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CHARAKTERIZACE PEPTIDOVÝCH HORMONŮ
KAPILÁRNÍ ELEKTROFORÉZOU



Veronika ŠOLÍNOVÁ

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Prohlašuji, že jsem pod vedením školitele doc. RNDr. Ivana Jelínka, CSc., a školitele konzultanta RNDr. Václava Kašičky, CSc., pracovala samostatně, veškerou použitou literaturu jsem citovala a že celá práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze 1. prosince 2006



Veronika Šolínová

Obsah

Předmluva a poděkování.....	9
Seznam zkratek.....	11
Seznam symbolů.....	13
Seznam původních prací.....	15
1 Kapilární elektromigrační metody.....	16
1.1 Úvod	16
1.2 Migrace iontů v elektrickém poli.....	17
1.3 Disperzní jevy.....	18
1.4 Separační účinnost a rozlišení.....	20
2 Kapilární elektroforéza peptidů.....	22
2.1 Úvod.....	22
2.2 Kvalitativní a kvantitativní analýza.....	22
2.2.1 Stanovení stupně čistoty.....	23
2.3 Detekce.....	24
2.3.1 Základní kritéria pro funkci detektoru.....	24
2.3.2 UV-absorpční detekce.....	25
2.3.3 Laserem indukovaná fluorescenční detekce.....	25
2.3.4 Elektrochemická detekce.....	26
2.3.5 Hmotnostní spektrometrická detekce.....	26
2.4 Separace strukturně podobných peptidů.....	26
2.4.1 Výběr optimálních separačních podmínek.....	26
2.4.2 Složení a pH základního elektrolytu.....	27
2.4.3 Další faktory ovlivňující výběr základního elektrolytu.....	27
2.5 Fyzikálně-chemická charakterizace peptidů.....	28
2.5.1 Efektivní a specifický náboj.....	28
2.5.2 Efektivní elektroforetická pohyblivost.....	30
2.5.3 Termodynamické acidobazické disociační konstanty.....	31
2.6 Vztahy mezi efektivní elektroforetickou pohyblivostí peptidů a jejich nábojem, velikostí a prostorovým uspořádáním.....	33
2.6.1 Semiempirické modely.....	33
2.6.2 Predikce sekundární struktury peptidů.....	35

3 Peptidové hormony.....	36
3.1 Neurohypofyzární hormony	36
3.2 Insulin.....	37
3.3 Enkefaliny a dalarginy.....	38
3.4 Hmyzí oostatické peptidy.....	39
3.5 Hormony uvolňující gonadotropiny	40
4 Výzkumné cíle.....	43
5 Experimentální část.....	45
5.1 Chemikálie.....	45
5.2 Úprava vzorku.....	45
5.3 Přístrojové vybavení.....	46
6 Výsledky a diskuse.....	48
6.1 Analýza a separace peptidových hormonů.....	48
6.1.1 Strategie volby experimentálních podmínek.....	48
6.1.2 Složení základních elektrolytů.....	54
6.1.3 Volba kapiláry.....	55
6.1.4 Citlivost detekce.....	55
6.1.4.1 UV-absorpční detekce.....	55
6.1.4.2 Porovnání citlivosti UV-absorpční detekce a laserem indukované fluorescenční detekce.....	57
6.1.5 Kvalitativní a kvantitativní analýza.....	58
6.1.6 Separace strukturně podobných peptidů.....	61
6.2 Fyzikálně-chemická charakterizace peptidů	67
6.2.1 Výpočet efektivního a specifického náboje.....	67
6.2.2 Stanovení efektivní elektroforetické pohyblivosti při standardní teplotě.....	68
6.2.3 Testování semiempirických modelů korelujících pohyblivost peptidů a jejich náboj, velikost a prostorové uspořádání.....	70
6.2.4 Predikce pravděpodobné struktury peptidů v roztoku.....	74
7 Závěr.....	76
8 Literatura.....	78
9 Seznam publikací.....	87
10 Dodatek.....	90
Příloha: Publikace I-VI	

Předmluva a poděkování

Dizertační práce je založena na třech původních publikacích v mezinárodních časopisech s impaktem faktorem, jedné práci přijaté do tisku a čtyřech kratších článků publikovaných ve sbornících konferencí.

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Seznam zkratek

ACN	acetonitril
AU	absorbanční jednotka
BGE	základní (nosný) elektrolyt
CAE	kapilární afinitní elektroforéza
CD	cirkulární dichroismus
CE-MS	spojení kapilární elektroforézy s hmotnostní spektrometrií
CE	kapilární elektroforéza, kapilární elektromigrační metody
CEC	kapilární elektrochromatografie
CIEF	kapilární izoelektrická fokusace
CITP	kapilární izotachoforéza
CMEKC	kapilární micelární elektrokinetická chromatografie
CTAB	cetyltrimethylammonium bromid
CZE	kapilární zónová elektroforéza
DLR	dalargin
ENK	enkefalin
EOF	elektroosmotický tok
ESI	elektrosprejová ionizace
GnRH	hormon uvolňující gonadotropiny
HPLC	vysokoučinná kapalinová chromatografie
CHAPS	3-[(3-choloamidopropyl)dimethylammonio]-1-propansulfonát
IČ	infračervený
i.d.	vnitřní průměr
IDAA	kyselina iminodioctová
IEC	iontově výmenná chromatografie
IOPs	hmyzí oostatické peptidy
LIF	laserem indukovaná fluorescence
LHRH	hormon uvolňující luteinizační hormon
LOD	mez detekce
LOQ	mez stanovitelnosti
MALDI	ionizace laserem za přítomnosti matrice
MS	hmotnostní spektrometrie

NBD	4-chloro-7-nitrobenzo[1,2,5]oxadiazol
NHPs	neurohypofyzární peptidy
NMR	jaderná magnetická rezonance
o.d.	vnější průměr
PEG	polyethylenglykol
RP-HPLC	vysokoúčinná kapalinová chromatografie s obrácenou fází
SDS	dodecylsulfát sodný
SEC	molekulová vylučovací chromatografie
TEAP	tetraethylammonium chloristan
TMOF	trypsinový modulační oostatický faktor
tricin	N-[tris(hydroxymethyl)methyl]glycin
tris	tris(hydroxymethyl)aminomethan
UV	ultrafialový
UV-Vis	ultrafialový-viditelný

Seznam symbolů

γ	aktivitní koeficient
ϵ_0	permitivita prostředí [Fm^{-1}]
ϵ_r	relativní permitivita prostředí [Fm^{-1}]
η	dynamická viskosity [Pa.s]
ζ	elektrokinetický potenciál, zeta potenciál [V]
κ	specifická elektrická vodivost [Sm^{-1}]
λ	vlnová délka [nm]
μ	elektroforetická pohyblivost [$\text{m}^2\text{V}^{-1}\text{s}^{-1}$]
μ_{ep}	efektivní elektroforetická pohyblivost [$\text{m}^2\text{V}^{-1}\text{s}^{-1}$]
$\bar{\mu}_{ep}$	průměrná efektivní elektroforetická pohyblivost [$\text{m}^2\text{V}^{-1}\text{s}^{-1}$]
μ_{eo}	elektroosmotická pohyblivost iontu [$\text{m}^2\text{V}^{-1}\text{s}^{-1}$]
μ_{lim}	limitní iontová pohyblivost [$\text{m}^2\text{V}^{-1}\text{s}^{-1}$]
θ	odporová konstanta nádobky
$\sigma_{l,i}$	směrodatná odchylka v prostorové doméně [m]
$\sigma_{t,i}$	směrodatná odchylka v časové doméně [s]
$\sigma_{d,i}^2$	střední kvadratická vzdálenost [m^2]
a_i	aktivita
a	průměr hydratovaného iontu [m]
A	plocha píku [AU.s nebo V.s nebo RFU.s]
A_c	korigovaná plocha píku [AU nebo V nebo RFU]
c	koncentrace látky [$\text{mol} \cdot \text{dm}^{-3}$]
c_i	látková (molární) koncentrace iontu i [$\text{mol} \cdot \text{dm}^{-3}$]
D_i	difúzní koeficient [m^2s^{-1}]
D_{lim}	limitní difúzní koeficient [m^2s^{-1}]
e	elementární náboj ($1,602177 \cdot 10^{-19}$ C)
E	intenzita elektrického pole [Vm^{-1}]
F	Faradayova konstanta ($96485,34 \text{ C mol}^{-1}$)
I	iontová síla roztoku [$\text{mol} \cdot \text{m}^{-3}$]
I_{el}	elektrický proud [A]

h	výška píku [AU nebo V nebo RFU]
K_a	termodynamická acidobazická disociační konstanta (konstanta acidity)
K_a^{mix}	smíšená acidobazická disociační konstanta
l_c	celková délka kapiláry [m]
l_d	efektivní délka kapiláry [m]
M_r	relativní molekulová hmotnost
n	šum detektoru [V nebo AU nebo RFU]
N_i	počet teoretických pater látky i
$N_{dif,i}$	počet teoretických pater pouze za působení podélné difúze
P_h	relativní výška píku
P_A	relativní plocha píku
P_{CA}	relativní korigovaná plocha píku
pK_a	záporný dekadický logaritmus acidobazické disociační konstanty (konstanty acidity)
q	efektivní náboj [e]
q_s	specifický náboj [e]
R	molární plynová konstanta ($8,31451 \text{ JK}^{-1}\text{mol}^{-1}$)
R	odezva detektoru [V nebo AU nebo RFU]
R_i	vnitřní poloměr kapiláry [m]
R_{ij}	rozlišení píků i a j
r_i	poloměr iontu i [m]
r	korelační koeficient
S	citlivost [$\text{V}.\text{mol}^{-1}\text{dm}^3$ nebo $\text{AU}.\text{mol}^{-1}\text{dm}^3$ nebo $\text{RFU}.\text{mol}^{-1}\text{dm}^3$]
t	teplota [$^\circ\text{C}$]
T	termodynamická teplota [K]
U	elektrické napětí [V]
$w_{i,i}$	šířka píku i při základně v časové doméně [s]
t_{eo}	migrační čas elektroneutrální látky [s]
t_m	migrační čas [s]
v_{eo}	rychlosť elektroosmotického toku [ms^{-1}]
x	molární zlomek
z	nábojové číslo iontu

Seznam původních prací

Základem této dizertační práce jsou následující publikace, které jsou dále označované římskými číslicemi (I-IV):

I. Analysis of Synthetic Derivatives of Peptide Hormones by Capillary Zone Electrophoresis and Micellar Electrokinetic Chromatography with Ultraviolet-Absorption and Laser-Induced Fluorescence Detection.

Šolínová V., Kašička V., Koval D., Barth T., Ciencialová A., Žáková L., *J. Chromatogr. B* 2004, 808, 75-82.

II. Analysis and Separation of Enkephalin and Dalargin Analogues and Fragments by Capillary Zone Electrophoresis.

Šolínová V., Kašička V., Barth T., Hauzerová L., Fanali S., *J. Chromatogr. A* 2005, 1081, 9-18.

III. Separation and Investigation of the Structure-Mobility Relationships of Insect Oostatic Peptides by Capillary Zone Electrophoresis.

Šolínová V., Kašička V., Koval D., Hlaváček J., *Electrophoresis* 2004, 25, 2299-2308.

IV. Separation and Investigation of the Structure-Mobility Relationships of Gonadotropin-Releasing Hormones by Capillary Zone Electrophoresis in Conventional and Isoelectric Acidic Background Electrolytes.

Šolínová V., Kašička V., Sázelová P., Barth T., Mikšík I., *J. Chromatogr. A*, v tisku

1 Kapilární elektromigrační metody

1.1 Úvod

Kapilární elektromigrační metody jsou analytické separační metody, které dělí látky a částice působením elektrického pole v kapiláře malého průměru naplněné kapalným roztokem základního elektrolytu. Kapilární elektroforéza (CE), přesněji označovaná jako kapilární zónová elektroforéza (CZE), kapilární izotachoforéza (CITP), kapilární izoelektrická fokusace (CIEF), kapilární micelární elektrokinetická chromatografie (CMEKC) či kapilární afinitní elektroforéza (CAE) jsou rychlé (s typickou dobou analýzy několik minut), vysoce účinné (10^5 - 10^6 teoretických pater na metr) a vysoce citlivé separační metody s malou spotřebou analytů (pikomoly až attomoly) v nano až pikolitrových objemech vzorku. Mají široké uplatnění při analýzách a separacích širokého spektra látek, od malých organických a anorganických iontů přes středně velké a vysokomolekulární látky až po buňky a mikroorganismy.

Vývoj elektroforetických metod v kapilárním měřítku začal v druhé polovině minulého století [1-5]. Novým impulsem pro rozvoj CE metod se na počátku 80. let staly práce Jorgenson a Lukacové [6, 7]. V současné době jsou CE metody stále více uznávaným protějškem, respektive doplňkem dosud nejrozšířenějších separačních metod – různých variant vysokoučinné kapalinové chromatografie (HPLC).

Základní experimentální sestava pro CE metody se skládá ze separační kapiláry, jejíž konce jsou ponořeny do elektrodových nádobek, ze zdroje vysokého napětí a z detektoru. Nejběžněji se používá křemenná kapilára o vnitřním průměru 25-75 μm a celkové délce 20-100 cm vně pokrytá polyimidem. Kapilára a nádobky jsou naplněny základním elektrolytem. V nádobkách jsou umístěny platinové elektrody (katoda a anoda), mezi které se vkládá konstantní separační napětí (obvykle do 30 kV) ze zdroje vysokého napětí. Separované zóny analytů jsou detekovány on-column, end-column nebo off-column detektorem. Mezi nejběžnější typy detekce patří UV-Vis absorpční, fluorimetrická, vodivostní, elektrochemická či hmotnostně spektrometrická.

Kapilární zónová elektroforéza je nejčastěji používaná elektromigrační metoda, která umožňuje dělit ionogenní látky podle rozdílných elektroforetických pohyblivostí v homogenním prostředí základního elektrolytu. Oproti tomu elektroneutrální látky můžeme dělit pomocí kapilární micelární elektrokinetické chromatografie. Tato nejmladší elektromigrační technika [8, 9] využívá jevy elektrokinetické (elektroforézu a

elektroosmózu) i principy chromatografické, tj. distribuci analyzovaných látek mezi pseudofázi tvořenou micelami ionogenního detergentu a vodnou fází základního elektrolytu a relativní pohyb těchto fází vůči sobě.

1.2 Migrace iontů v elektrickém poli

Pohyb iontu v kapalném prostředí působením elektrického pole se nazývá elektroforetická migrace. Ion se pohybuje v homogenním elektrickém poli konstantní elektroforetickou rychlostí, v_i , pro kterou platí vztah:

$$v_i = \mu_i \cdot E = \frac{|z_i|e}{6 \cdot \pi \cdot \eta \cdot r_i} \cdot E \quad (1.1)$$

kde μ_i [$\text{m}^2\text{V}^{-1}\text{s}^{-1}$] je elektroforetická pohyblivost iontu i definovaná jako rychlosť jeho pohybu v elektrickém poli o jednotkové intenzitě, z_i je nábojové číslo iontu i , e je elementární náboj, E [Vm^{-1}] je intenzita elektrického pole, η [Pa.s] je viskozita roztoku základního elektrolytu, r_i [m] je poloměr iontu i . Ze vztahu (1.1) vyplývá, že elektroforetická pohyblivost iontu i je přímo úměrná velikosti jeho náboje a nepřímo úměrná jeho poloměru a viskozitě prostředí.

