can be controlled by operating at a voltage where the heat can be effectively dispersed [65]. However, theoretical calculations have suggested that, for temperature-controlled capillaries with an inner radius of 50 σm or less, a centre-to-wall temperature difference of as much as 1.5 K in aqueous electrolyte will not cause a severe decrease in the plate numbers of the system [71]. Heating differences can be minimized by providing enough surface area to dissipate the generated heat. This can be done through use either of small inner-diameter capillaries or of long capillaries [62]. In addition, the resistance of the capillary can be increased by reducing the conductivity of the separation medium [72]. High viscosity decreases the mobility of the electrolyte ions minimizing the electrical current [65, 73]. Lower current also allows the use of increased ionic strength and electric field strength, which have a net positive effect on detection, efficiency and analysis time [73]. Higher efficiencies of the separations, with increased plate numbers, have been reported in non-aqueous than in aqueous conditions [65].

Electrophoretic dispersion occurs as a result of conductivity difference between the electrolyte solution and the analyte zone [65, 74]. However, electrophoretic dispersion tends to be negligible when the concentration of the analyte is more than two orders of magnitude lower than that of the background electrolyte [75]. The possibility for analyte–wall adsorption can be reduced through the use of lower sample concentration and with capillaries of larger inner radius, because this reduces the surface area-to-volume ratio [74]. Adsorption can also be minimized by optimization of electrolyte solution pH and its ionic strength. In addition, dynamic and permanent capillary coatings have been shown to be effective in reducing adsorption of analytes on the capillary wall [76]. To minimize injection contributions to the loss in efficiency, the injected plug length should be as short as possible; it is not recommended that it exceed 1% of the total capillary length [65].

4.2 Coating of the fused-silica capillary surface

Separation performance in CE will be impaired by nonreproducible EOF and by the adsorption of analytes to the capillary wall. In particular, cationic analytes have a tendency to interact with the anionic silanoate groups, resulting in peak broadening and peak distortion, which decrease separation efficiency and sensitivity. In addition, the magnitude of the EOF becomes unpredictable leading to poor repeatability of mobilities of analytes. Especially with large biopolymers like proteins, which have hydrophobic, electrostatic, hydrogen bonding and van der Waals interactions with the fused-silica capillary wall, the adsorption of the analytes considerably impairs the separation performance due to peak broadening, with poorer resolution as the result. The EOF can be suppressed or controlled at a certain pH, and analyte–wall interactions can be reduced or eliminated, by coating the active sites on the inner surface of the fused-silica capillary. The active sites comprise inert siloxane bridges, hydrogen bonding sites and ionizable vicinal, geminal and isolated silanol groups [61]. The surface of the fused-silica capillary wall has approximately 8.31 σmol/m^2 of silanol groups [63].

Dynamic and permanent coatings of the fused-silica capillary inner surface have been studied extensively, especially in the field of protein separations. Properties of the coated capillaries are commonly characterized by measuring the EOF and investigating its dependence on the pH of the electrolyte solution [64].

4.2.1 Dynamic coating

In dynamic coating, an electrolyte additive equilibrates with the capillary surface and alters the effective charge of the surface [53]. Dynamic coating is achieved by rinsing the capillary with a solution containing the coating reagent, which can be either a small-molecular-mass compound or a polymer. These additives interact strongly with the capillary, by Coulombic forces, hydrogen bonding and van der Waals forces, altering the effective charge on the surface [77]. Usually, a small amount of the coating reagent is added to the electrolyte solution as well, to maintain the coating [76]. The main advantages of dynamic coating are the simplicity and the renewable character. The dynamic coating is also easily removed from the capillary wall by rinsing. Some additives are only useful in a specific pH range, owing to their pH-dependent dissociation.

The additives may act as masking agents for the silanoate groups on the inner wall of the capillary. Or they may function as strong ion-pairing agents. Most additives alter the electric double layer at the interface between the capillary wall and the electrolyte solution, resulting in reduction, elimination of the EOF or its reversal from cathodic to anodic [61]. Ionic additives alter the hydrogen ion concentration of the electrolyte solution, which influences the charge density on the capillary wall resulting from protonation or deprotonation of the surface silanol groups, thus affecting the zeta potential [61]. The additives may also alter the ionic strength of the electrolyte solution and thereby affect the thickness of the diffuse layer [78]. At constant pH and ionic strength, electroosmotic mobility mainly depends on the surface density of the adsorbed cations in the Stern region of the electric double layer [60]. If the positive charge density exceeds the negative charge density on the capillary inner wall, the zeta potential becomes positive and the EOF is reversed from cathodic to anodic [61]. In addition, the incorporation of an additive in the electrolyte solution may alter the viscosity and dielectric constant of the bulk solution [61].

4.2.1.1 Amine compounds as coating reagents

Monoamines such as triethylamine (TEA), propylamine and morpholine; diamines such as ethylenediamine, 1,4-diaminobutane, 1,5-diaminopentane and 1,3-diaminopropane; and oligo- and polyamines such as triethylenetetramine, polyethyleneimine and chitosan are examples of dynamic coating reagents used in CE. The adsorption of amines has been proposed to involve several steps: the positively charged amines are first attracted to the negatively charged silica surface by Coulombic forces, and then retained by additional forces like hydrogen bonding between the silanols and the amino groups, and perhaps hydrophobic interactions between the alkyl chains and the siloxane groups [60, 79, 80]. The adsorption and ion-exchange effects at

the silica surface are then reduced, as is EOF. The reduction of EOF is reported to be directly proportional to the concentration and basicity of the additive and inversely proportional to the molecular size of the monovalent alkyl amines at a given pH and ionic strength [60, 81]. At higher amine concentration, the EOF is decreased and finally reversed by formation of a bilayer on the capillary surface [79, 82].

Coating coverage of 70% has been achieved with an amino phase [59]. The nonquantitative coating was explained by Coulombic repulsion between the charged amino groups, which prevents the amino groups from being bound closely on the silica wall. The order of efficacy of the amine compounds as coating reagents has been reported to follow the order of hydrophobicity: the more hydrophobic the amine, the more effective inhibitor it is of analyte–wall interactions [80]. Monovalent amines have found only limited application as additives in electrolyte solutions in protein separations, as they are poorly effective in reducing protein–silica wall interactions [80]. The drawback of amine compounds is that they lose their effectiveness at alkaline pH due to deprotonation of the amino group [83]. Quaternary ammonium alkanes, in turn, are presumed to be advantageous as a result of their non-pH-dependent ionization and the absence of pH alteration upon their addition to the electrolyte solution [84]. Much lower concentrations of quaternary ammonium alkyl compounds are needed than of their alkyl diamine counterparts for quenching protein–wall interactions [84]. Polyamines, such as spermine and tetraethylenepentamine, are presumed to be strongly adsorbed at the interface between the capillary wall and the electrolyte solution as a result of the multiple interaction sites of electrostatic and hydrophobic character possessed by these additives [61]. Polyamines can be added to any background electrolyte in such minute concentrations that the conductivity and ionic strength of the background electrolyte do not change [80]. In the presence of only 0.002% (w/v) of chitosan in the separation electrolyte, the direction of the EOF was reversed towards the anode [85]. The magnitude of the reversed EOF seemed to correlate with the charge density of the polymer [86].

4.2.1.2 Surfactants as coating reagents

Surfactants, such as cetyltrimethylammonium bromide, didodecyldimethylammonium bromide and various fluorosurfactants, are amphiphatic molecules possessing both hydrophilic and hydrophobic character. At low concentration in the electrolyte solution they may be associated as dimers, trimers or oligomers, depending on their chemical structure and on pH, ionic strength and composition and temperature of the electrolyte solution [61]. The adsorption of individual surfactant ions in the Stern layer results from the electrostatic attraction between the cationic surfactant and the silanoate group at the surface of the capillary wall. As the concentration of the surfactant in the solution is increased, so is the concentration of the adsorbed ions. Eventually a critical concentration is reached, at which the van der Waals forces between the hydrocarbon chains of adsorbed and free surfactant molecules in solution cause their association into hemimicelles. Hemimicelles are pairs of surfactant molecules with one cationic group directed towards the capillary wall and the other directed out into the solution [87, 88]. This leads to positive surface charge and to the reversal of EOF [89-91]. Once the micellar layer is

fully formed at the capillary wall, further increase in the surfactant concentration yields no change in the magnitude of the EOF [53]. Zwitterionic surfactants are of higher efficiency than neutral detergents [89], and they have been proposed to be adsorbed onto the silica surface in a bilayer fashion. The shielding efficacy of surfactants closely follows a hydrophobicity scale [89]. It has been proposed that the mechanism of adsorption of surfactants to the silica surface is driven by residual van der Waals forces, and amplified by additional hydrophobic interaction mechanisms once the compounds are deposited on the silica surface [83].

4.2.1.3 Polymers as coating reagents

Neutral polymers like propylene glycol, hydroxypropylmethyl cellulose and poly(ethyleneoxide) are believed to adsorb at the interface between the capillary wall and the electrolyte solution, shielding the silanol groups on the capillary surface and leading to increased local viscosity in the electric double layer [61, 77, 92]. The interactions involved may be hydrogen bonding between the surface silanols and the hydroxylic or ether oxygens of the polymers. Thus, the most stable coating has been obtained after rinsing the capillary with hydrochloric acid to increase the concentration of protonated hydroxyl groups available for hydrogen bonding [61]. The dynamic coating has not been as effective at pH over 5 owing to decreased concentration of protonated silanol groups [93]. Poly(acrylamine) and cellulose-based adsorbed coatings are easily washed off from the capillary wall because of their hydrophilicity [94, 95]. The use of neutral polymers as dynamic coating reagents is restricted to very low concentrations of the polymer in the electrolyte solution. Above an optimum polymer concentration, the chains of the polymers begin to overlap and interact with one another through van der Waals forces and hydrogen bonding, giving rise to a polymer network, which may act as a sieving matrix [96]. The adsorption of charged polymers introduces positive or negative charges on the capillary surface, changing the direction and/or the strength of the EOF, and reducing the analyte–wall interactions in the separation capillary [76].

4.2.2 Permanent coating

Covalently bonded capillaries require less maintenance than dynamically coated ones, but the preparation of reliable coatings can be challenging [76]. Preparation of a permanent wall coating typically consists of three steps: capillary pretreatment including etching and leaching, introduction of double bonds to the capillary wall by silylation, and binding of a polymer to this intermediate layer to form a chemically and mechanically stable capillary surface [76]. This multistep process is difficult and time-consuming and the coating may be nonreproducible as a result [97]. The coating may also be unstable outside a limited pH range [53].

To achieve a homogeneous coating surface, the capillary wall must be cleaned and activated by etching and/or leaching before the coating process [76]. Etching with sodium hydroxide removes impurities from the fused-silica capillary surface, and leaching with hydrochloric acid removes trace metals. This procedure produces reproducible silica surfaces in capillaries from different manufacturers and batches [97]. Attaching a polymer coating to the capillary wall can

be done with use of a reactive bifunctional silane reagent, such as ν-methacryloxypropyl-trimethoxysilane (MAPT) [98]. The silane groups of the reagent react with the surface silanols, and the other functional groups are used to attach and polymerize monomers to the capillary surface. In order to achieve reproducible silanization, the physically adsorbed water should be removed from the silica surface [97]. Other factors influencing the silanization are temperature, reaction time, the catalyst used for the reaction and the concentration of the silane reagent. Higher silanization yields have been obtained by using undiluted silane reagent, irrespective of the acidic or basic nature of the catalyst. For optimal results, the silanization should be carried out overnight at room temperature [97]. The main disadvantage of using silane reagents to deactivate the silica surface is that the resulting siloxane bond offers unsatisfactory hydrolytic stability at alkaline pH [76].

The attachment of the outer polymer layer is achieved through a free radical polymerization reaction with the established reactive olefinic sublayer on the silica surface. Polymerization conditions, including concentrations of the monomer, initiator and catalyst and their relative ratios, temperature and time, play an important role in controlling the properties of the final coating [76]. High monomer concentrations should yield large polymers, while increasing the initiator concentration should yield shorter polymeric chains [97]. A low monomer concentration can produce coatings formed by a small number of short polymer chains. Although the short polymer chains are attached to the capillary wall, they may be unable to eliminate the electrostatic interaction between the analyte and the remaining silanoate groups of the silica wall [99]. Polymerization also proceeds in the solution, not only on the capillary surface, and it has been shown that polymerization in the solution reduces the concentration of monomers available for the surface coating process. This can cause variability in the coating thickness [76].

Permanent coatings preattached to the silica wall can be divided into two types: (1) coatings that are not covalently attached but are adsorbed to the surface by physical or ionic forces; however, unlike dynamic coating reagents, they are not included in the electrolyte solution during separation; (2) coatings that are covalently attached to the capillary surface [77].

4.2.2.1 Permanent noncovalent coatings

Noncovalent permanent coatings are formed by compounds of hydrophilic nature. The adsorbed coatings are mainly of two types: aminated or polycationic polymers and hydroxylic or neutral polymers [77]. Aminated compounds such as chitosan, polybrene and polyethyleneimine are adsorbed to the silica wall by multi-site electrostatic interaction and create a positively charged capillary surface [85, 100, 101]. They make very stable coatings, which are useful over a wide pH range of 3-11.

Hydroxylic or neutral polymers such as poly(vinyl alcohol) and poly(ethyleneoxide) are fixed to the silica wall by weak interactions such as hydrogen bonds [93, 102]. Because these compounds are not charged, the coating is stable over almost the entire pH range. In the coating

procedure, the reagent is passed through the capillary in a suitable electrolyte solution. The hydroxylic polymers usually require thermal immobilization to become water insoluble. Before electrophoresis, the unbonded reagent is flushed from the capillary [77].

4.2.2.2 Permanent covalent coatings

In covalent coating, an olefinic compound is introduced to the fused-silica capillary wall via silanization. Olefinic compounds are hydrophobic and require a hydrophilic outer layer to ensure against hydrophobic interactions with analytes. This hydrophilic layer is of high viscosity, which makes it capable of suppressing the EOF [77]. If the wall is coated with a neutral polymer to eliminate electroosmosis, the analytes may be sterically prevented from contacting with the wall and therefore from being adsorbed. In this way, interactions other than the electrostatic ones are also suppressed [98].

Polyacrylamide coatings are reported to be superior to all other types of coating, and cannot be prepared by adsorptive methods [76]. However, polyacrylamide coatings bonded to the surface through Si–O–Si linkages suffer from long-term instability, since the siloxane linkage is prone to hydrolysis in basic conditions [103]. Increase in electrolyte concentration was observed to accelerate the hydrolysis of both the Si–O–Si network on the inner surface of the fused-silica and the Si–O–Si–C linkage to the polyacrylamide coating, but no effect was observed for Si–C linked capillaries [77]. The direct Si–C bond can be formed with use of Grignard reagent [104]. An alternative method involves catalytic hydrosilylation of olefins on a Si–H containing substrate. In the first step, a silicon hydride is formed, to which a terminal olefin is added [105].

Non-ionic surfactants, such as oxyethylene based surfactants of the Brij and Tween series, can be hydrophobically adsorbed onto an alkylsilane derivatized capillary surface to generate a hydrophilic layer that prevents analyte–capillary wall interactions and controls the electroosmotic flow [106]. In this hybrid coating possessing both permanent and dynamic character, the hydrophobic chain of the surfactant molecule probably interacts strongly with the alkylsilane derivatized surface, whereas the hydrophilic group of the surfactant creates a hydrophilic layer that masks the underlying alkylsilane and the residual free silanol groups [107]. For example, Tween 20 appears to exhibit a reasonable inhibitory power, since it is already quite effective at 3% concentration [89].

4.3 Capillary electrophoresis–electrospray ionization mass spectrometry

The driving force for coupling CE to mass spectrometry (MS) is the detailed structural information provided by MS. Capillary electrophoresis has been on-line coupled to MS with atmospheric pressure ionization techniques, namely electrospray ionization (ESI) [108] and atmospheric pressure chemical ionization [109]. Ionization has also been performed under lowered pressure, with continuous flow fast atom bombardment [110] and with laser vaporization ionization using UV laser [111]. An off-line coupling of CE and matrix assisted laser desorption ionization has been reported with continuous effluent deposition on matrix-

precoated cellulose membranes [112]. ESI is the most widely used of these ionization techniques. Advantages of ESI are simplicity, high ionization efficiency (50-100%) in terms of release of ions from charged droplets, and the ability to produce multiply-charged ions [113]. Mass spectrometers used in CE–MS coupling include quadrupole [114], ion trap [115], time of flight [116], Fourier transform ion cyclotron resonance [117] and magnetic sector [110, 118] instruments. The quadrupole mass filter has a slow scanning rate, which reduces resolution in fast separations. This can be avoided by using fast-scanning analysers, such as ion trap [119].

4.3.1 Coaxial sheath liquid coupling

The three techniques commonly used for interfacing CE and ESI-MS are the coaxial sheath liquid, the liquid junction and the sheathless nanospray coupling. The coaxial sheath liquid coupling, in which three coaxial capillaries are placed at the interface of CE and MS (Figure 2) was developed by Smith and co-workers in the late 1980's [108]. The innermost capillary is made of fused-silica, and is used for the CE separation. The middle capillary is the capillary for the coaxial sheath liquid, and is made of stainless steel, and the outermost capillary, also made of stainless steel, is for the introduction of nebulizing gas, which aids the droplet formation during the electrospray process [120]. The durability of the thin-wall CE capillary used in combination with the stainless steel sheath liquid capillary was observed to be poor due to electrodrilling, which destroys the capillary and interrupts the analysis [121]. The stainless steel capillary has accordingly been replaced with an aluminium-coated fused-silica capillary of similar diameter. A further advantage of fused-silica over stainless steel is the reduction of electrochemical processes near the tip of the capillaries [121]. However, the electrochemistry of aluminium in basic solutions results in limited usable lifetime of the sheath liquid capillary [121].

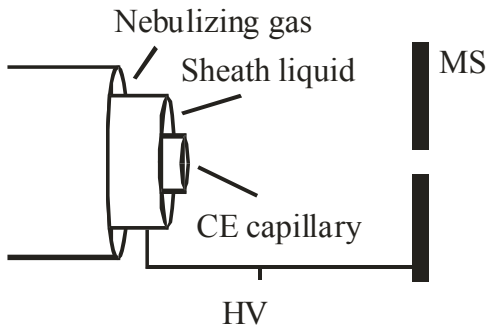


Figure 2. Coaxial sheath liquid coupling for CE–MS.

The sheath liquid consists of water, organic solvent such as methanol, and an electrolyte. The main function of the sheath liquid is to provide electrical contact between CE and MS [108]. In addition, it increases the total liquid flow to ESI, providing a stable ion production. The

electroosmotic flow rate from CE is only up to 0.3 σl/min [113], whereas a stable electrospray process requires a minimum flow rate of 0.5 σl/min [108]. Typical sheath liquid flow rates are 5-10 σl/min, but 2-30 σl/min has been reported to be practical [108]. The coaxial sheath flow coupling allows independent optimization of the CE and ESI flow rates [122]. However, the use of sheath liquid reduces the sensitivity of the signal due to dilution of the sample [123, 124]. Furthermore, the ionic species present in the sheath liquid are a source of chemical noise, lowering the detection sensitivity as they compete for the available charge at the spray tip [124]. Coaxial sheath liquid coupling is relatively easy to implement and use, but it is also rather demanding in terms of the optimization of operational parameters, such as sheath liquid composition, flow rate and capillary tip position [124].

4.3.1.1 Solution properties affecting electrospray performance

Several physical properties of the solvent, such as surface tension, conductivity, viscosity, dielectric constant, boiling point and heat of vaporization, are important in the ESI process (Table 4) [125]. Solvent properties influence the spray characteristics in a variety of ways, most notably in the dependence of the "onset potential" (i.e. the minimum potential required to form the characteristic Taylor cone) on surface tension, the spray current on conductivity and the droplet size on viscosity [125]. The post-electrophoretic addition of organic solvent, such as methanol, reduces the surface tension and viscosity of the CE electrolyte solution, and enables the formation of smaller charged droplets during electrospray [126]. This results in faster evaporation of the solvent, improved desorption efficiency of protonated analytes and thereby increased ion yield [127]. The improved desolvation process also enhances the stability of the electronebulization [128]. The lower surface tension permits droplet breakup at lower applied voltages, thus decreasing the tendency for unwanted electric discharges [127, 129]. Ions escape from droplets with greater ease when the dielectric constant of the solvent is low [130].

Table 4. Effects of physical and chemical properties of solutions on electrospray performance.

Change in solution property	Effect on electrospray performance
Decreasing surface tension	Decreased "onset potential" for Taylor cone formation
Increasing conductivity	Increased spray current, impaired spray stability due to electric discharge
Decreasing viscosity	Decreased droplet size and increased evaporation from the droplets
Decreasing dielectric constant	Increased ion escape from the droplets
Increasing amount of organic solvent	Reduced surface tension and increased evaporation from droplets
Adjustment of pH	Protonation/deprotonation of acids/bases in liquid phase
Increasing ionic strength of the electrolyte	Decreased analyte sensitivity
Increasing analyte concentration	Linearly increased signal sensitivity, after which decreased signal sensitivity

Likewise, chemical parameters such as solvent composition, solution pH, electrolyte additive, and analyte concentration, affect the ionization efficiency (Table 4) [131]. The solution chemistry strongly affects the ESI process, as components may suppress or enhance the ionization efficiency [108]. A solution with higher proportion of organic solvent enhances the ESI process, as discussed above. The pH of the solution sprayed into the MS can be modified by addition of volatile acids or bases to the sheath liquid [126]; and thereby, with post-electrophoretic modifications of the analyte or the electrolyte solution, the electrospray process and ionization of the analytes can be enhanced [108, 113]. However, the counterions of the sheath liquid electrolyte migrate through the separation capillary, replacing the counterions of the separation electrolyte. The exchange of ions can create ionic boundaries, where the pH and the conductivity of the electrolyte solution differ significantly, affecting migration times and separation of the analytes in CE [119].

The electrolytes used in ESI are generally volatile ones, such as ammonium acetate. Nonvolatile electrolytes affect the ESI process dramatically, since they will cause salt accumulation in the capillary vaporizer or the ion source. Nonvolatile electrolytes also increase the background noise markedly [132]. Improvements in interfaces, such as orthogonal sprayers creating an off-axis ion pathway, minimize the problem with nonvolatile electrolytes [133, 134]. Even an electrolyte solution containing 300 mM borate has been used with good MS response with off-axis spray [135]. Ionic and neutral species compete for available charge in the ESI process, thus lowering the maximum sensitivity obtainable. The consequences are reduction in stability of the electrospray, low sensitivity and at worst a total electrical breakdown [127, 129, 136]. The signal intensity of a protonated analyte has been demonstrated to decrease with increasing ionic concentration of the electrolyte in the sprayed sample solution, owing to competition of the electrolyte cations in the droplet to enter the gas phase [137]. The signal intensity of an analyte has also been found to decrease with increasing analyte concentration in the sample following a linear increase and plateau in the intensity curve [137].

Aqueous solutions with ionic concentration above 10^{-2} M cannot be efficiently sprayed by applying voltage, but electrolyte solutions with higher ionic strength are often used in CE separations. If high ionic strength electrolytes with high conductance are used in CE–MS, an excess of charge is created at the tip of the electrospray needle, resulting in electric discharge, and thus in instability in droplet formation [127, 138]. A current of 100 σA in CE is too high to allow coupling to MS [139]. If the CE and ESI currents are similar, sensitivity limits of the MS are maximized and the stabilities of CE and ESI are optimal [140]. The conductivity of the CE electrolyte solution can be lowered by diluting it with sheath liquid, and the optimal separation voltage can be determined by plotting the applied voltage versus the resulting current [132]. Non-aqueous CE is ideal for MS detection [141], since the generated electric current is lower in non-aqueous conditions [142]. However, non-aqueous CE–MS with methanol and methanol–acetonitrile has been observed to produce poorer peak shape, lower sensitivity and decreased stability relative to the aqueous electrolyte [141].