V reálných roztocích je pohyblivost iontu ovlivněna rovněž jeho elektrostatickými interakcemi s ostatními ionty, a proto je pohyblivost také funkci iontové síly roztoku. Iontová síla, I , je definovaná:

$$I = \frac{1}{2} \sum_i c_i z_i^2 \quad (1.2)$$

kde c_i [$\text{mol}\cdot\text{dm}^{-3}$] je molární koncentrace i -tého iontu a z_i je nábojové číslo iontu i . Pohyblivost iontu v roztoku při dané iontové síle se nazývá aktuální iontová pohyblivost. Obecně aktuální pohyblivost vzniká s klesající iontovou silou roztoku a při nekonečném zředění, tedy nulové iontové síle, dosahuje hodnoty, která je definována jako limitní iontová pohyblivost. Tato fyzikální veličina závisí na rozpouštědle a teplotě, pro běžné malé anorganické a organické ionty je tabelována. Efektivní elektroforetická pohyblivost slabého elektrolytu (látky) j , $\mu_{ep,j}$ [$\text{m}^2\text{V}^{-1}\text{s}^{-1}$], je definovaná jako pohyblivost této látky jako celku, tedy suma skládající se z příspěvků pohyblivostí jednotlivých iontových forem látky vážených molárním zlomkem těchto iontových forem v elektrolytu (látce) j :

$$\mu_{ep,j} = \frac{\sum_{i=1}^n c_i \mu_i sign(z_i)}{c_j} \quad (1.3)$$

kde c_j [mol·dm⁻³] je analytická koncentrace látky j , c_i je koncentrace i -té iontové formy látky j a μ_i [m²V⁻¹s⁻¹] je aktuální pohyblivost i -té iontové formy. Koncentrace jednotlivých iontových forem slabého elektrolytu, a tudíž i jeho efektivní pohyblivost, je silně závislá na pH prostředí, ve kterém se elektrolyt nachází.

Další velmi důležitý pohyb v kapiláře je elektroosmotický tok (EOF), který vzniká působením elektrického pole na volný povrchový náboj na vnitřní stěně kapiláry [10]. Rychlosť EOF, v_{eo} , se řídí von Smoluchovského rovnicí:

$$v_{eo} = \mu_{eo} \cdot E = \frac{\epsilon_r \cdot \epsilon_0 \cdot \zeta}{\eta} \cdot E \quad (1.4)$$

kde μ_{eo} [m²V⁻¹s⁻¹] je elektroosmotická pohyblivost definovaná jako rychlosť EOF, v_{eo} , v elektrickém poli o jednotkové intenzitě, E [Vm⁻¹], ϵ_r je relativní permitivita prostředí, ϵ_0 [Fm⁻¹] je permitivita vakua, η [Pa·s] je viskozita prostředí, ζ [V] je elektrokinetický (zeta) potenciál. EOF má rovinný rychlostní profil. Je závislý na pH a iontové síle základního elektrolytu a jeho rychlosť roste s intenzitou elektrického pole v kapiláře.

1.3 Disperzní jevy

Problematika disperzních jevů v kapilární elektroforéze je velmi významná, neboť ovlivňuje zejména citlivost a účinnost separace [11]. Disperze je způsobena několika faktory jako jsou podélná difúze, radiální i podélný teplotní spád v kapiláře (Jouleovo teplo), elektromigrační disperze, adsorpce na stěnu kapiláry, nerovnoměrný EOF a rozšíření zón v důsledku dávkování a detekce [12-15].

Podélná difúze je definovaná jako tok hmoty vyvolaný koncentračním gradientem, v jehož důsledku látky s vyšší koncentrací putují do míst s nižší koncentrací. V CZE nelze podélnou difúzi eliminovat. S prodlužujícím se časem dochází k většímu rozšíření zóny analytu. V praxi lze difúzi omezit zkrácením doby analýzy, např. vyšším separačním napětím nebo rychlejším elektroosmotickým tokem. Disperze zóny může být vyjádřena pomocí Einsteinovy rovnice pro difúzi v kapalinách

$$\sigma_{d,i}^2 = 2D_i t_{m,i} \quad (1.5)$$

kde $\sigma_{d,i}^2$ [m²] je střední kvadratická vzdálenost, tedy difúzní příspěvek disperze, D_i [m²s⁻¹] je difúzní koeficient a $t_{m,i}$ [s] je migrační čas i -tého analytu.

V roztocích o nekonečném zředění lze vztáhnout limitní elektroforetickou pohyblivost, $\mu_{lim,i}$, k limitnímu difúznímu koeficientu, $D_{lim,i}$, pomocí Nernstova-Einsteinova vztahu

$$D_{lim,i} = \frac{\mu_{lim,i} RT}{z_i F} \quad (1.6)$$

kde F je Faradayova konstanta, R molární plynová konstanta a T [K] je termodynamická teplota.

Jouleovo тепло vzniká uvnitř celé kapiláry při průchodu elektrického proudu základním elektrolytem. Důsledkem jeho nerovnoměrného odvodu ze separačního prostoru je parabolický teplotní profil uvnitř kapiláry a příčný a podélný teplotní gradient [16, 17]. Tento jev způsobuje změny ve viskozitě a pohyblivosti, a tím dochází k disperzi zóny analytu. Vliv Jouleova tepla lze potlačit volbou základního elektrolytu o nízké elektrické vodivosti a vhodnou konstrukcí aparatury [18].

Elektromigrační disperze způsobuje deformaci migrující zóny analytu v důsledku nelineární podstaty elektromigračních dějů. Dochází k deformaci Gaussova tvaru píku na trojúhelníkový. Vzniká v případech, kdy je dávkovaný analyt o takové koncentraci, že ovlivňuje vodivost nebo pH základního elektrolytu a tedy celkovou elektrickou vodivost v místě zóny analytu. Tvar píku iontů silných elektrolytů je ovlivněn pohyblivostí koiontů (iontů základního elektrolytu, které se pohybují stejným směrem a mají stejný typ náboje jako ion analytu). Pokud ion vzorku má vyšší pohyblivost než koiont základního elektrolytu, tak jeho pík bude rozmytý vpředu. Když bude pohyblivost iontu analytu nižší než pohyblivost koiontu základního elektrolytu, tak pík bude chvostovat, tedy bude rozmytý vzadu. V případě, že pohyblivosti iontu analytu i koiontu základního elektrolytu budou stejné, nebude docházet k deformaci [19, 20]. Pro slabé elektrolyty je situace složitější, protože jejich efektivní elektroforetická pohyblivost je ovlivněna také pH [12]. Proto je vhodné pro predikci možné elektromigrační disperze použít navržené počítačové simulační programy [21, 22].

Adsorpce na stěnu je další negativní jev v CZE. Vzniká interakcemi mezi analytem a stěnou kapiláry. Může docházet k reverzibilní adsorpci, která vede k deformaci tvaru píku a ke zpomalování rychlosti průchodu analytu kapilárou nebo k ireverzibilní adsorpci, kdy dochází ke ztrátě vzorku [10]. Je to jeden z hlavních problémů při analýze vysokomolekulárních látek [23, 24]. Omezit vliv adsorpce lze

pokrytím stěn kapiláry vhodným, nejčastěji polymerním materiélem, chemickým navázáním nebo fyzikální adsorpcí [25-27].

Nerovnoměrný elektroosmotický tok způsobuje rozšíření elektroforetických zón. Je způsoben rozdílnou hodnotou elektrokinetického potenciálu, ζ , podél kapiláry a tedy rozdílně rychlým elektroosmotickým tokem. Nejčastěji vzniká v důsledku podélných rozdílů v teplotě roztoku uvnitř kapiláry v důsledku nedokonalého chlazení celé kapiláry [16, 17], případně porušením pokrytí nebo adsorpcí nabitéch částic na povrchu kapiláry [28, 29].

Rozšíření zóny nastává při hydrodynamickém dávkování, kdy je roztok analytu dávkován pomocí rozdílu tlaků na koncích kapiláry. Dochází k deformaci pravoúhlé zóny analytu, která je způsobená hydrodynamickým tokem během dávkování. Téměř pravoúhlého dávkování zóny vzorku lze dosáhnout pomocí elektrokinetického dávkování, kdy je vstupní konec kapiláry ponořen do roztoku a ionty se dostávají po připojení stejnosměrného napětí na vstupní konec kapiláry vlastní elektromigrací a elektroosmózou. Zóna analytu může být také deformovaná neostrým či poškozeným koncem kapiláry [30], přítomností dodatečného hydrodynamického toku v kapiláře v důsledku rozdílných výšek hladin roztoků v elektrodotových nádobkách [31] nebo při použití CE-MS rozhraní [32].

1.4 Separační účinnost a rozlišení

Účinnost separace v CZE je určena analogicky jako v chromatografii, je tedy charakterizovaná počtem teoretických pater, N_i , a určuje, jak moc se zóny separovaných látek rozmývají.

$$N_i = \frac{l_d^2}{\sigma_{t,i}^2} = \frac{t_{m,i}}{\sigma_{t,i}^2} \quad (1.7)$$

kde l_d [m] je efektivní délka kapiláry, $t_{m,i}$ [s] je migrační čas analyzované látky i a $\sigma_{t,i}$ [m] a $\sigma_{t,i}$ [s] jsou směrodatné odchylky koncentračního gaussovského profilu v prostorové a časové doméně. V CZE počet teoretických pater dosahuje hodnot 10^5 - 10^6 . Do počtu teoretických pater se promítají všechny rozmývací jevy uvedené v kapitolách 1.2 a 1.3.

Pro zjednodušení, uvažujeme-li z disperzních jevů pouze podélnou difúzi a elektroosmotický tok je nulový, lze počet teoretických pater, $N_{dif,i}$, vyjádřit na základě Einsteinovy-Smoluchowského rovnice:

$$N_{dif,i} = \frac{\mu_{ep,i} U l_d}{2 D_i l_c} \quad (1.8)$$

kde $\mu_{ep,i}$ [$\text{m}^2 \cdot \text{V}^{-1} \cdot \text{s}^{-1}$] je efektivní elektroforetická pohyblivost analytu, U [V] je separační napětí, l_c [m] je celková délka kapiláry, a D_i [$\text{m}^2 \cdot \text{s}^{-1}$] je difúzní koeficient. Tato rovnice je omezeně použitelná; jak vyplývá z rovnice (1.6), platí pouze pro roztoky v nekonečném zředění. Pro látky s nevysokým nábojovým číslem a nízkou iontovou silou základního elektrolytu lze pro teplotu 25°C získat přibližný výraz

$$N_{dif,i} \approx 20 z_i U \frac{l_d}{l_c} \quad (1.9)$$

kde z_i je nábojové číslo analyzovaného iontu i . Z rovnice vyplývá, že vyšší účinnosti lze dosáhnout vyšším napětím, kdy se zkrácením doby analýzy omezí vliv difúze.

Veličina, která vyjadřuje separaci dvou analytů i a j , se nazývá rozlišení, R_{ij} . Charakterizuje relativní míru separace dvou píků. Je definována:

$$R_{ij} = \frac{2(t_{m,j} - t_{m,i})}{(w_{t,i} + w_{t,j})} \quad (1.10)$$

kde $t_{m,i}$ a $t_{m,j}$ [s] jsou migrační časy a $w_{t,i}$ a $w_{t,j}$ [s] jsou šířky píků při základně. Dva píky jsou úplně rozdělené, pokud je rozlišení větší než 1,5.

V kapilární elektroforéze se pro rozlišení uvádí vztah

$$R_{ij} = \frac{1}{4} \frac{\Delta \mu_{ep}}{\bar{\mu}_{ep}} \sqrt{\bar{N}} \quad (1.11)$$

kde $\Delta \mu_{ep} = \mu_{ep,i} - \mu_{ep,j}$ je rozdíl pohyblivostí analytů i a j , $\bar{\mu}_{ep}$ [$\text{m}^2 \text{V}^{-1} \text{s}^{-1}$] a \bar{N} jsou aritmetické průměry pohyblivostí a účinností píků analytů i a j .

2 Kapilární elektroforéza peptidů

2.1 Úvod

Peptidy představují početnou skupinu mimořádně významných a široce rozšířených biologicky aktivních látek: působí jako hormony, neurotransmitery, imunomodulátory, koenzymy nebo inhibitory enzymů, léčiva, toxiny a antibiotika a podílí se tak na řízení a regulaci mnoha životně důležitých procesů ve všech živých organismech. Přírodní peptidy jsou tvořeny ze 20 základních aminokyselin spojených peptidovými vazbami a někdy též disulfidovými můstky. Syntetické preparáty peptidů mohou obsahovat kromě kódovaných navíc ještě nekódované aminokyseliny a další typy vazeb. Výsledkem je velký počet aminokyselinových sekvencí v řetězcích peptidových molekul, které se liší svou velikostí (relativní molekulovou hmotností), elektrickým nábojem a prostorovým uspořádáním, tedy vlastnostmi, které způsobují jejich rozdílné elektroforetické pohyblivosti a umožňují jejich dělení kapilárními elektromigračními metodami.

V současné době význam separace peptidů vzrůstá především v oblasti proteomiky a peptidomiky, které představují hlavní směry při studiu biologických procesů a při vývoji nových léčiv. Separace, analýza, izolace a charakterizace peptidů patří mezi nejvýznamnější aplikační oblasti vysokoúčinných kapilárních elektromigračních separačních technik. Jejich využití v chemii peptidů bylo postupně shrnuto v přehledných článcích [33-35].

2.2 Kvalitativní a kvantitativní analýza

Peptidy a jejich analogy a fragmenty jsou izolovány z přírodních materiálů nebo jsou syntetizovány z jednotlivých aminokyselin nebo jejich bloků. V některých případech jsou připraveny semisynteticky, tzn. syntetický peptid je navázán na izolovaný nativní peptid nebo na jeho fragment. Takto připravené peptidy bývají často využívány v biochemii jako substráty a inhibitory enzymů při zjišťování mechanismu jejich katalytických účinků, k modelovým studiím interakcí antigenů s protilátkami, hormonů s receptory, peptidů a proteinů s nukleovými kyselinami a k mapování antigenních epitopů proteinů. Široké uplatnění nacházejí také ve farmaceutickém průmyslu, medicíně a veterinární praxi. V takových případech je velmi důležitá kontrola úspěšnosti izolace nebo syntézy, případně jednotlivých kroků celého procesu, neboť

získané produkty mohou obsahovat různé příměsi, jako jsou vedlejší produkty peptidové syntézy, které mají nežádoucí negativní účinky.

CZE analýza se běžně používá ke stanovení stupně čistoty [36, 37], stanovení nízkomolekulárních iontových příměsí [38, 39], k analýze peptidů o nízké koncentraci v komplexních biomatičních jako jsou biologické tekutiny, buněčné lysáty a tkáňové extrakty s využitím hmotnostní spektrometrie (MS) a laserem indukované fluorescenční (LIF) detekce [40, 41], ke sledování chemických a enzymatických reakcí a fyzikálních změn pomocí hmotnostní spektrometrie s indukcí laserem za přítomnosti matrice ve spojení s průletovým analyzátorem (MALDI-TOF-MS) [42, 43], k charakterizaci peptidů a proteinů sekvencí aminokyselin v polypeptidovém řetězci [44] a při peptidovém mapování, tj. při identifikaci proteinů prostřednictvím separace jejich peptidových fragmentů [45].

2.2.1 Stanovení stupně čistoty

Čistota peptidů a obsah jednotlivých složek ve vzorku mohou být stanoveny různými postupy. V případě, že standard peptidu je dostupný, můžeme množství peptidu v analyzovaném vzorku určit absolutně pomocí metody kalibrační křivky nebo metodou vnitřního standardu, tzn. porovnáním migračních časů, výšky a/nebo plochy píku standardu peptidu a peptidu v daném vzorku. Pokud standard peptidu není dostupný, tzn. když je peptid prvně syntetizován (izolován) a analyzován, pak stupeň čistoty i -té složky peptidového preparátu může být kvantifikován na základě vyhodnocení: i. relativní výšky píku, $P_h(i)$ (2.1), ii. relativní plochy píku, $P_A(i)$ (2.2), a iii. relativní korigované plochy píku $P_{CA}(i)$ (2.3):

$$P_h(i) = h(i) / \sum h(i) \quad i = 1 \dots n \quad (2.1)$$

$$P_A(i) = A(i) / \sum A(i) \quad i = 1 \dots n \quad (2.2)$$

$$P_{CA}(i) = A_c(i) / \sum A_c(i) \quad i = 1 \dots n \quad (2.3)$$

kde $h(i)$ je výška píku i -té složky peptidového preparátu, $A(i)$ je plocha píku i -té složky peptidového preparátu a $A_c(i)$ je korigovaná plocha píku i -té složky peptidového preparátu, n je počet složek vzorku peptidového preparátu. Korigovaná plocha píku je plocha píku vztažená k migrační rychlosti daného analytu a tj. je rovna ploše píku dělené migračním časem daného píku.