4.3.1.2. Effect of instrumental conditions on electrospray performance

The electrospray is defined by the physical dimensions of the capillaries in the coaxial sheath liquid CE–ESI-MS interface, which affect the performance of the system in terms of both sensitivity and stability [143]. The dimensions of the capillaries, i.e. their inner (i.d.) and outer (o.d.) diameters and hence wall thickness, have an influence on the spray formation, the degree of mixing of the sheath liquid and CE electrolyte solution at the tip of the ESI needle, the siphoning effect of the nebulizing gas, and thus the electrical environment at the cathode end of the separation capillary [120]. With reduced dimensions of the sheath and CE capillaries, better operation and increased sensitivity can be achieved for stable spraying with larger signal-to-noise ratio at lower flow rate [120, 144, 145]. Thinner wall and tapered capillary tip improve the wettability with the sheath liquid, so that ions are efficiently transferred to the electrospray, without unnecessary adsorption to the fused-silica surface. The stability of the electrospray is thereby improved [121, 146]. As the i.d. of the spray tip is decreased, the mass sensitivity as well as the absolute signal intensity are increased [145]. A small tip with i.d. of 5–10 σm provided gain in sensitivity relative to a larger capillary tip of 50–100 σm i.d. [147]. However, with a tip i.d. of 3 σm, the increased hydrodynamic resistance resulted in decreased flow, and no gain in sensitivity was observed relative to a 20 σm i.d. tapered ESI needle [147]. With reduced i.d. of the capillary sprayer, allowing its positioning closer to the ion sampling orifice, a much larger proportion of the spray cone can be sampled into the MS [147, 148].

The right positioning of the CE capillary relative to the coaxial sheath liquid capillary tip is very important for good electrical contact between CE and ESI, and thus for stable ESI operation [149]. Optimum sensitivity has been obtained with the CE capillary protruding 0.1–0.7 mm from the sheath liquid tube [128, 136, 138]. A capillary position beyond or below this range resulted in a decrease or breakdown of the ion current, mainly due to loss of electrical contact between CE and ESI [136]. The initial increase in ion current observed when the fused-silica capillary begins to protrude from the stainless-steel needle can be attributed to a decrease in mixing volume leading to less dilution by the sheath liquid. Furthermore, with the fused-silica capillary protruding slightly, it is likely that smaller droplets will be formed in the electrospray process, leading to more efficient ion production than where the electrospray occurs directly from the tip of a wide-bore needle. Beyond a certain point, however, the silica tip protrudes too far to enable adequate mixing [138]. Solvent mixing can be improved by removing a few millimetres of the polyamide coating of the CE capillary [128].

Likewise, the position of the sprayer relative to the ion sampling orifice needs to be carefully adjusted for optimum performance [130]. The positioning of the sprayer is instrument dependent [123]. Good sensitivity was obtained with the sprayer tip at a distance of 5–20 mm from the counter electrode [123, 130]. If the tip was located too close to the counter electrode, the stability and intensity of the signal were decreased, owing to the electric discharge occurring at the tip [132]. Positioning of the capillary 5–10 mm off-axis or at an angle of about 30∀ towards the sampling orifice increased the sample ion current and prevented droplets from

hitting the ion sampling orifice, decreasing the signals of cluster ions [123, 130]. Regardless of the capillary tip position, electrospray current increases with CE voltage [138]. The elevated temperature in the ion source aids in droplet desolvation. A temperature of 80 ∀C has been found suitable for water desolvation [128].

The nebulizing gas assists droplet formation and solvent evaporation, and it provides a certain amount of cooling for the CE capillary [120, 142]. The combination of pneumatic nebulizing and an electric field allows higher eluent flow rates and a higher percentage of water in the formation of a spray of charged droplets [130]. In addition, nebulizing makes the spray process independent of the position of the interface inside the ion source, allowing a greater distance between the spray capillary and its counter electrode. This in turn reduces the electric field at the tip and prevents electrical discharge [130]. The flow rate of the nebulizing gas has an effect on the CE separation. A high flow rate reduces both migration time and resolution due to pressure-induced flow, and a low flow rate produces distorted peaks [139, 142]. At low nebulizing gas pressure, large charged droplets are generated, which result in adduct ions in the spectra, decreased sensitivity and increased background [142, 150]. At too high nebulizing gas flow, a stable Taylor cone is not formed [151]. A drying gas is generally used to accelerate desolvation, increase MS sensitivity and avoid the entrance of undesirable ions to the MS. Sensitivity appears to be reduced with increase in the drying gas flow rate [142]. Drying gas flow rate can also contribute to retroelution through a backpressure effect [128].

The flow rate of the coaxial sheath liquid has an effect on spray characteristics. Too low sheath liquid flow rate leads to inadequate liquid feeding in the spray, resulting in a sputtering effect and decreased spray stability [128, 142]. However, higher flow rates will mean dilution of the separated compounds and lower signal-to-noise ratios [142]. In addition, degradation of peak intensity and shape may occur probably due to inefficient desolvation because of overfeeding of the spray with solvent [128]. Low coaxial sheath liquid flow yields a better signal-to-noise ratio, since the noise level is reduced and the intensity of the signal is increased; thus lower detection limits are achieved [121].

Another physical property affecting the CE–MS coupling is siphoning, which originates from a height difference between liquid levels in the inlet vial and outlet end of the separation capillary. A height difference of 8 cm, for example, creates a bulk flow of 50 nl/min by siphoning [152]. This laminar flow disturbs the flat flow profile in the CE capillary and degrades the separation efficiency [153]. However, a height difference of just 2.5 cm has been observed to enhance the functioning of the ESI source with increased signal-to-noise ratio [153]. Often a low constant pressure can be applied to the CE capillary to shorten analysis times and obtain additional electrolyte flow for the spray [123]. If the ESI source is operational during the injection phase, a reversed electroosmotic flow towards the CE inlet is formed and the hydrodynamically loaded analytes will migrate out of the capillary, leading to considerably reduced sensitivity [143, 149]. Likewise, it is not wise to turn on the nebulizing and drying gases during hydrodynamic injection, because this may decrease the introduced sample volume by altering the column pressure [128].

4.3.2 Liquid junction coupling

The additional make-up solution provided by the liquid junction to the CE–MS interface serves the same purpose as the coaxial sheath liquid; only the coupling structure is different (Figure 3). Henion and co-workers [154] developed the liquid junction coupling for CE–MS in the late 1980´s. The coupling was constructed from a tee piece made of stainless steel. The cathode end of the CE separation capillary was placed opposite the end of the capillary electrode of the ion spray interface, with a 10–25 σm gap between the two capillaries. The gap allows the make-up electrolyte solution to flow unrestricted into the interface from the surrounding reservoir, preventing suction from occurring at the end of the CE capillary. Flow rates up to 20 σl/min can be introduced to MS with the liquid junction. Later, an improved self-aligning liquid junction was described [155], where the make-up liquid was introduced to the junction of the capillaries at a controlled flow rate instead of by gravity. The self-aligning liquid junction interface provided higher degree of precision in the alignment and the spacing of the CE and spray capillaries and improved mixing of the CE electrolyte solution and the make-up liquid together, leading to better stability and increased signal-to-noise ratio.

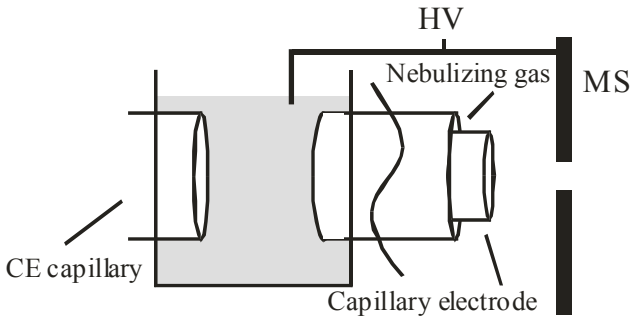


Figure 3. Liquid junction coupling for CE–MS.

The liquid junction is a very popular and versatile interface for CE–MS coupling [124]. It combines the advantages of independent optimization of the ESI and separation conditions with the advantage of a fine ESI tip for stable and sensitive ESI operation [147]. In a comparison of the liquid junction and coaxial sheath liquid interfaces both designs proved themselves capable of efficient coupling, but the coaxial configuration is more robust and reproducible [156]. A further advantage of coaxial coupling is that it provides the potential for flow injection analysis since the make-up liquid is delivered independently of the CE effluent. While the liquid junction coupling provides improved sensitivity and ion current stability when properly assembled, it is also more challenging to find the optimum setup of the system [156]. The advantages of the coaxial interface included zero dead volume and higher theoretical plate

numbers. However, problems are associated with high voltage arcing through the thin capillary wall [121].

4.3.3 Nanospray coupling

The nanospray source disperses liquid samples only by electrostatic means, with no sheath liquid flow or nebulizing gas to assist the spray formation [157]. The sheathless coupling of CE and MS is achieved by tapering the CE capillary tip and making an electrical contact between CE and MS (Figure 4). Several methods have been described for tapering the tip of a fused-silica capillary, including stretching the capillary in a methane–oxygen flame [158], etching with hydrofluoric acid [159, 160] and mechanical grinding with fine sandpaper [161]. The tips prepared by hydrofluoric acid etching work better than those made by mechanical grinding because the relatively rough surface created by grinding is easily wetted, eventually leading to a surface for crystallization of nonvolatile electrolytes [147]. The electrical contact is achieved by coating the CE capillary tip with a layer of gold or silver [157, 162, 163] or by attaching a narrow metal wire, such as gold-plated tungsten, in contact with the liquid, at the tapered tip outlet [159, 164, 165]. A more stable coating has been obtained with conductive silver paint than with sputtered gold layer [166]. The gold layer is often sputtered on the capillary tip using additional adhesive or protecting materials to enhance the stability of the coating [161, 166-168]. However, these coatings are susceptible to decomposition as a result of electrical discharges and electrochemical stress, which shortens the lifetime of the tip [165, 168, 169]. A polymeric nanospray needle emitter made of a conductive mixture of polypropylene and graphite is reported to be stable in use, and easy to produce [169]. A somewhat different approach to CE–MS nanospray coupling is to attach a separate gold-coated fused-silica spray tip to the CE separation capillary, to supply the high voltage at the junction between CE and MS [162, 170-172]. A stainless steel capillary has also been utilized as the nanospray coupling, functioning as both the CE cathode and electrospray needle, ensuring the electrical contact and initiating the electrospray [173].

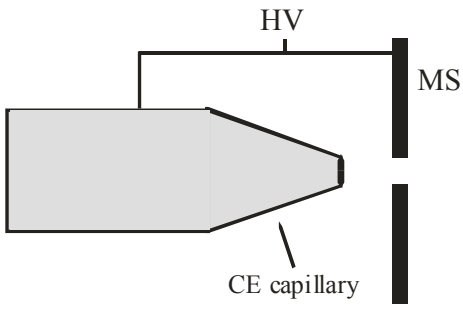


Figure 4. Sheathless nanospray coupling for CE–MS.

The diameter of the droplets formed in nanospray ionization depends on the flow rate of the electrolyte solution, on the capillary i.d. and on the field strength applied to the tip [168]. The lower the flow rate, the smaller the i.d. of the tip should be [166]. The reduction of the liquid flow rate yields smaller droplets with higher surface-to-volume ratio, which increases the evaporation efficiency [158] and thus signal sensitivity [162, 169]. Likewise, with lower flow rate of the electrolyte, and smaller capillary i.d., droplets are about 100–1000 times smaller than with conventional electrospray source [157]. A 25- to 50-fold improvement in sensitivity has been reported with nanospray relative to coaxial sheath liquid coupling of CE and MS [174, 175]. Furthermore, nanospray allows the use of lower electric fields to achieve stable spray so that the capillary tip can be positioned closer to the gas vortex entering the skimmer inlet of the MS. In this way, much higher sampling rates can be obtained. Also, the production of smaller micro-droplets results in a more efficient ion production and ion desorption [148]. However, if the conductivity of the electrolyte is increased, the electric field at the nanospray tip is increased, which can result in electrical discharge and thus in reduction in the signal intensity [158, 160]. The demands of the sheathless nanospray interface are low surface tension, a CE solvent of relatively low conductivity and high enough linear velocity [171].

4.4 Non-aqueous solvents in capillary electrophoretic separations

The recent introduction of non-aqueous media greatly extends the applicability of CE, which traditionally has been performed in aqueous electrolyte solution. Many solvent properties (Table 5) need to be considered in selecting the separation medium: dielectric constant, viscosity, polarity, autoprotolysis constant, acid-base chemistry, electrical conductivity, volatility, UV transparency and solvation ability [54, 56, 73, 176]. The solubility of hydrophobic organic compounds is increased in organic solutions [177], and the use of organic solvents may decrease the degradation of the analytes [178].

Table 5. Physical and chemical properties of organic solvents and their effects in CE.

Solvent property	Effect on
Dielectric constant	electrical properties of the solvent, stability of charged species in solvent, dissociation of analytes and background electrolyte components, ion solvation, double layer thickness
Viscosity	mobility of the analyte
Autoprotolysis constant	acidity/basicity of the solvent
Acid-base chemistry	dissociation of analytes and background electrolyte components, stability of charged species
Solvation ability	ion stability, dielectric friction, size of the solvated analyte

Solvents suitable for ionic analytes have high dielectric constants and dipole moments. The short chain alcohols and acetonitrile are frequently used in non-aqueous CE separations. Methanol is a rather similar solvent to water with a moderate dielectric constant (32.66 at 25 ∀C [179]) and capability for autoprotolysis. It is a neutral amphiprotic solvent, in which solvation is favoured due to the formation of hydrogen bonds [179]. The dielectric constant of acetonitrile

(35.94 at 25 ∀C [179]) is slightly higher than that of methanol owing to the strong dipole moment. Acetonitrile is also very different from methanol in being a protophobic dipolar aprotic solvent with a weak autoprotolysis constant. Other solvents used in non-aqueous separations include formamide, N-dimethylformamide and N,N-dimethylacetamide. However, the instability of these solvents due to hydrolysis and the strong UV absorbance at lower wavelengths decreases their usefulness in CE separations [54, 73, 176]. Propylene carbonate, with its high dielectric constant and viscosity, has been found suitable as a non-aqueous solvent for CE [180].

4.4.1 Effect of organic solvents on electrophoretic mobility

The dielectric constant is one of the key physical properties to consider in selecting a solvent for CE separation since it influences the electrical properties of the solvent [56]. The higher the dielectric constant of the solvent, the higher the number of dissociated ions present in the separation solution. The dielectric constant also affects the dissociation of the analytes. In general, the higher the value of the dielectric constant of the solvent, the less influence the solvent will have on the ionization of acids and bases [181], owing to the decreased electrostatic interactions between a solvent with high dielectric constant and ionized compounds [54, 182]. Ion solvation is characterized by dielectric constant and/or dipole moment of the solvent, but also by electron pair and hydrogen bond acceptance and donation abilities [182]. In aqueous solvents, the hydrophobic interactions dominate in solvation, whereas in non-aqueous solvents, electrostatic ion-dipole forces and donor-acceptor interactions are important [182]. It has been observed that ion-pair formation is favoured in organic solvents of low dielectric constants ($10<\kappa<30$) [183].

Electrophoretic mobility of an analyte is directly proportional to its effective charge and inversely proportional to its solvation radius [184]. Studies with mixed aqueous–non-aqueous media have shown that the electrophoretic mobilities of several analytes are decreased with increasing amount of organic solvent [56, 58]. Organic solvents influence the mobility of an analyte by changing the size of the solvated analyte and the viscosity of the bulk solution [181, 185, 186]. Solvent viscosity can be used to describe the effect of the solvent on the mobility of an analyte in CE, as Walden's rule states: The product of the actual mobility of an ion, at infinite dilution, and the viscosity of the pure solvent is constant and independent of the solvent. This rule assumes the ions to be spherical particles moving in a continuum. In a study with anilinium ions in methanol and acetonitrile, the products of actual mobilities and viscosities were constant within 7% [57]. The mobility of an analyte is also affected by the acid-base properties of the solvent, which affect the dissociation of the analyte and thus its pK_a value [177].

The analyte charge is dependent on the way the acid-base equilibrium and the ionization constant of the analyte are altered by the protolytic properties of the organic solvent [56, 181]. The pK_a values of weak acids are higher in many non-aqueous solvents than in water [54]. Thus, acidity of the acids is decreased. The pK_a values of weak bases are usually changed to a

lesser extent. In addition to acid-base properties of the solvent, the changes in pK_a depend on the stabilization of the deprotonated acid or the protonated base by the solvent and on the solubility of the molecular acid or base in the solvent [185]. Organic solvents are poor solvators for anions and thus negatively charged ions are less stabilized in organic solvents than in water [54, 187]. The variation in the pK_a values can be explained in terms of the transfer activity coefficient of the species [54].

4.4.2 Effect of organic solvents on electroosmotic mobility

Electroosmosis is dependent on the electrolyte composition and the physico-chemical properties of the contact surface [188]. A decrease in the electroosmotic mobility has been reported with increasing concentration of both protic and aprotic organic solvents [56, 58]. The double layer thickness decreases when the solvent has a low dielectric constant [56]. A pK_a value of 5.3 has been reported for the surface silanol groups in an aqueous system, but the value increases in organic solvents. For example, a pK_a value around 7 has been reported for a 50% methanol–water solution [58]. The decreased dissociation of the silanol groups decreases the electroosmotic mobility. In addition to the decrease in the dielectric constant of the solution, adsorption of ions on the silica surface may explain the observed decrease in the zeta potential with increasing fraction of organic solvent [58].

Organic solvents are applied in CE to enhance the separation selectivity of ionic analytes, which is based on the relative difference in the mobilities [54, 56]. Selectivity varies with the solvent because solvents affect the mobilities of analytes differently [177, 189, 190]. The differences in selectivity, and mobility, can be ascribed to differences in dissociation constants and solvation of the analytes in the different solvents [178].

5 EXPERIMENTAL

5.1 Chemicals and materials

Catecholamine and methoxycatecholamine standards, and the other chemicals and materials used in this study, are listed in Table 6. The purpose of each item is shortly noted.

Table 6. Standards, chemicals and materials used in the study.

Compound/material	Manufacturer/supplier	Notes	Paper
Adrenaline (A)	Sigma-Aldrich	Standard	II, V
3,4-Dihydroxybenzylamine (DHBA)	Sigma-Aldrich	Internal standard	I, II, V
Dopamine (DA)	Sigma-Aldrich	Standard	I-V
4-Hydroxy-3-methoxybenzylamine (HMBA)	Sigma-Aldrich	Internal standard	I, III-V
Metanephrine (MN)	Sigma-Aldrich	Standard	I-V
3-Methoxytyramine (3MT)	Sigma-Aldrich	Standard	I-V
Noradrenaline (NA)	Sigma-Aldrich	Standard	II, V
Normetanephrine (NMN)	Sigma-Aldrich	Standard	I-V
Acetone	Mallinckrodt	Solvent for MAPT	II
Acetonitrile	Rathburn	Sheath liquid constituent	III
Ammonia	Riedel-de Haën, Merck	25%, capillary conditioning	III, V
Ammonium acetate	Merck, Sigma-Aldrich	Electrolyte	I-V
Ammonium hydroxide	J.T. Baker	Capillary conditioning	II, V
Bind-Silane (MAPT, ν-metacryl-oxypropyltrimethoxysilane)	Pharmacia	Reagent for permanent coating	II
Dibutylamine	Fluka	Reagent for dynamic coating	I
Diisopropylamine	Fluka	Reagent for dynamic coating	I
Disodium hydrogenphosphate	Merck	Reagent in SPE	I, IV, V
EDTA (IDRANAL III)	Riedel-de Haën	In LC mobile phase	IV
Ethanol	Primalco	96 v-%, solvent	V
Glacial acetic acid	J.T. Baker, Mallinckrodt, Rathburn	For pH adjustment of CE electrolyte	I-V
Glycine	Merck	Reagent for dynamic coating	II
Helix pomatia juice	BioSepra	100000 Fishman Units of η-glucuronidase and 1000000 Roy Units of sulphatase	I, IV
Hydrochloric acid	Riedel-de Haën, Merck	37%, in acid hydrolysis	I, IV
Methanol	Rathburn, J.T Baker	Solvent	I-V
Morpholine	Fluka	Reagent for dynamic coating	II
Phosphoric acid	Riedel-de Haën	85%, for pH adjustment	II
1-Propanol	Riedel-de Haën	Solvent	V
2-Propanol	Rathburn	Sheath liquid constituent	III
Sodium acetate	Merck	In enzymatic hydrolysis	I, IV
Sodium hydroxide	J.T. Baker	Capillary conditioning	I-V
Sodium dihydrogenphosphate	J.T. Baker, Merck	In SPE, electrolyte	I, II, IV
Trichloroacetic acid (TCA)	Merck	In LC mobile phase	IV
Triethanolamine	Merck	Reagent for dynamic coating	I
Triethylamine (TEA)	Merck, Fluka	Reagent for dynamic coating	I, II
Water	Millipore	Milli-Q	I-V

AG MP-50	Bio-Rad Laboratories	Cation exchange resin	IV
Fused silica capillary	Composite Metal Services	50 σm id., 375 σm od.	I-V
Nanospray capillaries	New Objective Inc.	Tip 8∂1 σm id., multilayer conductive coating	V
Nucleosil 10C18	HPLC Technology Co	LC column	IV
Oasis HLB	Waters	SPE cartridges, 30 mg	I, IV
Quik-Snap Column	Isolab Inc.	Cation exchange columns (CEX)	IV

5.2 Instruments

CE, MS and LC instruments used in studies I-V are listed in Table 7. The CE instrument was used with a UV detector in studies I-II and IV-V. The CE–MS coupling was based on coaxial sheath flow in studies III and IV, and a sheathless nanospray coupling was used in study V. LC with EC detection was employed for the comparative analysis of urine samples (IV).

Table 7. CE, MS and LC instruments used in studies I-V.

Paper	Capillary electrophoresis	Mass spectrometry	Liquid chromatography
I	Beckman P/ACE MDQ		
II	Beckman P/ACE 5000 and Beckman P/ACE MDQ		
III	Beckman P/ACE MDQ	Micromass Quattro II	
IV	Beckman P/ACE 2200 and Prince Technologies PrinCE	Bruker Esquire	HP 1050 autosampler, LKB 2150 HPLC pump, Bio-Rad Laboratories HPLC column heater, Bioanalytical Systems LC-4B amperometric EC detector
V	Beckman P/ACE MDQ, Beckman P/ACE 2200 and Prince Technologies PrinCE	PE Sciex API 300	

5.3 Separation of catecholamines and methoxycatecholamines by CE and LC

CE separations of cationic catecholamines and methoxycatecholamines were performed in acidic conditions. Ammonium acetate was selected as the electrolyte for CE separations due to the buffering capacity at pH range 2.8-6.8. Owing to its volatility, ammonium acetate is also a suitable electrolyte for MS. A 50 mM ammonium acetate concentration with pH adjusted to 4.0 provided a baseline separation of the eight structurally similar compounds. The samples were hydrodynamically injected to the capillary, and separations were performed with normal polarity in the electric field. UV detection was at wavelength 200 nm. LC analyses of urinary NMN and MN with EC detection were routinely performed at pH 3.0 in the hospital laboratory (IV).