Takto stanovený stupeň čistoty je pouze přibližný, neboť molární absorpční koeficienty jednotlivých složek peptidového preparátu se mohou lišit. Nejčastěji

syntetické peptidy obsahují jako příměsi strukturně podobné látky výsledného produktu, které mají podobné molární absorpční koeficienty. Stanovení na základě relativní výšky píku (2.1) je vhodné použít v případech, kdy pásky mají podobný tvar a šířku. Stanovení na základě relativní plochy píku (2.2) se užívá při vyhodnocování píku, jejichž rozdílná šířka není způsobena různou migrační rychlostí, ale elektromigrační disperzí nebo adsorpcí na stěnu kapiláry. Nejpřesnější stanovení stupně čistoty je pomocí relativní korigované plochy píku.

Parametrem kvalitativní analýzy peptidu je elektroforetická pohyblivost, která je pro daný peptid charakteristická za definovaných experimentálních podmínek.

2.3 Detekce

2.3.1 Základní kritéria pro funkci detektoru

Mezi nejběžněji uváděné charakteristiky detektoru řadíme: citlivost, šum, mez detekce, mez stanovitelnosti, spolehlivost (opakovatelnost), charakter závislosti signálu na koncentraci (lineární dynamický rozsah) a selektivitu.

Citlivost, S , je definovaná jako poměr změny signálu detektoru a změny koncentrace nebo hmotnosti analytu. Citlivost je dána směrnicí kalibrační křivky v daném bodě. Optimální detektor má být co nejcitlivější v souladu s dalšími uvedenými kritérii.

Šum, n , je část měřené veličiny, která neobsahuje informaci o analytu. V ideálním detektoru by měl být šum nulový. Ve skutečných detektorech se měřená veličina skládá ze signálu (tj. odezvy na sledovanou látku) a dalších složek, které tvoří pozadí (základní linie). Nežádoucí efekty obsažené v pozadí jsou způsobené nečistotami v BGE, klidovými proudy zesilovače, síťovým nebo harmonickým kmitočtem, nevhodnou konstrukcí analyzátoru, driftem, poruchami v síťovém napětí, bublinkami v kapalině a jinými rušivými faktory. Většinu nežádoucích složek pozadí lze snáze či obtížněji potlačit, kompenzovat nebo odstranit. Je žádoucí, aby šum byl co nejnižší a poměr signálu k šumu co nejvyšší.

Mez detekce, LOD , je definovaná jako koncentrace (hmotnost) nebo látkové množství sledované látky, které lze kvalitativně postřehnout. Je určována statisticky, nejčastěji jako trojnásobek odhadu směrodatné odchylky šumu. Mez detekce se zmenšuje se vzrůstajícím poměrem signálu k šumu. Musíme vždy specifikovat metodu,

kterou jsme mezi detekce vypočítali, neboť hodnoty vypočítané různými metodami se liší. Mezi stanovitelnosti, LOQ , je definována jako nejnižší koncentrace (hmotnost), kterou lze stanovit.

Odezva detektoru, R , se vyjadřuje rovnicí $R = S \cdot c^x$, pak dynamický rozsah je interval koncentrace, ve kterém $x \neq 0$, tj. interval, ve kterém změna koncentrace analytu způsobuje změnu signálu.

2.3.2 UV-absorpční detekce

Nejčastěji používanou detekcí peptidů v CE je UV-absorpční detekce v rozsahu vlnových délek 200-220 nm, tj. v oblasti, ve které absorbuje peptidová vazba. Čím delší je peptidový řetězec a větší počet peptidových vazeb, tím intenzivnější je odezva signálu při stejně molární koncentraci [46]. Aby nedocházelo ke snižování signálu analytu, je vhodné používat jako základní elektrolyty takové, které v daném vlnovém rozsahu minimálně absorbují [47]. Tato podmínka je obtížně splnitelná pro BGE při detekování peptidů v oblasti 185-200 nm. Absorpce záření peptidovou vazbou je v dané oblasti vysoká, avšak absorpce BGE také, výjimkou jsou fosfátový a borátový pufr. Citlivost lze též ovlivnit konstrukcí detekční cely, byly navrženy cely ve tvaru Z, U [48], „sleeve“ [49] nebo bubliny [50], nebo s využitím prekoncentrace vzorku [51].

Peptidy obsahující v řetězci aromatické aminokyselinové zbytky mohou být detekovány při specifické vlnové délce 275-280 nm. Nejvyšší molární absorpční koeficient má tryptofan a tyrosin, slabší intenzitu vykazuje fenylalanin [52]. Intenzita signálu je velmi ovlivněna pH základního elektrolytu.

2.3.3 Laserem indukovaná fluorescenční detekce

Laserem indukovaná fluorescenční (LIF) detekce je nejcitlivější detekcí v CE, její citlivost je o dva až tři rády vyšší oproti UV-absorpční detekci. Nevýhodou této detekce je nutnost pre-, on-, nebo postcolumn derivatizace peptidů fluorescenčními značkami [53]. Navíc peptidy často obsahují více derivatizačních center v molekule, a tak proto výsledkem může být až několik produktů derivatizační reakce jedné analyzované látky. Mezi nejčastěji používaná derivatizační činidla reagující s aminoskupinou patří naftalen-2,3-dikarboxyaldehyd (NDA), furoylchinolin-3-karboxyaldehyd (FQ), fluorescein isothiocyanát (FITC) a (4-karboxybenzoyl)chinolin-2-karboxyaldehyd (CBQCA) [54]. Jen peptidy s aromatickými aminokyselinami s excitační vlnovou délkou 240-280 nm mohou být detekovány bez derivatizace.

Nezbytnou podmínkou pro excitaci peptidů je UV-laserový systém [55, 56], nebo multifotonová excitace např. v blízké infračervené oblasti [57].

2.3.4 Elektrochemická detekce

Mezi elektrochemickou detekci patří amperometrická [58], voltametrická [59] a vodivostní [60, 61]. Elektrochemická detekce není příliš častá v kapilární elektroforéze peptidů. Vysoko citlivá a selektivní amperometrická a voltametrická detekce se používá pro peptidy obsahující oxidovatelné a redukovatelné funkční skupiny, tedy pro peptidy obsahující aminokyseliny jako tyrosin, cystein nebo tryptofan [62-64]. Univerzální bezkontaktní vodivostní detekci lze detektovat všechny peptidy bez omezujících požadavků na jejich strukturu [65, 66].

2.3.5 Hmotnostní spektrometrická detekce

Hmotnostní spektrometrie (MS) je univerzální, citlivá i selektivní detekční technika pro CE [67]. Význam použití on- a off-line MS detekce v posledních letech s rozvojem v oblasti proteomiky, peptidomiky a peptidového mapování podstatně vzrostl [67-69]. Velmi významnou roli hraje MS při analýze a určování struktury peptidů a proteinů v komplexních směsích [70, 71]. Nejběžněji používaným iontovým zdrojem při spojení CZE-MS je elektrosprej (ESI) a ionizace laserem za přítomnosti matrice (MALDI), které umožňují nejen rychlou charakterizaci elektroforeticky separovaných peptidů jejich relativními molekulovými hmotnostmi [72], ale též poskytují důležité informace o struktuře a sekvenci aminokyselin v řetězci, o post-translačních modifikacích, peptidových mapách bílkovin [73] a o nekovalentních interakcích peptidů.

2.4 Separace strukturně podobných peptidů

2.4.1 Výběr optimálních separačních podmínek

Strategie pro racionální výběr podmínek pro CE separaci peptidů sleduje obecná pravidla pro výběr vhodných CE separačních podmínek [10, 74-77] a bere v úvahu specifické vlastnosti peptidů:

- i. amfoterní charakter: efektivní náboj a efektivní pohyblivost peptidů jsou silně závislé na pH, peptidy mohou být separovány jako kationty nebo anionty metodou CZE, CITP nebo jako neutrální látky pomocí CMEKC,

- ii. počet, rozložení a sekvence aminokyselin v řetězci a konformace peptidu, které ovlivňují rozpustnost a pohyblivost; ze struktury peptidu také vyplývá jeho ionogenní či neionogenní charakter, tedy i výběr vhodné separační metody (CZE nebo CMEKC),
- iii. biologickou aktivitu, specifické vazebné interakce, chemickou a teplotní stabilitu,
- iv. tendenci větších peptidů adsorbovat se na vnitřní stěny separační kapiláry.

2.4.2 Složení a pH základního elektrolytu

Z amfoterního charakteru peptidů vyplývá, že pro volbu CE separačních podmínek je důležité znát závislost efektivního a specifického náboje a/nebo efektivní elektroforetické pohyblivosti peptidů na pH, nebo alespoň jejich izoelektrické body (pI).

pH základního elektrolytu by mělo být nejméně 1-2 jednotky vzdálené od pI , protože elektroforetická pohyblivost a rozpustnost peptidu při pH blízkém pI je nízká. Také je důležité, aby pH základního elektrolytu bylo v rozsahu, kde specifický náboj je větší než $1\text{-}2 \cdot 10^{-4}$ e [47]. S přihlédnutím k tomu, že efektivní pohyblivost je přímo úměrná efektivnímu náboji a nepřímo úměrná relativní molekulové hmotnosti, a že rozdíly mezi pohyblivostí jednotlivých peptidů jsou maximální v oblasti největších rozdílů specifických nábojů jednotlivých peptidů, volíme jako optimální pH základního elektrolytu právě pH v oblasti maximálních rozdílů specifických nábojů.

Podle vhodně zvoleného pH vybíráme optimální složení základního elektrolytu (BGE). Jedna z významných vlastností BGE je pufrací kapacita při daném pH, pro optimální složení BGE splňuje pK_a pufrující složky podmínu $pH_{BGE} = pK_a \pm 0,5$. Typická iontová síla hlavní složky pufru se pohybuje v koncentračním rozsahu 10-200 mM. Těmto podmínkám vyhovuje několik pufrů. Výhodné je použití amfoterních, tzv. Goodových pufrů, dostupných v rozsahu pH 5,5-11, a kyselých izoelektrických pufrů dostupných v rozsahu pH 1,8-3,2, neboť navíc splňují podmínu relativně nízké elektrické vodivosti při dostatečné pufrací kapacitě [78, 79]. Mezi klasické pufry pro kyselou oblast patří fosfátový a acetátový a pro zásaditě pH borátový či fostátový pufr.

2.4.3 Další faktory ovlivňující výběr základního elektrolytu

Dalším důležitým faktorem ovlivňujícím výběr základního elektrolytu je rozpustnost látky. Analyzovaný peptid by měl mít minimální rozpustnost přibližně 1-0,1 mmol/l. Na rozpustnost peptidu má zásadní vliv pH, iontová síla a složení pufru, ale též různá aditiva. Pro zlepšení rozpustnosti bývá přidávána např. močovina a její deriváty

[80], neionogenní, zwitterionické, kationické a anionické detergenty [81] nebo cyklodextriny [82]. Tato činidla musí být přidávána opatrně, protože mohou měnit EOF, tvořit komplexy a nebo měnit mechanismus separace.

CZE separace peptidů jsou někdy prováděny v pufrech s přídavkem organického rozpouštědla jako acetonitrilu [83], methanolu [84], propanolu či jiných alkoholů. Organické rozpouštědlo může nejen ovlivnit rozpustnost, ale také změnit selektivitu a tím zlepšit nebo zhoršit rozlišení analytů [85].

Dále je nutné brát ohledy na chemickou a tepelnou stabilitu a biologickou aktivitu peptidů, aby nedocházelo k jejich nevratným změnám, jako např. denaturaci, kterým je třeba zabránit pokud jsou dále použity k biologickému testování. Biologická aktivita peptidu je vždy vázaná na určitou konformaci polypeptidové molekuly, která je stabilní jen za určitých podmínek (pH, rozpouštědlo, složení pufru). Chemická stabilita některých funkčních skupin v peptidovém řetězci musí být také zohledněna. Peptidy obsahující labilní skupiny, např. amidovou, disulfidovou či sulfhydrylovou, nemohou být analyzovány při vysokém pH, neboť by u nich mohlo docházet k nežádoucím hydrolytickým nebo transsulfidovým reakcím.

2.5 Fyzikálně-chemická charakterizace peptidů

2.5.1 Efektivní a specifický náboj

Peptidy jsou amfoterní elektrolyty obsahující různé typy ionogenních skupin jako například aminoskupinu na N-konci peptidového řetězce nebo na bočním řetězci lysinu nebo karboxylovou skupinu na C-konci peptidu či na bočních řetězcích kyseliny asparagové a glutamové. Pro vybrané peptidy jsou tabelovány jejich acidobazické disociační konstanty [86], pro ostatní peptidy bereme průměrné hodnoty acidobazických disociačních konstant jednotlivých ionogenních skupin aminokyselin [47] viz **tabulka 1**.

Efektivní náboj peptidů je roven sumě nábojů (včetně znaménka) všech ionogenních skupin přítomných v polypeptidovém řetězci. Nejčastěji používaný vztah pro výpočet efektivního náboje je odvozen od Henderson-Hasselbalchovy rovnice [87]. Efektivní náboj je funkcí pH roztoku základního elektrolytu a pK_a hodnot ionogenních skupin aminokyselinových zbytků obsažených v peptidu [87]:

$$q = \sum_{i=1}^m \frac{1}{1+10^{pH-pK_a(i)}} - \sum_{j=1}^p \frac{1}{1+10^{pK_a(j)-pH}} \quad (2.4)$$

kde m a p jsou integrální počty kationických a anionických skupin, $pK_a(i)$ a $pK_a(j)$ jsou záporné dekadické logaritmy acidobazických disociačních konstant i -tých a j -tých ionogenních skupin analytu. Z toho vyplývá, že pokud známe aminokyselinovou sekvenci peptidu, můžeme z disociačních konstant jednotlivých ionogenních skupin peptidu určit, která skupina bude disociována a jaký bude její příspěvek k efektivnímu náboji při daném pH.

Tabulka 1 Přibližný rozsah acidobazických disociačních konstant, pK_a , ionogenních skupin aminokyselinových zbytků v peptidovém řetězci.

Ionogenní skupina	Aminokyselinový zbytek	pK_a
-SO ₃ H	kyselina cysteová	1,3
α -karboxylová	C-konec peptidového řetězce	1,8-3,5
β - karboxylová	kyselina asparagová	3,5-4,5
-S-CH ₂ -COOH	S-karboxymethylcystein	3,4-4,0
γ - karboxylová	kyselina glutamová	4,0-4,5
imidazolová	histidin	5,6-6,9
α -NH ₃ ⁺	N-konec peptidového řetězce	7,5-8,6
-SH	cystein	9,0-10,5
ϵ -NH ₃ ⁺	lysin	9,0-10,8
δ -NH ₃ ⁺	ornitin	9,6-10,2
fenolová	tyrosin	9,8-11,0
guanidiniová	arginin	10,0-12,5

Pro výpočet efektivního a specifického náboje (efektivní náboj dělený relativní molekulovou hmotností) při daném pH byl navržen počítačový program, který je založen na matematickém modelu acidobazických rovnováh pro obecný amfolyt [88]. Ze závislosti náboje na pH základního elektrolytu je možné získat důležité informace, jako např. oblast minimálního a maximálního náboje a hodnotu pH, v jehož okolí se náboj blíží nule, či je roven nule, tzv. izoelektrický bod (pI) peptidu. V programu nejsou zohledněny vlivy sousedních aminokyselinových zbytků a stérické uspořádání v polypeptidovém řetězci, které mohou hrát důležitou roli v reálném prostředí. Podobný

přístup s přihlédnutím k vlivu prostředí na jednotlivé ionogenní skupiny byl publikován později [89]. Závislost efektivního náboje nebo efektivní pohyblivosti peptidu na pH lze určit též jinými metodami, jako např. acidobazickou titrační křivkou [90] nebo elektroforetickou titrační křivkou [91], případně pro malé oligopeptidy v rozmezí pH 4-10 kapilární izotachoforézou [92]. Izoelektrický bod peptidů lze též stanovit kapilární nebo gelovou izoelektrickou fokusací [74, 93] nebo CZE [94].