5.4 Optimization of CE–MS determination of catecholamines and methoxycatecholamines

The CE–MS studies on DA and methoxycatecholamines were performed with ESI in positive mode with triple quadrupole and ion trap MS instruments. Optimization of the CE separation,

the CE–MS coupling performance with the coaxial sheath flow interface and the MS parameters used with the triple quadrupole instrument are described in Paper III. The developed CE–MS method with coaxial sheath liquid coupling was adapted for ion trap MS and applied to quantitative urine sample analysis (IV). The CE–MS analysis of catecholamines and methoxycatecholamines with sheathless nanospray coupling was studied in non-aqueous separation conditions (V). The commercial nanospray capillaries had a conductive coating on the tapered tip. This method was tested for urine sample analysis (V).

5.5 Urine sample analysis

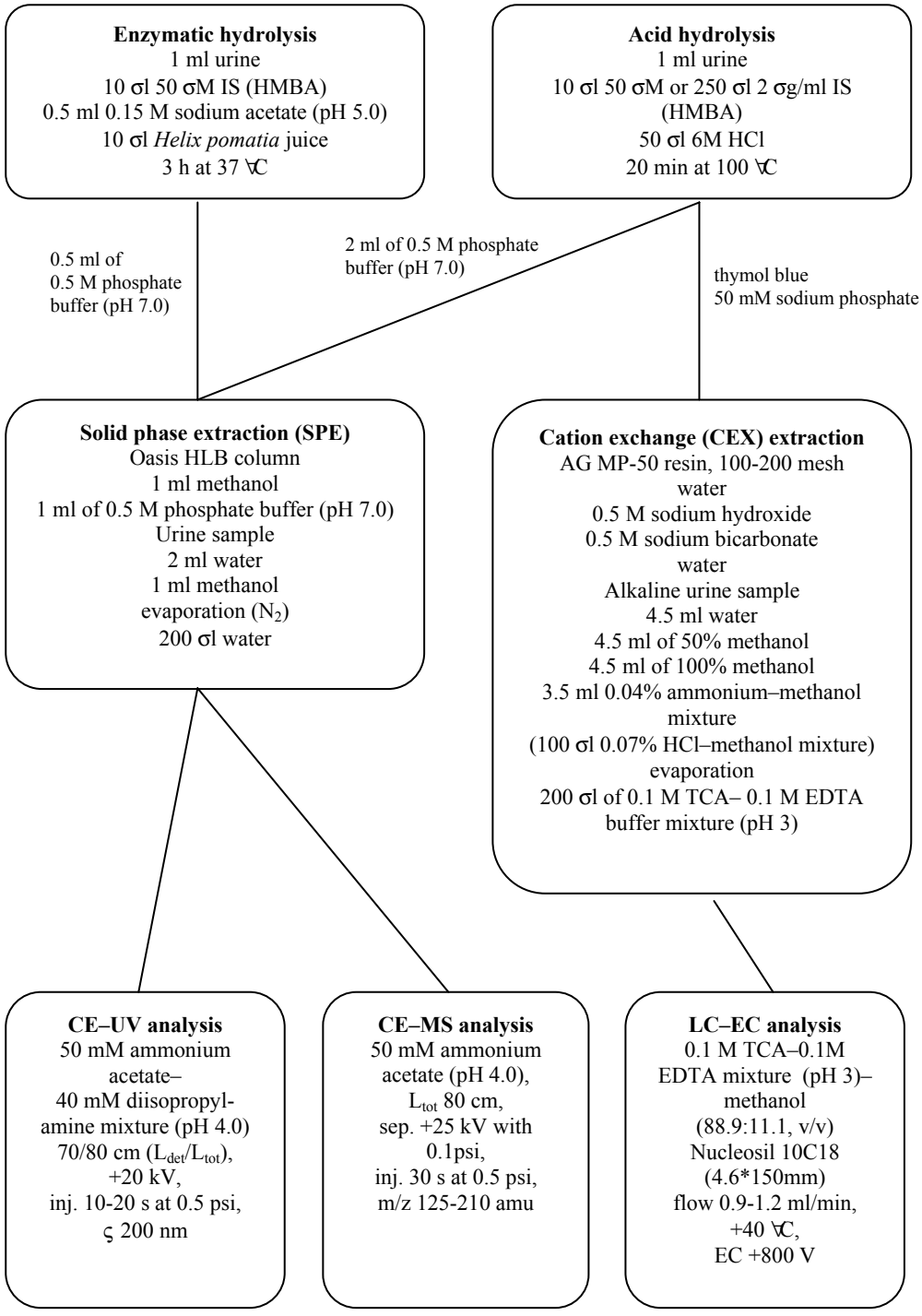
Method development for the determination of DA and methoxycatecholamines in urine was done with a pooled urine sample from healthy volunteers (I). The statistical concentrations of methoxycatecholamines in human urines of healthy Finnish people are below 1.2 σM, 4.0 σM, and 1.7 σM for 3MT, NMN and MN, respectively [46]. Also during method development, samples of a pooled urine spiked at two concentration levels were analysed in triplicate. In addition, patient urine samples obtained from the daily routine of a hospital laboratory and having a wide range of excreted amounts of catecholamines and methoxycatecholamines were analysed (IV). The results from stability studies of catecholamine and methoxycatecholamine standards in acid, base and enzymatic hydrolysis conditions were utilised in work with the urine samples (I). Enzymatic hydrolysis of buffered urine with *Helix pomatia* containing both η-glucuronidase and sulphatase activities (I, IV) and acid hydrolysis with hydrochloric acid (IV) were performed to deconjugate the sulphate and glucuronide conjugates of dopamine and the methoxycatecholamines. Purification and preconcentration of the hydrolysed urine samples buffered to pH 7.0 were performed with SPE based on a copolymer of N-vinylpyrrolidone and divinylbenzene (I, IV). Strong CEX extraction was used for comparison (IV). Pretreatment and analysis of patient urine samples are presented as a flow chart in Figure 5. Combinations of the hydrolysis, purification, analysis and quantitation methods (A-G) used in comparative studies (IV) are listed in Table 8.

Table 8. Combinations of hydrolysis, purification, analysis and quantitation methods used in the determination of urinary DA and methoxycatecholamines.

Method combination	Hydrolysis		Purification		Analysis			Quantitation	
	acid	enzyme	CEX	SPE	LC–EC	CE–UV	CE–MS	IS	ES
A	x		x		x			x	
B		x		x		x		x	
C	x			x		x		x	
D	x			x		x			x
E		x		x			x	x	
F	x			x			x	x	
G	x			x			x		x

IS: internal standard, ES: external standard

Figure 5. Pretreatment and analytical methods for the determination of DA and methoxycatecholamines in urine sample.



5.6 Dynamic and permanent capillary coating

Both dynamic and permanent coatings in fused-silica capillaries were studied. The dynamic coating was performed by addition of triethylamine (TEA), glycine or morpholine to ammonium acetate or sodium phosphate electrolyte solution (II). In view of the better performance of TEA, closer study was then made of amine reagents – diisopropylamine, dibutylamine, triethylamine and triethanolamine – in ammonium acetate electrolyte solutions (I). Paper II reports the separation of catecholamine and methoxycatecholamine standards, and Paper I the analysis of urine samples. A permanent coating with covalent bond formation was prepared with a silyl reagent (ν-acryloxypropyltrimethoxysilane, MAPT) (II). Again, ammonium acetate and sodium phosphate electrolyte solutions, at pH 4.0 and 3.0, respectively, were used for the separation of catecholamine and methoxycatecholamine standards. Resolution and electrophoretic mobility data for the catecholamines and methoxycatecholamines as well as the electroosmotic mobility and separation efficiency in terms of plate numbers were used in the evaluation of the preparation and performance of the coatings (II). Repeatability of the coating procedure was determined with three to five replicate capillaries with each coating type.

5.7 Non-aqueous solvents in CE studies of catecholamines and methoxycatecholamines

Separations of catecholamine and methoxycatecholamine standards were performed in aqueous electrolyte solution and in alcoholic electrolyte solutions of methanol, ethanol and 1-propanol, with anhydrous ammonium acetate as the electrolyte (V). Electrophoretic mobilities were determined with methanol or ethanol as the neutral EOF marker. The diffusion coefficients for the analytes were determined in aqueous and non-aqueous solutions using the stopped migration method [69]. In comparisons of the separation efficiency of the different solutions, plate numbers for catecholamines and methoxycatecholamines were determined as mean values of five determinations. In addition, the separation media were compared in terms of resolution between the analytes, selectivity and sensitivity in UV detection. The separation conditions for catecholamines and methoxycatecholamines were optimized in ethanol based solution in terms of ammonium acetate and acetic acid concentrations, capillary length and separation voltage. Analyses of spiked urine samples were performed with the optimized non-aqueous conditions.

6 RESULTS AND DISCUSSION

Analytical methods for the determination of DA and methoxycatecholamines were developed, including enzymatic hydrolysis of conjugates, purification with SPE and analysis with CE–UV and CE–MS. The new methods were compared with an existing LC–EC method with acid hydrolysis and CEX purification. In the development of the CE–MS method based on coaxial sheath liquid coupling, parameters such as CE separation conditions, CE–MS interface performance and MS voltages were optimized in order to obtain a method sensitive enough for the determination of DA and methoxycatecholamines in urine. In addition, the CE–MS behaviour of catecholamines and methoxycatecholamines was studied in non-aqueous electrolyte solutions with sheathless nanospray coupling of CE and MS.

Coating of the fused-silica capillary dynamically and permanently was studied as a means of enhancing resolution between catecholamines and methoxycatecholamines and matrix compounds in urine samples. Non-aqueous background electrolytes of methanol, ethanol and 1-propanol were investigated and compared with aqueous background electrolyte in terms of physico-chemical properties of the solvents and of analytical parameters such as resolution and separation efficiency. The results of these investigations are discussed, in order, below.

6.1 Determination of urinary dopamine and methoxycatecholamines

Catecholamines and their methoxy metabolites are metabolized in living organisms to form glucuronide and sulphate conjugates. In order for the total amount of catecholamines and methoxycatecholamines in urine to be determined, the conjugates must be hydrolysed to free the analytes. Urine is a complex matrix with many endogenous compounds that interfere with the analysis of catecholamines and methoxycatecholamines. A purification method is therefore needed to selectively extract the analytes from urine. The purification also serves as a preconcentration method to increase the concentration of the analytes in the sample before the separation with CE and detection with UV and MS.

Hydrolysis

The method development for urinary DA and methoxycatecholamines was started with stability studies on free amines in aqueous solutions under acid, base and enzymatic hydrolysis conditions (I). Strong acid conditions degraded the standards totally, and only in 5–10 mM hydrochloric acid with incubation below +38 ∀C were the analytes recovered in amounts over 70% (Table 9). In basic hydrolysis, the catecholamines having the 3,4-dihydroxy group degraded entirely irrespective of the conditions. The methoxycatecholamines were recovered in amounts of 80–90% in 5–20 mM base at room temperature. In enzymatic hydrolyses with *Helix pomatia*, the degradation of standards was linear up to 80% with increasing enzyme activity. Acid hydrolysis is efficient in deconjugating sulphate conjugates but inefficient for glucuronide conjugates [10, 11]. Therefore, as only enzymatic hydrolysis with activities of both η-glucuronidase and sulphatase is able to deconjugate both glucuronides and sulphates, enzymatic

hydrolysis was selected for the hydrolysis of urinary DA and methoxycatecholamine conjugates. The conditions for the hydrolysis were 3 h incubation at +37 ∀C with 1000 units of η-glucuronidase and 10 000 units of sulphatase per ml urine, at pH 5.0 (I). Lower concentrations of methoxycatecholamines were obtained after enzymatic hydrolysis than after acid hydrolysis (IV) (Table 10). The enzyme activity was then increased up to 20 000 U and 200 000 U of η-glucuronidase and sulphatase, respectively, and incubation time and temperature were increased. However, no increase was found in the amounts of free methoxycatecholamines. Thus, acid hydrolysis was applied to the urine samples (IV).