2.5.2 Efektivní elektroforetická pohyblivost

Efektivní elektroforetická pohyblivost peptidu je komplexní funkcí

- i. vlastností samotného peptidu (přítomné ionogenní skupiny, velikost, tvar),
- ii. vlastností základního elektrolytu, ve kterém se peptid nachází (složení, pH, iontová síla, viskozita a teplota),
- iii. interakcí peptidu se složkami média (solvatace, disociace, tvorba komplexu).

Z toho vyplývá, že pohyblivost musí být vždy vztažena k přesně definovaným podmínkám, jako je složení, pH a teplota základního elektrolytu.

Efektivní elektroforetickou pohyblivost peptidu v základním elektrolytu, μ_{ep} , lze získat z experimentálně určeného migračního času peptidu, t_m [s], a migračního času elektroneutrální látky – markeru EOF, t_{eo} [s], podle vztahu:

$$\mu_{ep} = \frac{I_c I_d}{U} \left(\frac{1}{t_m} - \frac{1}{t_{eo}} \right) \quad (2.5)$$

kde I_c [m] je celková délka kapiláry, I_d [m] je efektivní délka kapiláry, U [V] je vložené separační napětí.

Při analýzách v silně kyselých základních elektrolytech s velmi pomalým elektroosmotickým tokem způsobeným potlačenou disociací silanolových skupin na stěnách křemenné kapiláry byla efektivní elektroforetická pohyblivost stanovena pomocí tlakem urychleného měření elektroosmotického toku [95]. Vzorek byl dávkován společně se zónou A neutrálního elektroosmotického markeru do kapiláry naplněné základním elektrolytem. Poté bylo vloženo separační napětí, analyty byly separovány a neutrální marker zóny A se pohyboval elektroosmotickou rychlostí. Analýza byla přerušena, separační napětí vypnuto v přesně daném čase, t_u , poté co zóna peptidu prošla detektorem. Následně byla nadávkována druhá zóna B stejného neutrálního markeru, kapilára byla vrácena do nádobky se základním elektrolytem, a na nádobku byl vložen dávkovací tlak. Obě zóny markeru, A i B, byly registrovány detektorem

v daných časech, t_A a t_B . Potom elektroforetická pohyblivost byla stanovena podle vztahu:

$$\mu_{ep} = \frac{l_c l_d}{U} \left(\frac{1}{t_m} - \frac{1}{t_u} + \frac{t_A}{t_u t_B} \right) \quad (2.6)$$

kde l_c [m] je celková délka kapiláry, l_d [m] je efektivní délka kapiláry, t_m [s] je migrační čas peptidu, U [V] je vložené separační napětí.

2.5.3 Termodynamické acidobazické disociační konstanty

Stanovení termodynamické acidobazické disociační konstanty (konstanty acidity) (pK_a) pomocí CZE je založeno na měření závislosti efektivní elektroforetické pohyblivosti ionogenní látky na pH v sérii základních elektrolytů o konstantní iontové síle. Jednosytná slabá kyselina HA podléhá disociaci podle rovnice:



Pro zjednodušení zápisu nebyla uvažována tvorba hydrooxoniového kationtu ve vodném prostředí či obecně lyoniového iontu.

Poté termodynamická kyselá disociační konstanta, K_a , pro kyselinu HA je definovaná:

$$K_a = \frac{a_{H^+} a_{A^-}}{a_{HA}} = \frac{a_{H^+} c_{A^-} \gamma_{A^-}}{c_{HA} \gamma_{HA}} \quad (2.8)$$

a_i jsou aktivity jednotlivých částic, c_i jejich molární koncentrace [$\text{mol} \cdot \text{dm}^{-3}$] a γ_i jejich aktivitní koeficient. Aktivitní koeficient neutrální formy, γ_{HA} , je roven jedné.

Aktivitní koeficienty pro zředěné elektrolyty při standardní teplotě 25°C lze vypočítat z klasické Debye-Hückelovy rovnice:

$$-\log \gamma_i = \frac{0.5085 z_i^2 \sqrt{I}}{1 + 0.3281 \alpha \sqrt{I}} \quad (2.9)$$

kde I [$\text{mol} \cdot \text{dm}^{-3}$] je iontová síla (výpočet viz rovnice 1.2), z_i je náboj iontu i , α [m] je průměr hydratovaného iontu. Přesná hodnota průměru iontu nebývá známa, pro malé ionty se používá hodnota 0,5 nm.

Z praktického hlediska je vhodné zavést tzv. smíšenou disociační konstantu K_a^{mix} [96]:

$$K_a^{mix} = \frac{a_{H^+} c_{A^-}}{c_{HA}} \quad (2.10)$$

Ve výše uvedené rovnici vystupuje aktivita vodíkových kationů, a_{H^+} , která je přímo získána z měření pH základního elektrolytu ($pH = -\log a_{H^+}$), a poměr koncentrací disociované a nedisociované formy kyseliny HA, který lze získat z elektroforetického měření.

Efektivní elektroforetická pohyblivost slabého elektrolytu j , $\mu_{ep,j}$, je dána součtem iontových pohyblivostí, μ_i , všech iontových forem elektrolytu j vážených jejich molárním zlomkem x_i :

$$\mu_{ep,j} = \sum_i \mu_i x_i \quad (2.11)$$

$$x_i = \frac{c_i}{\sum_i c_i} \quad (2.12)$$

kde c_i je molární koncentrace i -té iontové formy elektrolytu j .

Efektivní elektroforetickou pohyblivost slabé jednosytné kyseliny, $\mu_{ep,HA}$, lze vyjádřit vztahem:

$$\mu_{ep,HA} = \left[\frac{1}{1 + 10^{(pK_a^{mix} - pH)}} \right] \mu_{A^-} \quad (2.13)$$

Aktuální iontová pohyblivost, μ_{A^-} , a smíšená disociační konstanta závisí na iontové síle a teplotě základního elektrolytu. V případě, že analýza je provedena při konstantní iontové síle a teplotě, tedy konstantních parametrech, efektivní elektroforetická pohyblivost, $\mu_{ep,HA}$, je pouze funkcí pH a parametru K_a^{mix} . Smíšené disociační konstanty se získají pomocí regresní analýzy linearizovaných forem nebo nelinearizovaných modelů závislosti efektivních elektroforetických pohyblivostí na pH získané z CZE analýz látky v základních elektrolytech o různém pH. Smíšená disociační konstanta se přepočte na termodynamickou disociační konstantu pomocí vztahů (2.8) a (2.10).

Metoda CZE byla použita jako alternativní metoda pro stanovení pK_a k tradiční potenciometrii pro široké spektrum látek jako aminokyseliny [96], peptidy [97-99], a imidazolové deriváty [100].

2.6 Vztahy mezi efektivní elektroforetickou pohyblivostí peptidů a jejich nábojem, velikostí a prostorovým uspořádáním

2.6.1 Semiempirické modely

Modely jsou založeny na Stokesově rovnici, která popisuje pohyb kulovité částice v kapalině, a na působení síly elektrického pole na nabitou částici. Semiempirické modely korelují efektivní elektroforetickou pohyblivost peptidů, μ_{ep} , s jejich efektivním nábojem, q , a velikostí jejich molekuly vyjádřenou pomocí relativní molekulové hmotnosti, M_r , nebo počtem aminokyselinových zbytků v polypeptidovém řetězci, n . Tyto modely rovněž popisují elektroforetické chování různých prostorových uspořádání molekul a mohou tak být využity při predikci sekundární nebo terciární struktury peptidů a proteinů v roztoku.

Prvně kvantitativně popsal tento vztah Offord [101] pro sérii oligo- a polypeptidů dělených papírovou elektroforézou:

$$\mu_{ep} = \frac{k_1 \cdot q}{M_r^{2/3}} \quad (2.14)$$

kde k je konstanta proporcionality. Tento vztah byl později použit v pracích dalších autorů, Issaq et al. [102], Rickard et al. [103] a Basak a Ladisch [104], Tessier et al. [105] a Jalali-Heravi [106] pro peptidy separované ve volném roztoku pomocí CZE.

Následující vztah byl odvozen pro rigidní sféricky tvarované molekuly v pufrech s nízkou iontovou silou [107]:

$$\mu_{ep} = \frac{k_1 \cdot q}{M_r^{1/3}} \quad (2.15)$$

Další semiempirický model byl navržen pro syntetické polymery s cylindrickým nebo tyčovitým tvarem molekul [108]:

$$\mu_{ep} = \frac{k_1 \cdot q}{M_r^{1/2}} \quad (2.16)$$

Cross [109] obměnil Offordův výraz tím, že jej převedl do logaritmické formy:

$$\log\left(\frac{\mu_{ep}}{q}\right) = k \cdot \log M_r \quad (2.17)$$

kde k je exponentem M_r v nelogaritmickém vztahu a může být určen jako směrnice této závislosti. Svou studii souboru peptidů upřesnil dalším zkoumáním konformačních stavů rozšířením o stanovení hydratačního obalu molekuly [110, 111].

Podobný výraz jako pro polymery navrhl Kim [112] pro svou sérii peptidů vzniklých enzymovým štěpením proteinů:

$$\mu_{ep} = \frac{k_1 \cdot q}{M_r^{0,56}} \quad (2.18)$$

Kombinací dvou modelů pro velké (2.14) a malé (2.15) molekuly získal Compton [113] další vztah:

$$\mu_{ep} = \frac{k_1 \cdot q}{k_2 \cdot M_r^{1/3} + k_3 \cdot M_r^{2/3}} \quad (2.19)$$

kde první člen ve jmenovateli, $M_r^{1/3}$, popisuje chování menších molekul, a druhý člen, $M_r^{2/3}$, chování větších molekul v pufru s nízkou iontovou sílou.

V dosud uvedených příkladech modelů byla použita jako parametr velikosti molekuly relativní molekulová hmotnost. Jinou možností vyjádření molekulové velikosti je počet zbytků aminokyselin v peptidovém řetězci, n . Tento přístup byl prvně použit Grossmanem et al. [114]:

$$\mu_{ep} = \frac{k_2 \cdot \ln(q+1)}{n^{0,43}} \quad (2.20)$$

Cifuentes a Poppe [89] představili prvně modifikaci Grossmanova klasického lineárního modelu, kde v logaritmické závislosti pohyblivosti na náboji bylo též vyjádřeno snížení vlivu náboje silně nabitého peptidu:

$$\mu_{ep} = \frac{k_2 \cdot \log(1+k_1 q)}{M_r^{0,411}} \quad (2.21)$$

Tento model byl také použit k charakterizaci fragmentů z enzymové hydrolýzy proteinu hovězího cytochromu c pomocí CE-MS [115]. Téměř stejná rovnice jenom s malým rozdílem v hodnotě exponentu M_r , 0,435 místo 0,411, byla navržena Adamsonem [116].

Sanz-Nebotová [117] kombinovala vztah (2.21) s q zohledňujícím snížení vlivu náboje silně nabitého peptidu a vztahy pro klasický semiempirický model (2.14), (2.15) a (2.16), porovnala jejich výsledky a vyhodnotila jako nejhodnější korelační model vztah:

$$\mu_{ep} = \frac{k_2 \cdot \ln(k_1 q + 1)}{M_r^k} \quad (2.22)$$

kde $k = 1/3$ pro malé molekuly, $k = 1/2$ pro středně velké polymery, $k = 2/3$ pro velké molekuly.

2.6.2 Predikce sekundární struktury peptidů

Vztahy korelující efektivní elektroforetickou pohyblivost peptidů s jejich efektivním nábojem a velikostí a tvarem jejich molekul lze použít pro určení pravděpodobné sekundární či terciární struktury peptidů a proteinů ve volném roztoku. Struktura molekuly je odvozena ze vztahu mezi elektroforetickou pohyblivostí a exponentem relativní molekulové hmotnosti, případně exponentem počtu aminokyselinových zbytků v peptidovém řetězci, jak je uvedeno v **tabulce 2** [107].

Tabulka 2 Vztah mezi absolutní hodnotu koeficientu, k , ve vztahu (2.17) a tvarem molekuly.

Tvar molekuly	Proporční vztah
kulovitý	$k \sim 1/3$
náhodné klubko – neuspořádaný řetězec	$k \sim 0,5-0,6$
tyčovitý	$k \sim 0,8$
zploštělý disk	$k \sim 2/3$
Free-draining spirála	$k \sim 1,0$

Pro určení molekulové konformace je velmi důležité najít správnou korelační rovnici, ve které je elektroforetická pohyblivost vztažena k velikosti molekul. Také musíme brát v úvahu délku peptidového řetězce, neboť krátké peptidy se chovají v roztoku jako jednoduché organické ionty. Molekuly peptidů s delšími řetězci tvoří více uspořádané sekundární struktury, které jsou silně závislé na prostředí, ve kterém se nacházejí [118]. α -helikální nebo β -listové konformace se za běžných podmínek používaných pro CZE analýzy vyskytují častěji než náhodná klubka. Ve velkém rozsahu molekulových hmotností polypeptidy mohou tvořit odlišně organizované struktury [119]. Struktura je závislá na rozpouštědle, iontové síle a pH základního elektrolytu [120, 121]. Struktura syntetických peptidů byla studována CE s MS detekcí [122]. Znalost peptidové struktury pomáhá i při určování tvarů proteinových molekul v roztoku [123] a při peptidovém mapování proteinů [124].

3 Peptidové hormony

Biologicky aktivní peptidy reprezentují širokou skupinu syntetických nebo v přírodě se vyskytujících biomolekul, mezi nimiž jednu z nejpočetnějších a nejdůležitějších tříd tvoří hormony a peptidy, které hormony uvolňují.

3.1 Neurohypofyzární hormony

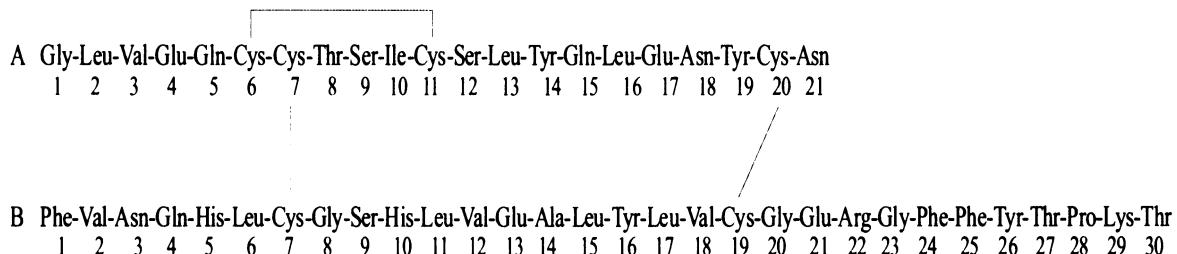
Mezi nejvýznamnější neurohypofyzární hormony patří vasopressin a oxytocin [125]. Oxytocin způsobuje stahy dělohy a vypuzování mléka při kojení. Vasopressin a jeho analogy jsou antidiuretické hormony [126], stimulují vylučování vody z ledvin a také zvyšují krevní tlak, aby umožnily lepší prokrvení ledvin [127]. Analogy antidiuretických hormonů byly různě modifikovány pro farmaceutický průmysl a vykázaly až 30krát větší účinnost antidiuretické aktivity a výrazný vzrůst účinku na krevní tlak. Pomocí studia 3D-struktury vasopressinu bylo dokázáno, že mezi dvěma kruhy Tyr a Phe jsou vytvořeny intramolekulární hydrofobní vazby. Díky této interakci tvoří molekula oxytocinu přibližně čtverec a vasopressinu lopatku. Tvar molekuly má zásadní význam pro vazbu daného hormonu k příslušnému receptoru na povrchu buňky a ovlivňuje také jeho elektroforetickou pohyblivost.

Strukturně podobné analogy vasopressinu a jiných biopeptidů byly separovány metodou CZE v kyselém základním elektrolytu 150 mM kyselině fosforečné, pH 1,8, a metodou RP-HPLC a iontově výměnnou chromatografií (IEC) [128, 129]. Oxytocin, vasopressin a další neurohypofyzární peptidové hormony byly analyzovány pomocí CMEKC ve čtyřech různých micelárních systémech – kationickém, anionickém, zwitterionickém a neutrálním [130]. Ke kontrole čistoty syntetických biologicky aktivních peptidů jako angiotensinu, oxytocinu, bradykininu či hormonu uvolňujícího gonadotropiny (GnRH) byla vyvinuta on-line prekoncentrační CE spojením kapiláry naplněné chromatografickým sorbentem s C18 reverzní fází a separační vnitřně nepokryté křemenné kapiláry při pH 3,5 [131]. Srovnání a výhody použití metody CEC na kolonách C8, C18 a C18/SCX oproti HPLC a CZE v kyselém prostředí acetonitrilu a tetraethylammonium chloristanu (ACN-TEAP), pH 3,0, bylo demonstrováno na oxytocinu, desmopressinu a karbetocinu [132]. Vliv složení BGE a předúpravy vzorku na účinnost separace v lidské plazmě byla testována na skupině čtyř peptidů včetně vasopressinu v tris-fosfátovém pufru, pH 2,0 [133]. Metoda imunoanalytického testování založeného na kompetitivní reakci volného vasopressinu a vasopressinu

vázaného v komplexu s fluorescein-isothiokyanátem (FITC) v mozkomíšní tekutině byla vyvinuta na modelové směsi [134].

3.2 Insulin

Insulin je peptidový hormon produkovaný pankreatem, reguluje metabolismus sacharidů, proteinů a mastných kyselin [125]. Molekula insulinu se skládá ze dvou polypeptidových řetězců, A a B, obsahujících 21 a 30 aminokyselinových zbytků viz. obrázek 1. Dva řetězce insulinu jsou vázány dvěma disulfidickými vazbami a třetí disulfidická vazba je na řetězci A. Relativní molekulová hmotnost je 5808, izoelektrický bod, pI , je 5,4-5,5.



Obrázek 1 Sekvence aminokyselin lidského insulinu

CZE se často využívá k analýze lidského insulinu a jeho derivátů. Stabilní deriváty insulinu s anhydridem kyseliny exo-3,6-epoxy-1,2,3,6-tetrahydroftalové (ETPA) byly stanoveny pomocí CE a iontově výměnné HPLC [135]. CZE s UV detekcí byla použita při analýze insulinu v CHES-acetátovém elektrolytu v přítomnosti acetonitrilu (ACN), pH 7-8, [136] a následně porovnána s HPLC analýzou ve fosfátovém pufru s přídavkem ACN jako mobilní fázi. Metoda CE byla použita k validaci HPLC analýzy insulinu pro kvalitativní kontrolu pro farmaceutické účely [137]. Lidský insulin, proinsulin a mezoformy byly zároveň kvantitativně určeny pomocí CE v kyselině N-[tris-(hydroxymethyl)-methyl]-3-aminopropansulfonové, diethylentriaminu a methanolu po jejich předchozí purifikaci chromatografickými metodami [138]. Pro imunoanalytické stanovení insulinu pomocí CZE se nejčastěji používá fluorescenční detekce, kde se insulin předem musí fluorescenčními značkami derivatizovat nebo lze též využít nativní fluorescence (excitace při 275 nm, emise při 305 nm) [139]. Insulin s navázanou

fluorescenční značkou NN 382 byl analyzován v 50 mM fosfátu a 25 mM sulfátu draselném, pH 7,0, s multifotonovou LIF detekcí v blízké infračervené oblasti [140].

3.3 Enkefaliny a dalarginy

Enkefaliny (ENKs), [Leu^5]enkefalin ([Leu^5]ENK), pentapeptid H-Tyr-Gly-Gly-Phe-Leu-OH, a [Met^5]enkefalin ([Met^5]ENK), pentapeptid H-Tyr-Gly-Gly-Phe-Met-OH, a dalargin (DLR), hexapeptid H-Tyr-D-Ala-Gly-Phe-Leu-Arg-OH, jsou biologicky aktivní peptidy, působící jako ligandy pro opiatové receptory v mozku [126]. Enkefaliny jsou fragmenty přírodního opioidního hormonu dynorfinu A (17 aminokyselin) a dynorfinu B (13 aminokyselin). Enkefaliny a dynorfiny jsou produkované ze dvou velkých prekurzorových proteinů, preproenkefalinu a preprodynorfinu [126]. Mají významnou roli při vzniku stresu, zmírnění bolesti a jsou zapojeny do teplotní regulace a dýchání. Tyto peptidy a jejich analogy jsou také aplikovány při léčbě některých mentálních chorob jako chronická schizofrenie nebo senilní demence Alzheimerova typu.

Enkefaliny a dalargin byly často analyzovány kapilární elektroforézou, většinou byla kontrolována čistota syntetických preparátů, ale CE byla též použita pro stanovení těchto peptidů v biologických tekutinách jako sérum, plazma nebo mozkomíšní mok. Racionální přístup při výběru a optimalizaci důležitých experimentálních parametrů jako složení a pH základního elektrolytu, průměr kapiláry či sběr frakcí v analytické a preparativní CZE opioidních peptidů včetně dynorfinových a enkefalinových analogů v kombinaci s off-line hmotnostním spektrometrem byla shrnuta v práci autorů Lee a Desiderio [141]. [Leu^5]ENK, [Met^5]ENK a [$\text{desTyr}^1,\text{Leu}^5$]ENK byly analyzovány s vysokou citlivostí (s detekčním limitem 3-11 attomolů) metodou CZE s hmotnostním spektrometrem ESI-TOF-MS v citrátovém a fosfátovém BGE [142]. Analogy ENKs a DLR a dalších peptidů jako desmopressin a vasopressin byly separovány CZE v silně kyselém BGE, 150 mM kyselině fosforečné, pH 1,8 [128]. Stejné analogy byly též analyzovány a děleny CMEKC s micelární pseudofází tvořenou anionickým detergentem dodecylsulfátem sodným (SDS) v alkalických BGES (20 mM tetraborát, pH 9,2, a 20 mM fosfát, pH 8,8) nebo s kationickým detergentem cetyltrimethylammonium bromidem (CTAB) v kyselém BGE (50 mM fosfát, pH 4,1) [128, 129, 143]. Chirální separace dalarginových analogů s D- a L-izomery Phe na čtvrté pozici v peptidovém řetězci byly prováděny v 50 mM fosfátovém pufru, pH 2,5

s 10 mM β -cyklodextrinem [128]. [D -Tle^{2,5}]DLR byl analyzován CZE v 0,5 M kyselině octové, pH 2,5, a poté přečištěn preparativně pomocí průtokové zónové elektroforézy [144]. Šest analogů a fragmentů enkefalinů bylo rozděleno a stanovenou v biologických matricích pomocí multidimenzionálního separačního systému tvořeného on-line spojením molekulově vylučovací chromatografie (SEC), koncentrační kolony s reverzní fází C18 a CZE [145, 146] s následnou hmotnostní spektrometrickou detekcí ESI-MS-MS-MS [147]. Dynorfinové peptidové analogy byly separovány CMEKC s anionickým, SDS, kationickým, CTAB, a zwitterionickým, CHAPS, surfaktantem [148]. Peptidy odvozené od enkefalinů byly derivatizovány fluorescein izothiokyanátem a následně děleny CMEKC v borátovém BGE s SDS a detekovány fluorescenčním detektorem [149]. [D -Pen^{2,5}]ENK (D -Pen je D -penicilamin neboli D -3-merkaptovalin) a [D -Ser²,Thr⁶]DRL byly analyzovány v krysím séru pomocí CZE ve fosfátovém (pH 2,4) a borátovém (pH 8,3) pufu [150, 151]. Enkefaliny a jejich fragmenty byly elektrochemicky detekovány jako komplexy mědi (oxidace mědi na dvojmocnou) vznikající v kapiláře po CZE separaci [64]. Analogy enkefalinů byly separovány v tris-fosfátovém a Na-fosfátovém BGE, pH 2,5, v kapiláře modifikované nekovalentně vázanou dvojvrstvou opačně nabité polymerů [152]. [Leu^5]ENK, [Met^5]ENK a jiné peptidové hormony byly separovány ve třech BGEs o pH 2,61; 2,85 a 10,0, a jejich pK_a byly stanoveny v široké pH oblasti 2-12 [153], a následně analyzovány hmotnostním spektrometrem ESI-MS v 50 mM kyselině octové a 50 mM kyselině mravenčí, pH 2,85 [154]. Úspěšná separace deseti peptidů – enkefalinů a jejich fragmentů a derivátů byla provedena ve fosfátovém pufu, pH 3,11, s UV detekcí [155]. [Leu^5]ENK, [Met^5]ENK a [D -Ala²]ENK byly též děleny pomocí CE v nevodném prostředí s elektrochemickou detekcí v nepokrytých křemenných a polyethylenglykolem (PEG) pokrytých kapilárách [156]. Fluorescenční detekce byla využita při vývoji imunoanalytického stanovení založeného na kompetitivní reakci volného [Met^5]ENK a [Met^5]ENK vázaného v komplexu s fluoresceinem [157].

3.4 Hmyzí oostatické peptidy

Hmyzí oostatické peptidy (insect oostatic peptides, IOPs) se řadí do skupiny antigenadotropních hmyzích hormonů vyskytujících se v různých živočišných druzích jako například mouše masařce *Neobellieria bullata* nebo komároví *Aedes aegypti*. Tyto peptidy bývají též někdy nazývány trypsinové modulační oostatické faktory (TMOF).

Jelikož ovlivňují reprodukční procesy hmyzu, jsou považovány za možný nový typ biologicky odbourávatelných insekticidů. TMOF vyskytující se u komára *Aedes aegypti* (*Aed*-TMOF) je přírodní decapeptid, který má sekvenci H-Tyr-Asp-Pro-Ala-Pro-Pro-Pro-Pro-Pro-OH. Byl prvně popsán Borovskym [158] jako inhibitor biosyntézy trypsinových enzymů ve středním střevě komára, kde zabraňuje degradaci přijaté krve a tím nepřímo zastavuje růst oocytů [159]. Borovsky také navrhl pravděpodobnou sekundární strukturu decapeptidu jako levotočivou α -helikální strukturu [159]. Později analogy se zkracujícím se C-koncem řetězce, s cyklickou strukturou a prolinem nahrazeným dehydroprolinem či tyrosinem nahrazeným homotyrosinem byly syntetizovány na pevné fázi [160-162] a charakterizovány NMR a cirkulárním dichroismem [162, 163]. Biologická aktivita a mechanismus působení analogů byly studovány na mouchách masařkách *Neobellieria bullata* a myších. Zatímco u masařek byl v závislosti na struktuře pozorován 20-80% pokles reprodukčního procesu [164, 165], u myší žádný efekt na reprodukci zjištěn nebyl [166].

3.5 Hormony uvolňující gonadotropiny

Hormon uvolňující gonadotropiny (GnRH), dříve nazývaný hormon uvolňující luteinizační hormon (LHRH), je fyziologicky významným decapeptidem (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂), který je syntetizován v neurosekrečních buňkách v hypotalamu a reguluje reprodukční funkce a podporuje druhotné pohlavní znaky. Agonisté GnRH jsou používány k léčení rakoviny prsu a prostaty.

Syntetické GnRH a jejich analogy a fragmenty jsou používány v CE jako modelové analyty nebo ve směsích společně s dalšími peptidy při vývoji metod pro reálné vzorky, při testování nové CE instrumentace [154, 167, 168] nebo při hledání optimálních experimentálních podmínek pro separaci [153, 169] nebo analýzu biopeptidů [131]. Úspěšná kombinace CE-MS bez zmlžujícího plynu byla demonstrována na gonadorelinu (syntetický lidský GnRH) [167]. On-line prekoncentrační CE-MS systém umožnil při imunoanalytickém stanovení vysokou citlivost, dobrou opakovatelnost a snadnou automatizaci. Jiný on-line prekoncentrační CE systém byl optimalizován pro kontrolu čistoty syntetických biologicky aktivních peptidů včetně GnRH ve fosfátovém BGE, pH 3,5 [131]. Membránový prekoncentrační CE-MS/MS systém byl navržen pro sekvenování biologicky aktivních peptidů na úrovni sub-100 femtomolů [170]. Devět modelových peptidů bylo analyzováno v 2 mM

acetátu ammoném a 1% v/v kyselině octové. Úspěšná CE a CEC separace směsi peptidů a proteinů byla provedena v křemenných kapilárách modifikovaných kladně nabitym alkylaminosilylovým monomerem s UV a MS detekcí [171]. Rychlá separace pěti peptidů včetně GnRH byla získána v 5 mM kyselině octové s 50% v/v ACN. Spojení CE a nano-ESI rozhraní, kde elektrické porézní spojení a vstřikovací špička byly integrovány na vlastní křemennou kapiláru, bylo otestováno na separaci směsi devíti peptidů a proteinů včetně GnRH v 1 M kyselině octové, pH 2,4 [172]. Různé typy off-line a on-line spojení CZE-ESI-TOF-MS s pozlaceným rozhraním byly zkonztruovány a použity při separaci a analýze standardní směsi pěti peptidů a proteinů v 5 % v/v kyselině octové [173]. GnRH a jeho čtyři analogy, [D-Ala⁶]GnRH, [D-Lys⁶]GnRH, [D-Phe²,D-Ala⁶]GnRH a [Gly-OH¹⁰]GnRH, byly analyzovány v kapiláře pokryté 3-(aminopropyl)trimethylsilanem v 0,01 M kyselině octové, pH 3,5, jako modelové směsi při testování vhodnosti CE-ESI-MS pro další identifikaci toxinů v hadím jedu [174]. CE využívající polem zesílené dávkování vzorku byla použita při stanovení vedlejších produktů po syntéze buserelinu, [Des-Gly¹⁰,D-Ser(tBu)⁶,Pro-NHEt⁹]GnRH, [175], jako optimální experimentální podmínky byly nalezeny kyselé BGEs s pH menším než 3,5. Úspěšné off-line spojení CE a MALDI-MS bylo předvedeno na analýze čtyř standardních peptidů, proteinů a reálného vzorku slz v 50 mM acetátu ammoném, pH 7,4 [176]. Na základě známých nebo dříve stanovených charakteristik (acidobazické disociační konstanty ionogenních skupin) byly stanoveny vhodné separační podmínky pro CE analýzu sedmi terapeutických peptidů včetně buserelinu a triptolerinu, [D-Trp⁶]LHRH, [153]. Optimalizace podmínek byla prováděna v širokém rozmezí pH 2-12, nejlepší separace byla dosažena v BGE složeném z 50 mM kyseliny octové a 50 mM kyseliny mravenčí, pH 2,85 bylo upraveno pomocí amoniaku. Stejná sada peptidů byla též separována a charakterizována v organovodném elektrolytu, 2-propanol, kyselina octová a kyselina mravenčí, pomocí CE-ESI-MS s komerčním [154] a v laboratoři vyvinutým rozhraním pro stínící roztok [168]. Dále bylo provedeno srovnání predikovaných a experimentálně získaných hodnot rozlišení, elektroforetické pohyblivosti a retenčních faktorů v CE a HPLC [169]. Úplné separace GnRH a jeho pěti analogů bylo dosaženo pomocí RP-HPLC v mobilní směsi složené z ACN a fosfátového pufru, pH 2,5, CZE ve fosfátovém a borátovém pufru a CMEKC s přidáním CTAB, CHAPS a Triton X-100 do BGEs použitých v CZE [177]. Užití alternativní bezkontaktní vodivostní detekční techniky k UV-absorpční detekci bylo demonstrováno na analýze směsi devíti peptidů ve fosfátovém pufru, pH 2,5 [65], kde mez detekce se

pohybovala na úrovni μM a rozlišení dosahovalo vysokých hodnot. Nativní GnRH se používá ke studiu biologických regulačních procesů jako je kinetika a mechanismus účinku hormonů v těle [175, 178-180]. Rychlá CE metoda byla vyvinuta a validována pro měření stability lidského a lososího GnRH a jejich analogů v přítomnosti střevních enzymů [178], analýza byla prováděna v kyselině octové, pH 4,0. Stanovení syntetického GnRH v plazmě bylo prezentováno pomocí on-kapilárního prekoncentračního CE systému [180], k potlačení adsorpce na stěny kapiláry a pro zvýšení citlivosti a selektivity MS detekce byla kapilára pokrytá 3-aminopropyl-triethoxsilanem. Dalším příkladem použití CE je stanovení proteinů během studie vlivu GnRH v lidské ovariální tekutině a séru při in-vitro oplodnění [179].