Table 9. Validation data for the determination of urinary DA and methoxycatecholamines.

	HMBA	DA	3MT	NMN	MN
Recovery in acid hydrolysis (%)					
In water (10 mM HCl, 30 ∀C, 30min)	-	73	87	84	93
In urine (286 mM HCl, 100 ∀C, 20 min), with SPE	30	13	99	26	79
Recovery (%) in SPE					
1.0 σM spiking	96	109	113	106	105
5.0 σM spiking	102	98	123	104	124
Repeatability (%RSD) in SPE					
5.0 σM spiking	5.0	5.3	4.7	5.4	3.7
LOD (σM)					
CE–UV (MDQ); 10 nl inj.	0.4	0.7	0.5	0.6	0.4
CE–UV (P/ACE 2200); 21 nl inj.	-	0.7	0.5	0.5	0.8
LC–EC; 10 σl inj.	-	-	0.2	0.1	0.1

- not determined

Purification

SPE purification was developed to remove the urinary matrix compounds interfering with the UV detection (I). pH and ionic strength of the phosphate buffer were adjusted to obtain good retention of DA and the methoxycatecholamines in the non-ionizable polymer resin. The poor recovery of DHBA in the SPE required a change in the internal standard to HMBA (I). Good recoveries were then obtained with spiked samples (I), and further optimization of the final dissolution procedure resulted in good repeatabilities of the method (IV) (Table 9). Poor recoveries were obtained for HMBA, DA and NMN from acid hydrolysed urine samples with SPE purification (IV) (Table 9). Possibly this was due to the degradation of these compounds in acid hydrolysis conditions, though a good acid stability has been reported for DA [10]. Another reason for the poor recovery could have been poor retention of HMBA, DA and NMN in the SPE phase after acid hydrolysis since, during SPE method development, the ionic strength of the sample was observed to have a major effect on the retention of the analytes (I). The higher ionic strength of the sample after acid hydrolysis than after enzymatic hydrolysis may have reduced the retention of HMBA, DA and NMN in the SPE phase (IV). The SPE purification was highly efficient and, besides DA and the methoxycatecholamines, only a few matrix compounds were seen in the UV electropherograms (I). Purification of urine samples for LC–EC analysis with CEX extraction was even more efficient in removing the matrix compounds

sensitive to the detector, but the high salt concentration of the sample purified by CEX made it unsuitable for CE analysis (IV). Likewise, the SPE purification developed for CE could not be used with LC analysis.

Table 10. Results for the determination of NMN, MN, 3MT and DA (σmol/l) in patient urine samples by Methods A-G (see Table 8).

		Method						
Sample		**A**	**B**	**C**	**D**	**E**	**F**	**G**
1	NMN	1.21	0.27	1.75	0.54	<0.20	0.93	0.54
	MN	0.78	0.51	3.68	1.09	nd.	2.44	1.41
	3MT	na.	0.66	2.93	0.88	0.96	2.02	1.20
	DA	na.	2.71	5.70	1.68	2.72	3.13	1.69
2	NMN	0.95	0.70	1.15	0.83	0.24	0.72	0.43
	MN	0.50	<0.19	1.13	0.81	0.62	1.66	0.95
	3MT	na.	0.72	0.63	0.46	0.67	0.80	0.49
	DA	na.	1.37	1.60	1.14	1.20	2.40	1.34
3	NMN	0.54	nd.	nd.	nd.	nd.	nd.	nd.
	MN	0.29	1.03	31.47	1.87	nd.	nd.	nd.
	3MT	na.	0.90	1.98	0.15	0.71	nd.	nd.
	DA	na.	1.01	5.13	0.34	nd.	nd.	nd.
4	NMN	1.38	0.41	1.04	0.77	0.22	0.82	0.85
	MN	0.62	0.43	1.15	0.84	nd.	1.33	1.35
	3MT	na.	0.57	0.77	0.58	0.63	0.79	0.84
	DA	na.	2.55	3.59	2.57	2.67	5.07	5.09
5	NMN	2.79	0.38	1.00	0.82	0.73	2.92	1.11
	MN	2.09	0.90	3.44	2.73	1.24	10.66	4.28
	3MT	na.	1.06	1.06	0.87	1.15	2.96	1.11
	DA	na.	1.03	0.55	0.48	0.80	2.53	0.71
6	NMN	1.66	0.32	1.90	0.41	nd.	0.75	<0.20
	MN	0.71	0.71	2.20	0.45	nd.	1.60	0.35
	3MT	na.	0.77	1.91	0.40	0.57	0.79	0.21
	DA	na.	2.72	3.13	0.63	3.26	2.21	0.42

nd. not detected; na. not analysed

Analysis by CE–UV, CE–MS and LC–EC

A baseline separation of DA and the methoxycatecholamines with CE was obtained with 50 mM ammonium acetate (pH 4.0) electrolyte solution (I). However, some urine matrix compounds interfered with the separation even after SPE, and the electrolyte solution was modified with the addition of diisopropylamine to form a dynamic coating on the capillary wall. This decreased the EOF and increased the resolution between the analytes and matrix compounds (I). Sufficient limits of detection were obtained for DA and the methoxycatecholamines, even for the determination of these compounds in urine samples of healthy persons (Table 9). The repeatability of the peak areas between injections and of migration times with CE–UV were %RSD 0.3–10 and 0.3–0.4, respectively. The LC–EC analysis of methoxycatecholamines was more sensitive than CE–UV owing to the larger

injection volume and the more catechol-specific detector (IV). The repeatabilities of peak heights and retention times in LC–EC were %RSD 5.6–9.4 and 0.8–1.2, respectively. When DA and methoxycatecholamines in spiked urine samples were determined by CE–UV in ethanolic instead of aqueous conditions, only a few matrix peaks appeared in the electropherograms (V). The ethanol based electrolyte solution provided a faster analysis than aqueous solution with dynamic coating reagent. However, the non-aqueous method was not applied to real urine samples.

The determination of urinary DA and methoxycatecholamines by CE–MS was a robust method with aqueous conditions in CE and coaxial sheath liquid coupling (III, IV). A highly specific detection was provided by MS, eliminating the interfering matrix compounds observed in the electropherograms with UV detection. Dynamic coating reagent was not added to the separation electrolyte with MS detection, and the migration times fluctuated slightly in the bare fused-silica capillary (%RSD 2.8-3.0); however, the relative migration times were highly repeatable (%RSD 0.2-0.3) (IV). The power of CE–MS for analysis of urinary DA and methoxycatecholamines was also demonstrated with ethanolic conditions in CE and sheathless nanospray coupling (V). No interfering compounds were detected, but the analysis time was considerably longer than in the aqueous separation without dynamic coating reagent. The method also was not robust enough for routine analysis.

Comparison of methods

Inspection of the results obtained with the different methods for the determination of urinary DA and methoxycatecholamines – with acid or enzymatic hydrolysis, with CEX extraction or SPE based on polymer resin, with LC–EC, CE–UV or CE–MS analysis and with quantitation based on internal (IS) or external standard – showed that the different variables could not be adjusted so as to obtain similar results (IV) (Table 10). The differences in the results could nevertheless be interpreted as originating from (1) the efficiency of the hydrolysis, the acid hydrolysis being more efficient in deconjugation, (2) the stabilities of the free amines in the hydrolysis conditions and their recoveries in the purification methods, especially the recovery of IS, (3) the sensitivity, with LC–EC being more sensitive than CE–UV or CE–MS, and (4) the specificity of the detection, with MS being the most specific and UV the least specific detection method.

6.2 Determination of catecholamines and methoxycatecholamines by CE–MS with coaxial sheath liquid and sheathless nanospray couplings

Mass spectra for DA and the methoxycatecholamines were measured with triple quadrupole and ion trap instruments by direct infusion into MS. All the analytes produced a protonated molecular ion $[M+H]^+$, where the positive charge is located at the amine group (III). In addition, the spectra showed fragments representing the loss of ammonia and water. For HMBA, DA and 3MT, the first fragmentation in MS was the cleavage of ammonia. For NMN and MN, however, the cleavage at lowest energy was the loss of water molecule, producing a resonance stabilized benzyl cation. HMBA and DA are of same molecular weight and produce fragments with the

same m/z values but with different relative abundances. The lower relative abundance of the molecular ion of HMBA is due to the further fragmentation to a resonance stabilized benzyl cation, a stabilization that is not possible for DA. The same ions were present in the spectra obtained with the different mass analysers but with some differences in the relative ion abundances (Table 11). Lower relative intensities were obtained for the $[M+H]^+$ ions and higher relative intensities for the fragment ions with the ion trap instrument. No attempt was made to explain the results in terms of the different ion optics of the two instruments. Base peaks (100%) were used in quantitation.

Table 11. Ion fragments (m/z) and abundances (%) for DA and methoxycatecholamines with triple quadrupole (QqQ) and ion trap (QIT) MS instruments.

		$[M+H]^+$		$[M+H-X]^+$	
Compound	**Analyser**	**m/z**	**%**	**m/z**	**%**
HMBA	QqQ	154	17	137	100
	QIT	154	3	137	100
DA	QqQ	154	100	137	11
	QIT	154	100	137	49
3MT	QqQ	168	100	151	16
	QIT	168	100	151	78
NMN	QqQ	184	44	166	100
	QIT	184	17	166	100
MN	QqQ	198	100	180	56
	QIT	198	51	180	100

X: NH_3 for HMBA, DA and 3MT, and H_2O for MN and NMN

Coaxial sheath liquid coupling

In optimization of the CE–MS performance, sheath liquid composition of 2-propanol–water (50:50, v/v) gave stronger DA and methoxycatecholamine signals than methanol or acetonitrile with water at the same percentage (III). Increasing the amount of organic solvent in the sheath liquid was expected to increase the signal intensities, as the droplet formation and evaporation in the electrospray process become easier [126]. However, increasing the amount of 2-propanol in the sheath liquid caused the DA and methoxycatecholamine signals to decrease. This could have been due to the high viscosity of 2-propanol [134]. Increase in the amount of methanol, on the other hand, was accompanied by a considerable increase in the signal intensities (Figure 6) (III). As the spray was not stable with 100% methanol, a compromise was made between the signal intensity and the stability of the spray, and methanol–water mixture (80:20, v/v) was chosen as the sheath liquid. Since the separation electrolyte was ammonium acetate, the formation of moving ionic boundaries in the separation capillary was avoided by choosing acetic acid as the additive for the sheath liquid. The concentration of acetic acid was optimized in the range of 0.1–1.0% with only minor effect on the intensities of the signals (III).

The effect of coaxial sheath liquid flow rate on signal intensity, on peak-width at base and on the resolution of HMBA and DA, as well as DA and 3MT, was evaluated (III). The strongest signals and the narrowest peak-widths at base in the electropherograms were obtained with the

flow rate of 6 σl/min (Figure 7) (III). Also, a baseline separation between HMBA and DA was achieved with this flow rate. Maximum signals for DA and methoxycatecholamines were obtained with 4.0 kV voltage at the electrospray needle. As both $[M+H]^+$ and fragment ions were detected for DA and methoxycatecholamines, an increase in the cone voltage decreased the signal intensities for $[M+H]^+$ ions and increased them for the fragment ions.

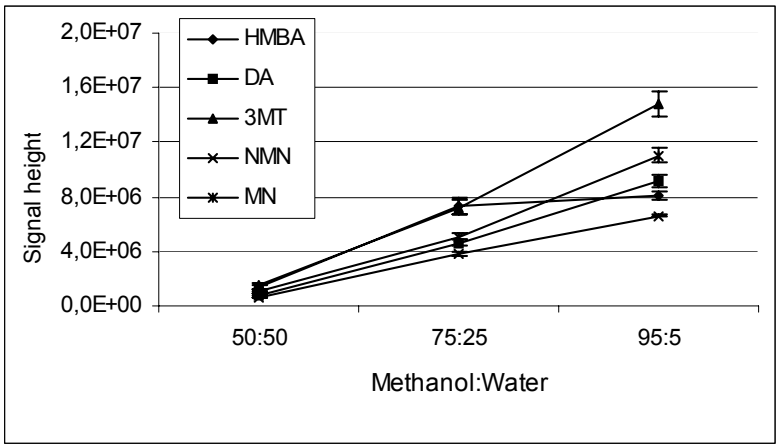


Figure 6. Effect of sheath liquid composition (methanol–water mixtures) on signal intensities of DA and methoxycatecholamines.