4 Výzkumné cíle

Cílem dizertační práce bylo vyvinout nové metody pro analýzu, separaci a fyzikálně-chemickou charakterizaci vybraných peptidových hormonů a jejich analogů a fragmentů kapilárními elektromigračními technikami, zónovou elektroforézou a micelární elektrokinetickou chromatografií. Při analýze a separaci strukturně podobných peptidů byla výzkumná činnost zaměřena na vývoj vhodných experimentálních podmínek, tedy na volbu vhodného módu v závislosti na ionogenním či neionogenním charakteru peptidu, na výběr optimálního složení a pH základních elektrolytů, na úpravu vzorku a na volbu vhodného způsobu detekce. Při fyzikálně-chemické charakterizaci peptidů bylo záměrem spočítat jejich efektivní a specifické náboje, co nejpřesněji stanovit jejich efektivní elektroforetické pohyblivosti při standardní teplotě a pomocí semiempirických modelů studovat korelace mezi pohyblivostí, nábojem a velikostí peptidů a určit nejpravděpodobnější tvar jejich molekul v roztoku.

V oblasti CE analýzy a separace peptidů dílcími cíli bylo:

- Zvolit optimální separační podmínky na základě strategie pro racionální výběr podmínek pro ionogenní a neionogenní peptidy (**I, II, III, IV**).
- Nalézt vhodné složení základních elektrolytů pro analýzu a separaci v kyselém prostředí o pH < 2,5 a ve slabě alkalickém prostředí (pH ~ 8), s cílem minimalizovat adsorpci peptidů na stěnu kapiláry (**I, II, III, IV**).
- Porovnat mez detekce a citlivost UV-absorpčního detektoru s polem diod v komerčním CE analyzátoru P/ACE MDQ (Beckman Coulter) s mezí detekce a citlivostí fluorescenčního detektoru (argon ion LIF detektor, excitace/emise 488/520 nm) v tomtéž zařízení a s mezí detekce a citlivostí UV-absorpčního detektoru v laboratorním CE analyzátoru vlastní konstrukce (**I, II**).
- Kvalitativně a kvantitativně stanovit čistotu syntetických a semisyntetických preparátů peptidových hormonů a jejich analogů a fragmentů (**I, II, III, IV**).
- Porovnat CZE separaci strukturně podobných peptidových hormonů v klasických a izoelektrických pufrech jako základních elektrolytech (**II, III, IV**).

V oblasti fyzikálně-chemické charakterizace peptidů dílčími cíli bylo:

- Spočítat efektivní a specifický náboj peptidů na základě průměrných hodnot acidobazických disociačních konstant ionogenních skupin přítomných v jejich řetězcích (**I**, **II**, **III**, **IV**).
- Určit experimentálně efektivní elektroforetické pohyblivosti peptidů při standardní teplotě 25°C (**III**, **IV**).
- Ověřit pomocí semiempirických modelů vztahy mezi pohyblivostí peptidů a jejich nábojem, velikostí a prostorovou strukturou a určit pravděpodobný tvar jejich molekul v roztoku (**II**, **III**, **IV**).

5 Experimentální část

5.1 Chemikálie

Všechny použité chemikálie dosahovaly stupně čistoty p. a. Tris (tris(hydroxymethyl)aminomethan) byl dodán firmou Serva (Heidelberg, Německo), kyselina fosforečná, kyselina octová a dimethylsulfoxid (DMSO) byly získány z Lachemy (Brno, ČR), kyselina iminodioctová (IDAA) byla dodána od Bachemu (Bubendorf, Švýcarsko), isoforon (3,5,-trimethyl-2-cyklohexen-1-on) a dodecylsulfát sodný (SDS) byly od Fluky (Buchs, Švýcarsko) a tricin ([tris(hydroxyl-methyl)-methyl]-glycin) byl získán od firmy Merck (Darmstadt, Německo).

5.2 Úprava vzorku

Analogy oxytocinu, vasopressinu, oktapeptidu B23-B30 z C-konce B-řetězce lidského insulinu (HI), enkefalinů, dalarginu, lineárních hmyzích oostatických peptidů a hormonů uvolňujících gonadotropin byly syntetizovány na pevné fázi [181] v Ústavu organické chemie a biochemie AV ČR (ÚOCHB). Struktura dalarginu byla obměněna substitucí leucinu v pozici 5 za D-terciární leucin [182], glykosidací [183] nebo jodizací tyrosinu v pozici 1, derivatizací na karboxylové skupině C-konci argininu a kombinací těchto modifikací. Cyklické hmyzí oostatické peptidy byly cyklovány z lineárních řetězců, boční řetězce byly chráněny [162]. Syntetické peptidy byly čištěny preparativní HPLC nebo molekulovou vylučovací chromatografií (SEC) a charakterizovány pomocí aminokyselinové analýzy, hmotnostní spektrometrie a analytické HPLC.

Lidský insulin a jeho B23-B30 oktapeptid byl pro fluorescenční detekci značen pomocí derivatizačního činidla, 4-chloro-7-nitrobenzo[1,2,5]oxadiazolu (NBD). NBD reagoval s volnou ϵ -amino skupinou Lys B29 v oktapeptidu HI, přičemž jeho α -amino skupina Lys byla chráněna Fmoc chránící skupinou [184]. Fluorescenčně značené molekuly HI byly připraveny semisynteticky trypsinem katalyzovanou syntézou peptidové vazby mezi C-koncem Arg B22 desoktapeptidu HI a N-koncem NBD-značeného oktapeptidu B23-30 HI [185].

Rozpustnost peptidů je dána jejich aminokyselinovým složením a sekvencí. Při CE analýze se peptidy nejčastěji rozpouštějí v deionizované vodě nebo v základním elektrolytu. Rozpustnost peptidu ve vodě je výhodná z hlediska možnosti použít stejný vzorek v různých elektrolytových systémech a dosáhnout lepšího zkonzentrování

rozpuštěného vzorku. Na druhou stranu použití základního elektrolytu jako rozpouštědla vzorku zaručí konstantní separační podmínky, pohyb v BGE během celého pokusu, což je důležité při měření fyzikálně chemických vlastností. Protože nejdříve bylo důležité nalézt vhodné experimentální podmínky, většina peptidových hormonů byla rozpuštěna v deionizované vodě, ve vodě nerozpustné peptidy byly rozpuštěny v základním elektrolytu. Analyzované peptidy byly rozpuštěny v koncentračním rozsahu 0,01-1,5 mg.ml⁻¹ pro UV-detekci a 10-1000krát nižší pro LIF detekci.

5.3 Přístrojové vybavení

Měření byla prováděna ve dvou přístrojích, v laboratorním CE-analyzátoru sestrojeném v ÚOCHB AV ČR (ÚOCHB-CE) [186] a v komerčním zařízení P/ACE MDQ System (Beckman-Coulter, Fullerton, CA, USA).

CE analyzátor sestrojený v ÚOCHB byl obsluhován manuálně, sběr a zpracování dat bylo prováděno chromatografickou stanicí Clarity (DataApex, Praha, ČR). Křemenné kapiláry z vnějšku pokryté polyimidem s vnitřním průměrem 50 µm a vnějším průměrem 200 µm, které měly celkovou délku 30 cm a efektivní 19 cm, byly vyrobeny v Ústavu skelných a keramických materiálů, resp. v Ústavu radiotechniky a elektroniky AV ČR (ÚRE, Praha, ČR). Jednotlivé experimenty byly prováděny při laboratorní teplotě 22-24°C bez aktivního chlazení kapiláry. Dávkování vzorku bylo hydrodynamické. Látky byly detekovány UV detektorem s pevnou vlnovou délkou 206 nm, zdrojem záření byla bezelektrodová vysokofrekvenčně buzená jódová výbojka (LKB-Pharmacia, Uppsala, Švédsko), a UV-senzitivní křemíková fotodioda s vestaveným předzesilovačem (Hamamatsu Photonics Deutschland, Herrsching, Německo).

Komerční CE analyzátor byl plně automatický přístroj, jeho řízení, sběr a zpracování dat byly prováděny softwarem P/ACE System MDQ, verze Karat (Beckman Coulter, Fullerton, CA, USA). Křemenné kapiláry z vnějšku pokryté polyimidem byly dodané firmou Polymicro Technologies (Phoenix, AR, USA), měly vnitřní průměr 50 µm nebo 75 µm a vnější průměr 375 µm, celkovou délku 39-40 cm a efektivní délku (k detektoru) 29-30 cm, přesné rozměry jsou udány u jednotlivých měření. Kapilára byla chlazena kapalinou nastavenou na standardní teplotu 25°C. Dávkování vzorku bylo hydrodynamické. Látky byly detekovány UV-detektorem s diodovým polem (PDA) s vlnovým rozsahem 190-600 nm a nebo fluorescenčním detektorem s argonovým

laserem indukovanou fluorescencí (LIF) s excitační vlnovou délkou 488 nm a emisní vlnovou délkou 520 nm.

Nová kapilára byla postupně promývána, nejprve vodou, poté 1 M hydroxidem sodným, opět vodou a nakonec základním elektrolytem. Každý krok trval 10 minut. Následně byly vnitřní stěny kapiláry a elektroosmotický tok stabilizovány vloženým vysokým napětím po dobu 20 minut. Mezi jednotlivými měřeními se stejnými podmínkami byla kapilára promývána základním elektrolytem 2 minuty. Při každé změně základního elektrolytu byla kapilára promývána 0,1 M NaOH 5 minut a poté byl stabilizační krok opakován jako pro novou kapiláru. Separační napětí bylo v rozsahu 10-25 kV. Vzorky byly dávkovány tlakem 5-15 mbar po dobu 5-15 s. Základní elektrolyty byly filtrovány přes 0,45 µm filtr (Millipore, Bedford, MA, USA).

6 Výsledky a diskuse

6.1 Analýza a separace peptidových hormonů

Ke kvalitativní a kvantitativní CE analýze a následné separaci strukturně podobných peptidů a k jejich fyzikálně-chemické charakterizaci bylo vybráno pět různě velkých skupin peptidových hormonů. První skupinu tvoří osm neurohypofyzárních peptidů (NHPs), jako oxytocinu (OT), vasopressinu (VP) a hmyzího diuretického hormonu (IDH) a jejich derivátů (**tabulka 3**), druhá skupina obsahuje pankreatický hormon lidský insulin (HI) (**obrázek 1**, kapitola 3.2) a jeho fragment B23-30. Třetí 12-členná skupina obsahuje enkefaliny (ENKs) a dalargin (DLR) a jejich fragmenty a deriváty (**tabulka 4**), čtvrtá, nejpočetnější, je skupina 20 hmyzích oostatických peptidových hormonů (IOPs) (**tabulka 5**) a pátou 12-člennou skupinu tvoří hormony uvolňující gonadotropin (GnRHs) a jejich analogy a fragmenty (**tabulka 6**).

6.1.1 Strategie volby experimentálních podmínek

Strategie pro racionální volbu podmínek pro CE separaci peptidů, která bere v úvahu specifické vlastnosti peptidů, byla použita při výběru experimentálních podmínek pro jejich analýzu. Charakteristické vlastnosti peptidů vyplývají z jejich aminokyselinového složení a sekvence, které určují jejich elektrický náboj, velikost, tvar, hydrofobicitu a specifické vazebné schopnosti. Ze struktury též vyplývá ionogenní či neionogenní charakter látky, který hraje důležitou roli při volbě separačního módu metody. Pokud jsou peptidy nabité alespoň v určité oblasti pH, můžeme zvolit metodu CZE, pokud ne, volíme metodu CMEKC.

Analyzované peptidy (**tabulky 3-6**) obsahují 2 až 10 aminokyselinových zbytků, jejich relativní molekulová hmotnost je v rozmezí od 238,2 do 1262,5. Výjimku tvoří lidský insulin, který má ve dvou řetězcích 51 aminokyselinových zbytků, relativní molekulovou hmotnost 5808 a izoelektrický bod 5,4-5,5. Ze struktury peptidů vyplývá, že většina peptidů obsahuje nejméně jednu ionogenní skupinu, nejčastěji volný N-konec ($\alpha\text{-NH}_3^+$ skupina) nebo C-konec ($\alpha\text{-COOH}$ skupina) peptidového řetězce. Oxytocin, vasopressin a všechny jejich deriváty, DLR-NHEt, a většina GnRHs a jejich fragmentů a analogů mají amidované karboxylové skupiny na C-koncích peptidových řetězců, deriváty OT, VP a IDH mají na N-konci kyselinu merkaptopropionovou, všechny GnRHs kromě třech mají v pozici 1 kyselinu pyroglutamovou (pGlu) a PacDLR má N-konec derivatizovaný fenylacetátem. Kromě IDH a jeho derivátů, dvou fragmentů

Tabulka 3 Zkratky, sekvence a relativní molekulové hmotnosti, M, neurohypofyzárních hormonů a derivativovaného B23-30 fragmentu lidského insulínu

Peptid	Zkratka	Sekvence v třípismenném kódu	M _r
Oxytocin	OT	H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-GlyNH ₂	1007,2
Deamino-oxytocin	dOT	Mpa-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-GlyNH ₂	992,2
Arg ⁸ -vasopressin	AVP	H-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-GlyNH ₂	1084,2
(Deamino-D-Arg ⁸)-vasopressin	dDAVP	Mpa-Tyr-Phe-Gln-Asn-Cys-Pro-D-Arg-GlyNH ₂	1069,3
Hmyzí diuretický hormon	IDH	H-Cys-Leu-Ile-Thr-Asn-Cys-Pro-D-Arg-GlyNH ₂	975,4
(Deamino-D-Arg ⁸)-IDH	dDA-IDH	Mpa-Leu-Ile-Thr-Asn-Cys-Pro-D-Arg-GlyNH ₂	960,4
(D-Arg ⁸)-IDH	DA-IDH	H-Cys-Leu-Ile-Thr-Asn-Cys-Pro-D-Arg-GlyNH ₂	975,4
(Deamino-D-Arg ⁸)-vasopressin	dAVP	Mpa-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-GlyNH ₂	1069,3
NBD-derivát B23-B30-lidského insulínu. fragmentu NBD-B23-30-HI			H-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-(NBD)-Thr-OH
NBD = 4-chloro-7-nitrobenzo[1,2,5]oxadiazol, Mpa – lysína merkaptopropionová			1175,7

Tabulka 4 Zkratky, sekvence a relativní molekulové hmotnosti, M_r , enkefalinů, dalarginu a jejich fragmentů a analogů

Peptid	Zkratka	Sekvence v třípismenném kódu	M_r
[Leu ⁵]jenkefalin	[Leu ⁵]ENK	H-Tyr-Gly-Gly-Phe-Leu-OH	555,8
[Met ⁵]jenkefalin	[Met ⁵]ENK	H-Tyr-Gly-Gly-Phe-Met-OH	573,8
ENK dipeptidový fragment	YG	H-Tyr-Gly-OH	238,3
ENK tripeptidový fragment	YGG	H-Tyr-Gly-Gly-OH	295,4
Dalargin	DLR	H-Tyr-D-Ala-Gly-Phe-Leu-Arg-OH	726,1
DLR dipeptidový fragment	YA	H-Tyr-Ala-OH	252,3
DLR tripeptidový fragment	YaG	H-Tyr-D-Ala-Gly-OH	309,4
[D-Tle ⁵]dalargin	[D-Tle ⁵]DLR	H-Tyr-D-Ala-Gly-Phe-D-Tle-Arg-OH	726,1
Dalargin ethylamid	DLR-NHEt	H-Tyr-D-Ala-Gly-Phe-Leu-Arg-NH-Et	749,0
[Tyr(I ₂)dalargin	I ₂ DLR	H-(3,5-di-I)-Tyr-D-Ala-Gly-Phe-Leu-Arg-OH	960,8
[D-Ser ² ,Thr ⁶]dalargin	[D-Ser ² Thr ⁶]DLR	H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH	689,9
N-fenylacetyl-dalargin	PacDLR	C ₆ H ₅ -Ac-Tyr-D-Ala-Gly-Phe-Leu-Arg-OH	859,1

Tabulka 5 Zkratky, sekvence a relativní molekulové hmotnosti, M_r , hmyzích oostatických peptidů

Zkratka	Sekvence v třípísmenném kódu	M_r
YDPAP ₆	H-Tyr-Asp-Pro-Ala-Pro-Pro-Pro-Pro-Pro-OH	1044,1
YDPAP ₅	H-Tyr-Asp-Pro-Ala-Pro-Pro-Pro-Pro-Pro-OH	950,0
YDPAP ₄	H-Tyr-Asp-Pro-Ala-Pro-Pro-Pro-Pro-OH	852,9
YDPAP ₃	H-Tyr-Asp-Pro-Ala-Pro-Pro-Pro-OH	755,8
YDPAP ₂	H-Tyr-Asp-Pro-Ala-Pro-Pro-OH	658,7
YDPAP	H-Tyr-Asp-Pro-Ala-Pro-OH	561,6
YDPA	H-Tyr-Asp-Pro-Ala-OH	464,5
YDP	H-Tyr-Asp-Pro-OH	393,4
YD	H-Tyr-Asp-OH	296,3
c(YDPAP) ₂	Tyr-Asp-Pro-Ala-Pro-Tyr-Asp-Pro-Ala-Pro []	1087,2
c(YDPAP)	Tyr-Asp-Pro-Ala-Pro []	543,6
c(YDPA)	Tyr-Asp-Pro-Ala []	446,5
YDPAP ₅ ΔP	H-Tyr-Asp-Pro-Ala-Pro-Pro-Pro-Pro-Pro-Δ-Pro-OH	1044,5
YDPAΔP	H-Tyr-Asp-Pro-Ala-Δ-Pro-OH	559,6
YDΔPA	H-Tyr-Asp-Δ-Pro-Ala-OH	462,5
YDΔP	H-Tyr-Asp-Δ-Pro-OH	391,4
DPAP	H-Asp-Pro-Ala-Pro-OH	399,0
DPA	H-Asp-Pro-Ala-OH	301,3
DΔPA	H-Asp-Δ-Pro-Ala-OH	299,3
HtyDPA	H-Hty-Asp-Pro-Ala-OH	476,6

Hty – homotyrosin, Δ-Pro – dehydroprolin

Tabulka 6 Zkratky, sekvence a relativní molekulové hmotnosti, M_r , GnRHs a jejich fragmentů a analogů

Peptid	Zkratka	Sekvence v třípísmenném kódu	M_r
Lidský GnRH	hGnRH	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH ₂	1181,5
[β Ala ⁶ -ProNHEt ¹⁰]hGnRH	β ANHEt-hGnRH	pGlu-His-Trp-Ser-Tyr- β -Ala-Leu-Arg-Pro-Pro-NHEt	1262,5
[Des-Pro-Gly-NH ₂]hGnRH	dPGNH ₂ -hGnRH	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-OH	1028,3
[Des-Arg-Pro-Gly-NH ₂]hGnRH	dAPGNH ₂ -hGnRH	pGlu-His-Trp-Ser-Tyr-Gly-Leu-OH	872,1
Fragment hGnRH 1-4	pGluHWS	pGlu-His-Trp-Ser-OH	538,6
Fragment hGnRH 4-10	SYGLRPG-NH ₂	H-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH ₂	766,0
Fragment hGnRH 7-10	LRPG- NH ₂	H-Leu-Arg-Pro-Gly-NH ₂	440,6
Lososí GnRH	sGnRH	pGlu-His-Trp-Ser-Tyr-Gly-Trp-Leu-Pro-Gly-NH ₂	1211,5
[Des-Gly-NH ₂]sGnRH	dGNH ₂ -sGnRH	pGlu-His-Trp-Ser-Tyr-Gly-Trp-Leu-Pro-OH	1155,4
[Des-pGlu ¹ -D-Om ⁶]sGnRH	dPyrDOm-sGnRH	H-His-Trp-Ser-Tyr-D-Om-Trp-Leu-Pro-Gly-NH ₂	1058,3
Kurečí GnRH I	cGnRH I	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Gln-Pro-Gly-NH ₂	1180,4
[Des-Gly-NH ₂]cGnRH II	dGNH ₂ -cGnRH II	pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-OH	1179,4
<i>pGlu – kyselina pyroglutamová</i>			

hGnRH obsahují GnRHs fenolovou skupinu tyrosinu. OT, VP, DLR, lidské GnRHs a jejich deriváty a fragmenty mají ve svých řetězcích guanidinylovou skupinu argininu, GnRHs kromě dvou fragmentů mají imidazolovou skupinu histidinu a analog fragmentu lososího GnRHs obsahuje δ -aminoskupinu ornitinu. Všechny neurohypofyzární hormony obsahují disulfidové můstky tvořené mezi cysteiny nebo mezi cysteinem a kyselinou merkaptopropionovou. NBD značený lidský insulin obsahuje 15 volných ionogenních skupin, guanidinylovou skupinu argininu, čtyři fenolové skupiny tyrosinu, dvě imidazolové skupiny histidinu, čtyři γ -karboxylové skupiny kyseliny glutamové a dvě volné aminoskupiny na N-koncích a dvě volné karboxylové skupiny na C-koncích. Pomocí průměrných hodnot acidobazických disociačních konstant jednotlivých ionogenních skupin byla spočtena závislost efektivního a specifického náboje na pH a určeny optimální oblasti pH pro analýzu a separaci, tj. oblasti pH, kde jsou tyto peptidy nabité a rozdíl v jejich specifických nábojích je maximální.

Všechny peptidové hormony kromě deamino-oxytocinu a cyklických oostatických peptidů bylo možné separovat v kyselých základních elektrolytech, NHPs bylo možné analyzovat jako kationty při pH < 8, ENKs a DLR při pH < 4, IOPs při pH < 3,5, GnRHs při pH < 5 a GnRHs s amidovaným C-koncem až do pH 10. Přesné měření pohyblivosti v silně kyselých pufrech při pH nižším než 2 je složité, neboť vzniklá iontová síla a prudce roste vodivost BGE a dochází k nárůstu teploty roztoku v kapiláře. Všechny výše uvedené peptidy kromě deamino-oxytocinu a cyklických oostatických peptidů bylo možné analyzovat jako kationty v pH rozmezí 2-3,5. Vhodné podmínky pro analýzu všech hormonů jako aniontů v zásaditém prostředí nebylo možné najít. NHPs jsou v alkalické oblasti nenabité nebo záporně nabité až při pH vyšším než 11. Chemická labilita disulfidového můstku ovšem vylučuje použití silně alkalické oblasti pH. GnRHs s volným C-koncem lze analyzovat jako anionty již při pH > 7, ale GnRHs s blokovaným C-koncem až při pH > 12, což není výhodné z důvodu vysoké koncentrace hydroxidových aniontů. Dochází k podobným nežádoucím efektům jako při vysoké koncentraci vodíkových iontů. Insulin a jeho derivát B23-30-HI, které jsou značené pomocí NBD, lze analyzovat pouze v oblasti s pH nižším než 8,5, protože NBD dosahuje svého excitačního a emisního maxima právě v oblasti pH 3-8,5. Enkefaliny, dalarginy a IOPs bylo možné bezproblémově analyzovat jako anionty v alkalické oblasti při pH 8,1.

6.1.2 Složení základních elektrolytů

Základní elektrolyt byl vybírána tak, aby splňoval několik kritérií. Bylo nezbytné, aby zaručoval požadované pH v zóně putujícího analyzovaného peptidu, tedy musel mít dostatečnou pufrační kapacitu. To znamenalo, že pH základního elektrolytu bylo co nejméně vzdálené od konstanty acidity (pK_a) pufrující složky a vodivost základního elektrolytu byla co nejnižší. Proto jsme většinou volili uni-univalentní základní elektrolyt, složený z jednoho kationtu a jednoho aniontu, aby se omezily rušivé vlivy nadbytečných systémových zón. Dále pohyblivost složek BGE byla zvolena tak, aby docházelo k co nejmenší elektromigrační disperzi zón analytu a aby nedocházelo k interakcím analyzovaného iontu se složkami BGE nebo s látkami dodatečně přidanými do BGE ovlivňujícími EOF. V úvahu byla též brána použitá detekční technika.

Tabulka 7 Složení a pH základních elektrolytů.

Zkratka	Složení	pH
BGE I	2 M kyselina octová	2,18
BGE II	0,1 M H_3PO_4 ; 0,05 M tris	2,25
BGE III	0,1 M kyselina iminodioctová	2,30
BGE IV	0,2 M kyselina iminodioctová	2,32
BGE V	0,05 M kyselina iminodioctová	2,40
BGE VI	0,5 M kyselina octová	2,50
BGE VII	0,04 M tris; 0,04 M tricin	8,10
BGE VIII	0,005 M H_3PO_4 ; 0,02 M tris; 0,05 M SDS	8,80

Na základě strategie pro racionální volbu experimentálních podmínek byly pro CE analýzu a separaci peptidů vybrány základní elektrolyty v kyslé oblasti, pH 2,18-2,50, a slabě alkalický pufr, pH 8,1, pro metodu CZE a alkalický pufr, pH 8,8, pro metodu CMEKC. Jejich přesné složení a pH společně s jejich zkratkou jsou uvedeny v tabulce 7. Pro kyselou oblast byly použity základní elektrolyty složené ze středně silných či slabých kyselin jako jsou kyselina trihydrogenfosforečná, ($pK_{a1} = 2,15$; $pK_{a2} = 7,21$; $pK_{a3} = 12,32$) nebo kyselina octová ($pK_a = 4,76$) a izoelektrický pufr, kyselina iminodioctová ($pK_a = 2,23$), který navíc splňuje podmínu relativně nízké elektrické vodivosti při dostatečné pufrační kapacitě. Pro alkalickou oblast byl vybrán amfoterní, tzv. Goodův pufr, tricin ($pK_a = 8,15$) v kombinaci s bází trisem ($pK_a = 8,3$), a

již zmiňovaný fosfátový pufr. Jako konstituent micelární pseudofáze byl použit aniontový detergent dodecylsulfát sodný (SDS). Všechny uvedené elektrolyty byly použity pro UV-absorpční detekci. Pro fluorescenční detekci byla zvolena kyselina octová, pH 2,50.

6.1.3 Volba kapiláry

Pro výběr vhodné separační kapiláry byla důležitá dvě hlediska, geometrické parametry a vnitřní povrch kapiláry. Délku kapiláry jsme volili tak, aby v rozumně krátké době bylo možné úplné rozdělení analyzovaných látek. Rozměr vnitřního průměru kapiláry byl důležitý při kontrole teploty základního elektrolytu a citlivosti optických detekcí.

Vzhledem k vybrané oblasti pH a složení základních elektrolytů, peptidové hormony byly v kyselém prostředí kladně nabité a postupně se zvyšujícím pH přecházely do neutrální až záporně nabité formy kromě výše zmíněných výjimek. Bylo možné použít vnitřně nepokryté křemenné kapiláry s polyimidem na vnější straně s vnitřním průměrem 50 nebo 75 μm a vnějším průměrem 375 μm , které měly celkovou délku 39-40 cm a efektivní délku 29-30 cm nebo kapiláry o rozměrech 50 μm i.d. a 200 μm o.d., s celkovou délkou 30 cm a efektivní délkou 19 cm. Při analýze peptidových hormonů jako kationtů v nepokrytých křemenných kapilárách v kyselých základních elektrolytech bylo využito snížení EOF a adsorpce díky potlačení disociace silanolových skupin. Rychlý katodický EOF se poté uplatnil při analýze všech peptidových hormonů, které se vyskytly v aniontové formě při alkalickém pH 8-9.

6.1.4 Citlivost detekce

6.1.4.1 UV-absorpční detekce

Nejběžněji používaná detekce peptidů v CE je UV-absorpční detekce v oblasti 200-220 nm, tj. v oblasti absorpce UV-záření peptidovou vazbou.

Porovnáním dvou UV-absorpčních detektorů v přístrojích se zabývá kapitola v publikaci I. Srovnání citlivosti detektorů bylo demonstrováno na CZE analýze dvou neurohypofyzárních nonapeptidů, oxytocinu a Arg⁸-vasopressinu v kyselém tris-fosfátovém pufru, pH 2,25, BGE II. První absorpční detektor se skládal z vysokofrekvenčně buzené jódové výbojky s emisní vlnovou délkou 206 nm jako zdroje světla a UV-senzitivní křemíkové fotodiody s vestaveným předzesilovačem

(ÚOCHB detektor) a byl zabudován do CE-analyzátoru vyvinutého v ÚOCHB. Druhý detektor, detektor s polem fotodiod s vlnovým rozsahem 190-600 nm a s deuteriovou lampou (PDA detektor), byl pro srovnání také nastaven na konstantní vlnovou délku 206 nm. Tento detektor byl součástí komerčního přístroje Beckman P/ACE MDQ. Charakteristiky obou detektorů jako šum, n , odezva detektoru, R , poměr signál/šum, R/n , mez detekce, LOD , a citlivost, S , jsou uvedeny v **tabulce 8**. Uvedené hodnoty byly získány jako průměry dvou následných analýz s maximálním rozdílem 2% v odezvě detektoru. Z tabulky vyplývá, že hodnoty poměrů R/n a meze detekce pro OT a AVP byly u obou detektoru srovnatelné.

Tabulka 8 Charakteristiky detektorů stanovené při CZE analýze peptidů v BGE II.