A zero potential at the electrospray needle during sample injection and 30 s thereafter increased the signal intensities of DA and the methoxycatecholamines considerably (III). The signals obtained with zero voltage at the needle during injection were 14 times stronger than the signals obtained with 4.0 kV voltage. The 4.0 kV positive voltage at the end of the CE capillary caused a change in the direction of the EOF towards the inlet of the capillary, which induced the driving of the sample out of the capillary. With zero potential for 30 s, the analytes migrated further into the separation capillary, and no loss of the injected sample took place. This effect was not studied with the ion trap instrument, in which the spray capillary with orthogonal configuration is grounded and a negative potential is applied to the counter electrode (IV). The siphoning effect due to 10 cm height difference between the liquid levels at the CE capillary ends with the inlet end at a lower position also resulted in considerable decrease in signal heights of the analytes (III). Signal intensities with 10 cm height difference were only 16% of those with no height difference between the capillary ends.

The optimized CE–MS conditions were separation in 50 mM ammonium acetate electrolyte solution (pH 4.0) with 25 kV separation voltage and 0.1 psi pressure. Sheath liquid composition was methanol–water (80:20, v/v) with 0.5% acetic acid, introduced at 6 σl/min with nebulizing gas rate of 35 l/h and ion source temperature of 70 ∀C. Potential at the electrospray needle during injection was avoided, as was the siphoning effect.

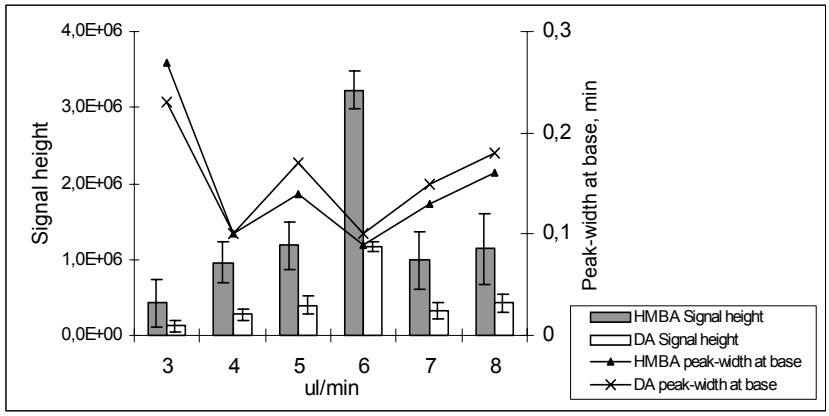


Figure 7. Effect of sheath liquid flow rate (σl/min) on signal height and on peak-width at base of HMBA and DA.

Sheathless nanospray coupling

The CE–MS analysis of catecholamines and methoxycatecholamines was studied with sheathless nanospray coupling with water, methanol, ethanol and 1-propanol as the background electrolyte solvent (V). In coaxial CE–MS coupling the ionization process is dominated by the sheath liquid. With sheathless nanospray coupling, only the CE electrolyte solution affects the ionization, and a non-aqueous electrolyte solution can have an important effect. With the nanospray coupling, the ammonium acetate concentration in the electrolyte solution had to be decreased to obtain stable spray when water, methanol and ethanol were used as the electrolyte solvents (V). Even lower concentration was needed with 1-propanol, owing to the poor solubility of ammonium acetate in 1-propanol, which resulted in a slight precipitation of ammonium acetate at the tip of the nanospray capillary. In addition, the electrolyte solutions prepared with water and 1-propanol needed to be modified with 40% and 10% of methanol, respectively, to decrease the surface tension and viscosity and so obtain a stable spray. The suitable electrospray needle voltages for water, methanol, ethanol and 1-propanol solutions were between 1300 and 2000 V. Lower voltages were sufficient for methanol and ethanol because of their lower surface tension and viscosity compared with those of water and 1-propanol. Unlike in non-aqueous solutions, the higher conductivity in aqueous solution easily led to electric discharge from the capillary tip. Only minor differences in sensitivity of the analyses with aqueous and non-aqueous solutions were noticed.

Sensitivity comparisons

Limits of detection (LOD) for DA and the methoxycatecholamines with aqueous CE separation and coaxial sheath liquid coupling using triple quadrupole (III) and ion trap (IV) analysers, and with sheathless nanospray coupling using the triple quadrupole analyser in non-aqueous ethanolic separation conditions (V), are presented in Table 12. Also included are the linearity data for coaxial sheath liquid methods. Significant differences were not observed in the LOD

values, despite the 25–50-fold gain in sensitivity reported in the literature for nanospray, relative to coaxial sheath liquid coupling [174], evidently because a larger fraction of the analyte is converted into gas phase ions. Migration times for catecholamines and methoxycatecholamines in aqueous separations with coaxial sheath liquid coupling were from 12.1 to 13.3 minutes, and from 22.1 to 25.2 minutes with sheathless nanospray coupling in ethanol based separation solution.

Table 12. Limits of detection (LOD) (σM) and linearity correlation (R^2) for DA and methoxycatecholamines in CE–MS analyses with triple quadrupole (QqQ) and ion trap (QIT) analysers using coaxial sheath liquid coupling (SL) and sheathless nanospray coupling (N).

	HMBA	DA	3MT	NMN	MN	V(inj.), nl
LOD						
QqQ-SL	4.1	1.9	1.1	3.2	3.2	22
QIT-SL	nd.	1.2	0.9	0.7	1.4	23
QqQ-N	0.5	1.3	0.8	0.9	1.1	18
Linearity, R^2						
QqQ-SL	0.98	0.97	0.97	0.92	0.96	22
QIT-SL	nd.	0.94	0.97	0.95	0.95	23

nd., not determined; V(inj.), injection volume

6.3 Separation of catecholamines and methoxycatecholamines by CE with dynamically and permanently coated capillaries

The effect of dynamic and permanent coatings of the fused-silica capillary on electrophoretic mobility, resolution and efficiency of catecholamine and methoxycatecholamine separations was studied with TEA, glycine and morpholine as dynamic coating reagents and MAPT as permanent coating reagent (II). Ammonium acetate (50 mM, pH 4.0) and sodium phosphate (50 mM, pH 3.0) were used as the electrolyte solutions.

Dynamic coating

In dynamic coating, the apparent electrophoretic mobilities of the catecholamines and methoxycatecholamines in phosphate electrolyte solution were reduced most with TEA as the coating additive (Figure 8). Mobilities were also lowest with TEA as the additive in acetate electrolyte solution, and mobilities were lower with morpholine than with glycine. The adsorption of TEA and morpholine on the silica wall via the positively charged amine resulted in more or less neutral capillary wall, and with reduced EOF, the electrophoretic mobilities of catecholamines and methoxycatecholamines were reduced. However, with glycine adsorbing on the silica wall, the capillary surface was still negatively charged due to the anion group of the acid, and the EOF was altered only slightly. The instability of the coating on the capillary wall was detected as fluctuation in migration times of the analytes. The most effective stabilization of the coating was obtained with TEA in acetate buffer. TEA was a flexible enough molecule to

produce an even layer on the capillary wall in two conditioning runs. The ring structure of morpholine makes the molecule rigid and a uniform coating was only slowly achieved. Best resolution between the analytes was obtained with TEA as the modifier (Table 13). TEA enhanced the resolution by reducing the EOF in the capillary. In addition, the ionic strength of the electrolyte solution with ionic additive was increased relative to the unmodified electrolyte in the bare fused-silica capillary, likewise decreasing the EOF. The highest plate numbers – over 300 000 plates/m – were obtained with TEA (40 mM) modified ammonium acetate buffer (50 mM, pH 4.0). The electrophoretic mobilities of catecholamines and methoxycatecholamines were very slow in TEA modified phosphate electrolyte solution; migration times were very long and the efficiency of the separation was poor.

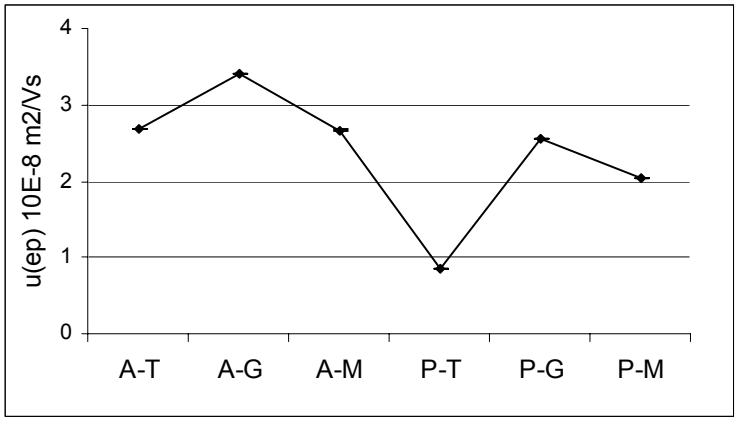


Figure 8. Effect of dynamic coating on electrophoretic mobilities of DHBA. Analytes behaved similarly to DHBA. (A: ammonium acetate (50 mM, pH 4.0); P: sodium phosphate (50 mM, pH 3.0); T: triethylamine; G: glysine; M: morpholine)

Since use of TEA as the dynamic coating reagent gave the best enhancement in resolution (II), other alkyl amines, namely diisopropylamine, dibutylamine and triethanolamine in ammonium acetate electrolyte solutions were studied in the hope of obtaining still better resolution between the analytes and matrix compounds in urine samples (I). Diisopropylamine (40 mM) performed marginally better and was selected for use in separation of DA and methoxycatecholamines in urine samples.

Permanent coating

ν-Methacryloxypropyltrimethoxysilane (MAPT) is a reactive bifunctional silane reagent that reacts through the silane group with the surface silanols in fused-silica capillary wall. The double bond introduced by the MAPT reagent usually is used to attach and polymerize monomers to the capillary surface. However, no polymerization was performed in our studies, as we wished to investigate solely the effect of silanization of the capillary wall on the separation of catecholamines and methoxycatecholamines (II). The free silanol groups at the capillary wall were reduced due to the silanization leading to decreased EOF. In addition, the

hydrophobic chain of MAPT was able to shield the capillary wall from the analytes, reducing analyte–wall interactions and increasing separation efficiency.

The MAPT reagent was used in 1–50% concentrations in the coating reaction. Better separation of the catecholamines and methoxycatecholamines was achieved with the modified capillaries than with bare fused-silica capillaries. EOF was reduced in capillaries coated with 30-50% MAPT. EOF was also stabilized, producing highly repeatable mobilities of catecholamines and methoxycatecholamines. However, the EOF was not totally eliminated in MAPT capillaries, indicating the presence of some unchanged silanol groups in the capillary wall. Higher electrophoretic mobilities were obtained in acetate than in phosphate based electrolyte. The residual EOF was more reduced in phosphate electrolyte solution than in acetate electrolyte solution owing to the lower pH and higher ionic strength of the electrolyte solution. Best resolution of the catecholamines and methoxycatecholamines was obtained in a capillary coated with 30% MAPT, with phosphate buffer (Table 13), but the best efficiency of the separation in terms of plate numbers was achieved in a capillary coated with 1% MAPT, with 50 mM phosphate electrolyte solution (pH 3.0).

Table 13. Comparison of mean resolutions (R_m) for catecholamines and methoxycatecholamines in dynamically and permanently coated capillaries. Concentration of dynamic modifiers was 40 mM.

		Uncoated	**Dynamic coating**			**Permanent coating with MAPT**		
R_m		FS	TEA	glycine	morpholine	1%	10%	30%
DHBA-DA	A	1.68	2.53	1.67	2.07	2.16	2.17	2.69
	P	2.93	7.40	2.64	3.66	2.72	3.38	3.55
DA-3MT	A	1.03	1.10	1.02	1.07	1.13	1.05	1.38
	P	1.02	1.56	0.89	0.84	0.98	1.10	1.46
3MT-NA	A	2.44	3.08	2.15	3.38	2.26	2.36	2.44
	P	3.42	11.9	3.58	5.50	3.35	4.44	2.24
NA-NMN	A	0.88	1.04	0.92	1.01	1.05	0.98	1.22
	P	0.83	1.31	0.81	0.77	0.80	1.05	1.14
NMN-A	A	1.92	2.54	1.79	2.44	1.78	1.90	1.90
	P	2.23	8.32	2.72	3.58	2.11	3.13	2.14
A-MN	A	0.81	1.10	0.87	0.96	0.99	0.87	1.06
	P	0.81	1.23	0.83	0.74	0.87	1.00	1.27

(n=3); A: ammonium acetate (50 mM, pH 4.0); P: sodium phosphate (50 mM, pH 3.0); FS: fused-silica; TEA: triethylamine; MAPT: ν-metacryloxypropyltrimethoxysilane

In conclusion, TEA showed the best performance among the dynamic coating reagents, and treatment with 30% MAPT produced the best resolution of catecholamines and methoxycatecholamines in permanent coating. Dynamic coating is easier and faster, and the coating is renewable, unlike the permanent coating.

6.4 Effect of non-aqueous solvents on separation of catecholamines and methoxycatecholamines by CE

Separation of catecholamines and methoxycatecholamines by CE was performed in aqueous medium and in non-aqueous media of methanol, ethanol and 1-propanol with UV detection (V). The analyses were compared in terms of electrophoretic mobility, resolution, plate numbers, diffusion coefficients and sensitivity. The electrophoretic mobilities of the catecholamines and methoxycatecholamines decreased when the electrolyte solvent was changed from water to methanol, ethanol and 1-propanol (Figure 9). The dielectric constants of the solvents decrease from water to 1-propanol (Table 14), and the dissociation of the analytes decreases in the same order. In addition, the pH and the apparent pH (pH*) of the electrolyte solution was increased from 3.7 to 6.4 from water to 1-propanol (Table 14) due to decrease in the dielectric constant of the solvent. The decreased dissociation was also observed as lower effective charge, which was calculated from mobility and diffusion coefficient data. The effective charges decreased on average from 0.96 (water) to 0.26 (1-propanol).

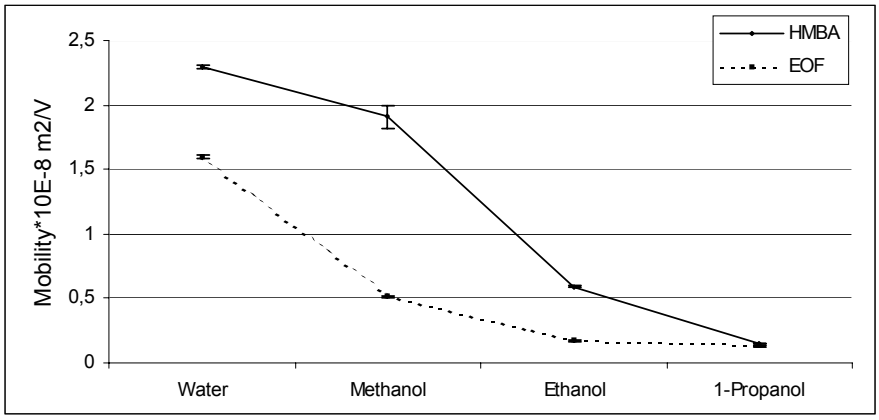


Figure 9. Electrophoretic mobility of HMBA and electroosmotic mobility in aqueous and non-aqueous solutions of 20 mM ammonium acetate in ROH:acetic acid (99:1, v/v) with ROH being water, methanol, ethanol or 1-propanol. Analytes behaved similarly to HMBA.

Viscosities of the solvents increase from water to 1-propanol (Table 14). The hydrated radius of the catecholamines and methoxycatecholamines increased on average from 4.2 Å to 9.5 Å as the water molecules in the hydration sphere were replaced by the bulkier alcohol molecules. These two factors (viscosity and hydrated radius) increase the frictional force of the analytes, decreasing their mobilities. Reduction in the electroosmotic mobility was observed when the solvent was changed from water to 1-propanol (Figure 9). The decrease in dielectric constant of the solvent (Table 14) decreased the ionization of the silanol groups in the capillary wall reducing the electroosmotic mobility.

Table 14. Physical and chemical properties of the solvents [from Refs. 54, 191, 179] with measured pH and apparent pH (pH*) of the electrolyte solutions of 20 mM ammonium acetate in ROH:acetic acid (99:1, v/v), with ROH being water, methanol, ethanol or 1-propanol.

Solvent	κ	ξ (mPas)	κ/ξ	pK_{auto}	pH/pH*
Water	78.3	0.890	87.9	14.0	3.74
Methanol	32.7	0.545	60.1	17.2	5.94
Ethanol	24.6	1.089	22.6	18.9	6.28
1-Propanol	20.3	1.956	10.4	19.4	6.35

κ, dielectric constant; ξ, viscosity; $pK_{auto,}$ autoprotolysis constant

The dependence of the selectivity of the separation on the solvent was observed as changes in the migration order of the catecholamines and methoxycatecholamines. These changes reflected the dependence of the dissociation and solvation of the analytes on the dielectric constants and hydrogen bonding abilities of the solvents. The plate numbers, indicating the efficiency of the separation, were determined in aqueous and non-aqueous conditions and were found to be higher in non-aqueous than in aqueous solvents (Figure 10). Also, the diffusion coefficients for the catecholamines and methoxycatecholamines were determined in the solvents (Figure 10) by stopped migration method, and were found to correlate inversely with the viscosity of the solvent (Table 14). Owing to the low diffusion, the highest plate numbers were achieved in ethanol. The limits of detection were determined for the catecholamines and methoxycatecholamines with UV detection. Sensitivity did not change in any significant degree from solvent to solvent.

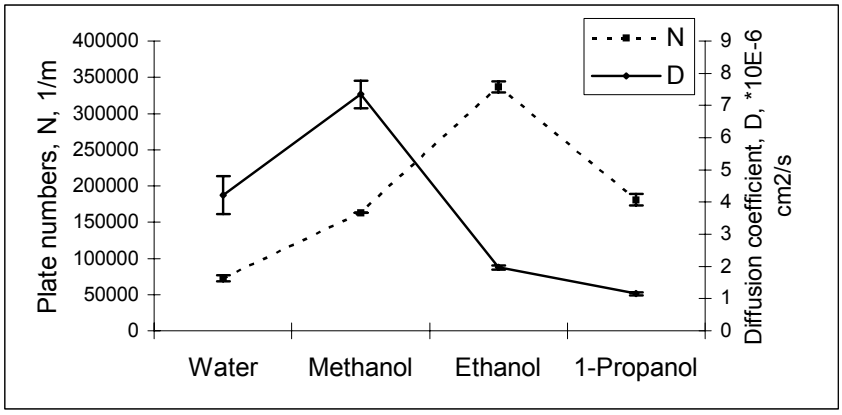


Figure 10. Plate numbers (N) and diffusion coefficients (D) for HMBA in aqueous and non-aqueous solutions of 20 mM ammonium acetate in ROH:acetic acid (99:1, v/v), ROH being water, methanol, ethanol or 1-propanol. The analytes showed similar behaviour to HMBA.

In summary, the electrophoretic mobilities of the catecholamines and methoxycatecholamines were strongly affected by the nature of the electrolyte solvent, the effect being due to changes in the hydrodynamic size of the analytes, their dissociation, the magnitude of EOF and the viscosity of the electrolyte solution. The selectivity of the separation could be changed through choice of a different solvent. Non-aqueous separations were more efficient than aqueous, and ethanol was the best of the non-aqueous solvents in terms of resolution and efficiency.

7 CONCLUSIONS

The enzymatic hydrolysis of catecholamine and methoxycatecholamine conjugates with *Helix pomatia* proved to be inefficient. More efficient deconjugation was obtained with acid hydrolysis. The purification method based on SPE on a polymer-based resin was satisfactory in removing interfering matrix compounds from urine samples with good recoveries for DA and methoxycatecholamines, and with good repeatabilities. However, this purification method, which was optimized for enzymatically hydrolysed urine samples, was not directly applicable for the purification of acid hydrolysed samples, since the ionic strength of the sample had a major effect on the retention of the analytes on the sorbent. The polymer-based SPE developed for CE analysis, and the CEX extraction developed for LC analysis, were found not to be interchangeable but suitable only for the analytical method for which they were developed.

A baseline separation of catecholamines and methoxycatecholamines was obtained with 50 mM ammonium acetate (pH 4.0) electrolyte solution. The resolution between the analytes and matrix compounds was successfully increased by dynamically coating the fused-silica capillary wall with diisopropylamine to suppress the EOF. The optimized CE–UV analysis was sensitive enough for determination of DA and methoxycatecholamines in urine samples. Selectivity of the analysis was different in ethanol based non-aqueous CE conditions and aqueous conditions, and further validation of the non-aqueous analysis might lead to a faster determination of catecholamines and methoxycatecholamines in urine samples.

The catecholamines and methoxycatecholamines could be ionized for MS detection. Careful optimization of the CE–MS performance led to a sensitive and highly specific method for the determination of urinary DA and methoxycatecholamines. Conditions were 50 mM ammonium acetate electrolyte solution (pH 4.0) with 25 kV separation voltage and 0.1 psi pressure, coaxial sheath liquid composition of methanol–water (80:20, v/v) with 0.5% acetic acid, at flow rate of 6 σl/min, 4.0 kV ESI voltage and 20 V cone voltage. Unexpectedly, no improvement in sensitivity was achieved with the use of sheathless nanospray coupling. Methanol and ethanol, with their high volatility and low surface tension, were found to be suitable solvents for sheathless nanospray CE–MS analysis. Water and 1-propanol were not successfully sprayed at 100% composition. In addition, the high conductivity in aqueous electrolyte solution resulted in discharge and impaired analysis. The analysis of spiked urine samples was demonstrated with ethanol based separation and MS detection with sheathless nanospray coupling. However, the coaxial sheath liquid coupling was found to be more robust than sheathless nanospray coupling.

Both dynamic and permanent coating of the fused-silica capillary enhanced the resolution between catecholamines and methoxycatecholamines, and increased the separation efficiency. TEA produced a more uniform layer on the capillary wall than did morpholine or glycine. Best resolution of the analytes and highest plate numbers were obtained in dynamic coating with TEA as the electrolyte solution additive. Permanent coating with MAPT, which is used to provide double bonds for the fused-silica capillary wall, proved to be an efficient coating agent

even without polymerization reactions. Treatment of the capillaries with 30–50% MAPT resulted in only a minor residual EOF. Separation of catecholamines and methoxycatecholamines was enhanced in both acetate and phosphate electrolyte solutions. The results of the studies with dynamic coating were further optimized, and successfully adapted for use in urine sample analysis.

The effects of replacing water as the electrolyte solvent with methanol, ethanol and 1-propanol were studied in CE separations of catecholamines and methoxycatecholamines. The physical properties of the solvents had a great influence on the separation performance. The electrophoretic mobilities of catecholamines and methoxycatecholamines, as well as the electroosmotic mobility, were decreased from water to 1-propanol. Major factors influencing this response were dielectric constant and viscosity of the solvent. The selectivity of the separation was greatly influenced by the solvent, which could be observed as change in the migration order of the analytes. The separation efficiency was higher in non-aqueous conditions, and owing to the low diffusion, the highest plate numbers were achieved in ethanol based analysis.

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