Peptid	Detektor	c [$\mu\text{mol} \cdot \text{dm}^{-3}$]	R [mV] ^a [mAU] ^b	R/n	S [V $\cdot \text{mol}^{-1} \cdot \text{dm}^3$] ^a [AU $\cdot \text{mol}^{-1} \cdot \text{dm}^3$] ^b	LOD [$\mu\text{mol} \cdot \text{dm}^{-3}$]
OT	ÚOCHB	89,1	64,0	89,0	717,7	3,00
	PDA	85,0	43,8	109,3	51,4	2,34
AVP	ÚOCHB	84,4	141,8	197,3	1680,6	1,28
	PDA	92,0	67,2	167,6	72,9	1,65

Průměrná hodnota šumu pro detektor ÚOCHB byla 0,72 mV a pro detektor PDA 0,04 mAU; ^a – ÚOCHB detektor; ^b – PDA detektor.

Vliv absorpce základního elektrolytu na citlivost detektoru s polem fotodiod (PDA), mez detekce a mez stanovení byl studován v práci I a II.

Porovnání citlivosti detektoru s využitím tris-fosfátového pufru (BGE II) a kyseliny octové (BGE VI) bylo ukázáno na analýze oxytocinu a Arg⁸-vasopressinu. Kyselina octová absorbuje více světla a píky peptidů byly proto menší, nižší však byl i šum. Separace peptidů v kyselině octové byla také ovlivněna elektromigrační disperzí, píky byly širší a méně symetrické. Hodnoty jednotlivých vlastností detektoru jako poměru signál/šum, R/n , meze detekce, LOD , citlivosti, S , a meze stanovitelnosti, LOQ , jsou uvedeny v **tabulce 9**. Citlivost detektoru poklesla přibližně 2,5krát v kyselině octové, ve stejném poměru se zvýšila mez detekce. Tyto rozdíly nejsou tedy příliš dramatické a lze říci, že kyselina octová může být používána jako BGE v CZE.

Porovnání dvou kyselých pufrů tris-fosfátového (BGE II) a izoelektrického (BGE III) pufru bylo demonstrováno na sérii enkefalinů a dalarginu a jejich derivátů a

fragmentů. Byla použita stejná kritéria pro hodnocení citlivosti detektoru jako pro NHPs (viz **tabulka 9** a publikace II, tabulka 4). Z tabulky vyplývá, že více absorbující kyselina iminodioctová poskytuje srovnatelné charakteristiky detektoru jako trifosfátový pufr. Negativní vliv absorpce základního elektrolytu BGE III je v tomto případě kompenzován rychlejší analýzou, užšími píky a vyšší separační účinností.

Tabulka 9 Vlastnosti UV-absorpčního detektoru PDA při vlnové délce 206 nm.

Peptid	BGE	c [mmol.dm ⁻³]	R/n ·10 ⁻³	S [AU.mol ⁻¹ .dm ⁻³]	LOD [µmol.dm ⁻³]	LOQ [µmol.dm ⁻³]
OT	II	0,85	1,09	51,4	2,34	7,80
	VI	0,91	0,86	18,0	3,18	10,6
AVP	II	0,92	1,68	72,9	1,65	5,50
	VI	0,86	1,29	28,4	2,02	6,73
[Leu ⁵]ENK	II	2,08	4,02	42,6	1,55	5,17
	III	2,01	4,06	46,5	1,48	4,95
[Met ⁵]ENK	II	1,61	5,46	74,7	0,88	2,95
	III	1,49	7,41	114,4	0,60	2,01

6.1.4.2 Porovnání citlivosti UV-absorpční detekce a laserem indukované fluorescenční detekce

V práci I byla srovnávána citlivost UV-absorpčního PDA detektoru s fluorescenčním detektorem s argonovým laserem indukovanou fluorescencí (LIF) s excitační vlnovou délkou 488 nm a emisní vlnovou délkou 520 nm v komerčnímu přístroji Beckman P/ACE MDQ. CZE analýzy byly provedeny s derivatizovaným lidským insulinem a s jeho B23-30 oktapeptidovým fragmentem, oba peptidy byly derivatizovány fluorescenční značkou NBD. NBD-HI a NBD-B23-30-HI byly analyzovány v kyselém základním elektrolytu 0,5 M kyselině octové, BGE VI. Fluorescenční značka NBD je vázána na ε-aminoskupinu Lys B29, excitační maximum dosahuje při 475-478 nm a emisní maximum při 530-534 nm. Hlavní charakteristiky obou detektorů jsou uvedeny v **tabulce 10**. Z hodnot mezí detekce vyplývá, že fluorescenční detekce je o 1 až 2 rády citlivější.

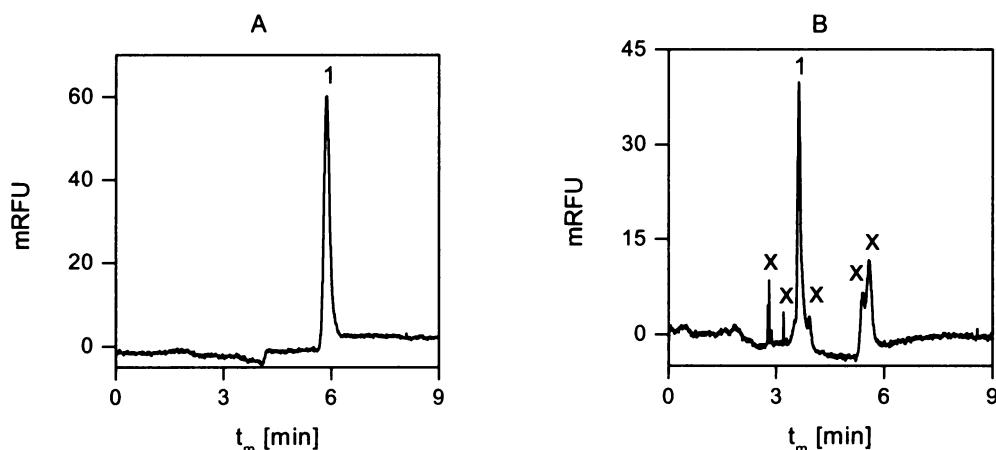
Elektroforegramy CZE analýz NBD-B30-30-HI a NBD-HI v kyselině octové, pH 2,5 s LIF detekcí jsou na **obrázku 2**. Zatímco pro NDB-B23-30-HI byl detekován

jeden pík (**obr. 2A**), při analýze NBD-HI kromě hlavního píku analyzované látky bylo nalezeno několik příměsí (**obr. 2B**). Je pravděpodobné, že fluorescenční činidlo nebylo zcela odstraněno z preparátu oktapeptidového fragmentu a reagovalo následně s nechráněnými skupinami aminokyselinových zbytků v desoktapeptidu HI.

Tabulka 10 Charakteristiky UV-absorpčního PDA detektoru nastaveného na 206 nm a LIF detektoru (488/520 nm) stanovené z CZE analýz insulinu a jeho oktapeptidového fragmentu v BGE VI

Peptid	Detektor	c [$\mu\text{mol} \cdot \text{dm}^{-3}$]	R [mAU] ^a [mRFU] ^b	R/n	S [AU $\cdot \text{mol}^{-1} \cdot \text{dm}^3$] ^a [RFU $\cdot \text{mol}^{-1} \cdot \text{dm}^3$] ^b	LOD
NBD-HI	PDA	63,2	2,77	184,7	44,1	1,02
	LIF	5,09	505,7	136,6	99331	0,112
NBD-B23-30	PDA	729	8,57	571,1	11,7	3,83
	LIF	0,89	629,1	169,9	706590	0,016

Průměrná hodnota šumu pro LIF detektor byla 3,70 mRFU a pro PDA detektor 0,02 mAU; ^a – PDA detektor; ^b – LIF detektor.



Obrázek 2 CZE analýzy fluorescenčně značených peptidů s LIF detekcí. (A) NBD-B23-30-HI, $0.05 \mu\text{g} \cdot \text{mL}^{-1}$, (B) NBD-HI, $0.5 \mu\text{g} \cdot \text{mL}^{-1}$. Analýzy byly prováděny v BGE VI, dávkování 10 mbar, 10 s, separační napětí 15 kV, proud 20–21 μA ; 1 – hlavní syntetický produkt; x – neidentifikované příměsi.

6.1.5 Kvalitativní a kvantitativní analýza

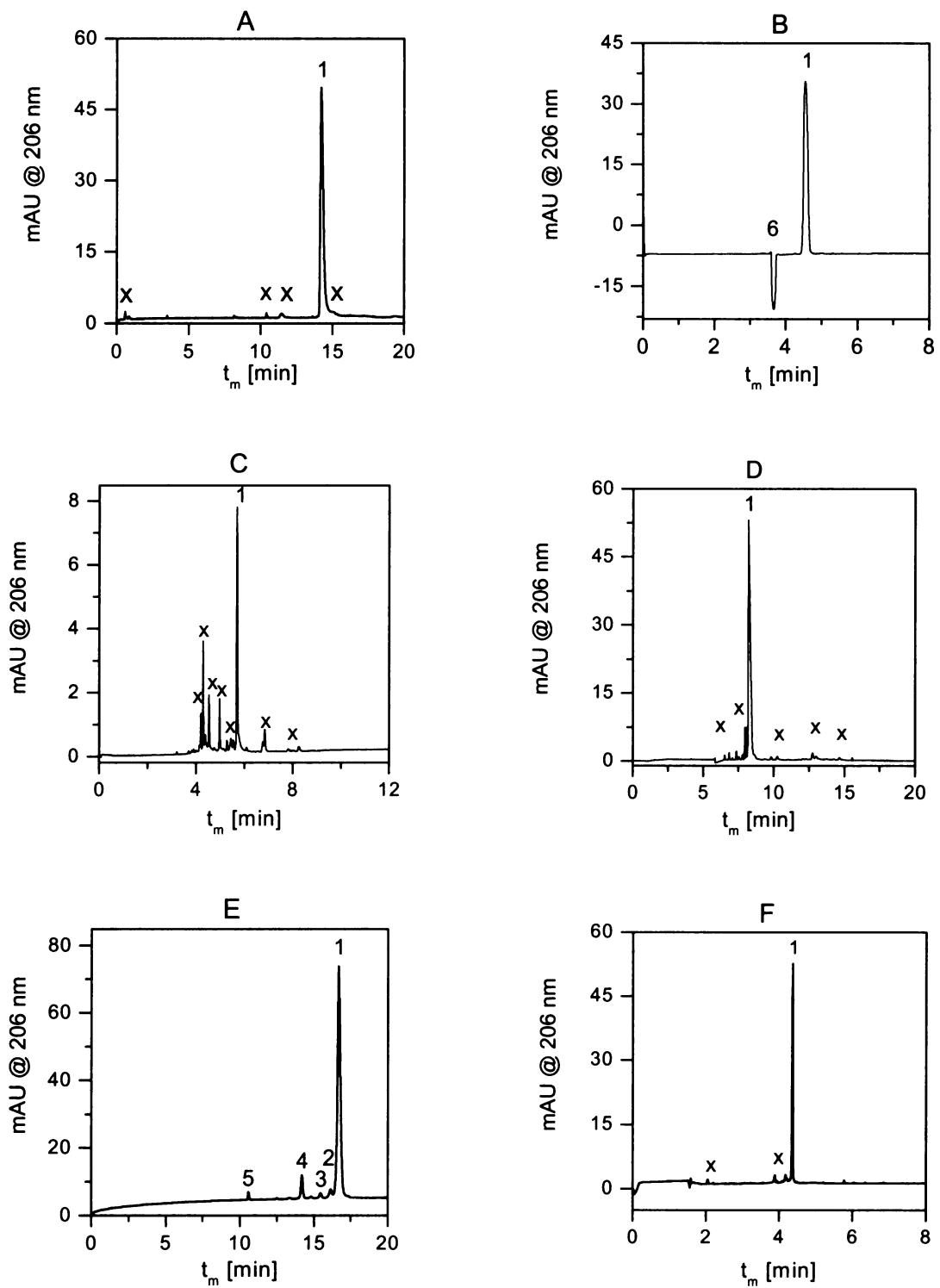
Kvalitativní a kvantitativní analýza byla prvním a základním krokem při studiu nových peptidů. Pro úplnou charakterizaci peptidových preparátů, zvláště pak těch, jež

byly dále užívány pro biologické testy, bylo důležité znát obsah příměsí vznikajících nejčastěji při jejich syntéze a následných čistících operacích.

Stupeň čistoty syntetických a semisyntetických peptidových hormonů čištěných chromatografickými metodami jako RP-HPLC a/nebo SEC byl stanoven v publikacích I, II, III a IV. Peptidy byly analyzovány v základních elektrolytech vybraných podle strategie pro racionální volbu podmínek pro separaci peptidů. Standardy nově připravených peptidů nebyly dostupné, takže nebylo možné je stanovit metodou kalibrační křivky či vnitřního standardu. Proto byl stupeň čistoty určen pomocí relativní výšky píku, P_h , relativní plochy píku, P_A , a relativní korigované plochy píku P_{CA} (viz kapitola 2.1.1). Většinou se hodnoty stupňů čistoty pohybovaly mezi hodnotami 85-99%, pro NHPs 80-96% (přesné hodnoty viz tabulka 2, publikace I), ENKs a DLR 85-99,9% (přesné hodnoty viz tabulka 3, publikace II), IOPs 95-99% a GnRHs 88-99%. Stupně čistoty peptidových hormonů byly stanoveny ze dvou měření, která se lišila o méně než 1%.

Vybrané příklady CZE a CMEKC analýz jsou uvedeny na obrázku 3, ostatní viz publikace I-IV. CZE analýzy peptidů s vysokým stupněm čistoty jsou demonstrovány na oxytocinu (viz obrázek 3A) stanoveném jako kation v kyselém elektrolytu, BGE II, a na [Leu⁵]enkefalinu (viz obrázek 3B) analyzovaném jako anion v alkalickém prostředí, BGE VII. Opačným příkladem je elektroforegram [Des-Gly-NH₂]cGnRH II (viz obrázek 3C) s mnoha neidentifikovanými příměsmi, kde stupeň čistoty dosáhl hodnoty pouze 55,1%. Všechny tyto tři příklady poskytovaly podobné hodnoty stupně čistoty stanovené vsemi třemi možnými způsoby vyhodnocení, tj. podle relativní výšky, plochy a korigované plochy. Na příkladu CZE analýzy dalarginu je ukázáno (viz obrázek 3D), že mohou být velké rozdíly ve stupni čistoty v závislosti na použitém způsobu vyhodnocování. Stupeň čistoty stanovený pomocí relativní výšky píku byl o 10-15% menší než stupeň čistoty stanovený pomocí relativní plochy píku nebo relativní korigované plochy píku v závislosti na použitém základním elektrolytu v důsledku relativně velké výšky a malé plochy ostrého píku hlavní příměsi. Příkladem kvantitativní analýzy peptidu s vysokým stupněm čistoty metodou CMEKC je stanovení deamino-oxytocinu v alkalickém prostředí BGE VIII (viz obrázek 3F). Z uvedených příkladů analýz je zřejmé, že získaný stupeň čistoty závisí na experimentálních podmínkách a také na způsobu jeho stanovení.

V práci III je na příkladě dekapeptidu YDPAP₆ ukázána identifikace minoritních příměsí. Na elektroforegramu dekapeptidu (viz obrázek 3E) jsou jasně



Obrázek 3 CZE (A)-(E) a CMEKC (F) analýzy peptidových hormonů: (A) oxytocin, $0,95 \text{ mg.ml}^{-1}$, BGE II, $U = 10 \text{ kV}$, $I_{el} = 69\text{-}70 \mu\text{A}$; (B) $[Leu^5]$ enkefalin, $1,25 \text{ mg.ml}^{-1}$, BGE VII, $U = 10 \text{ kV}$, $I_{el} = 13,2\text{-}14,2 \mu\text{A}$; (C) $[Des-Gly-NH_2]cGnRH$ II, $1,10 \text{ mg.ml}^{-1}$, BGE II, $U = 15 \text{ kV}$, $I_{el} = 43,2 \mu\text{A}$; (D) dalargin, $1,25 \text{ mg.ml}^{-1}$, BGE III, $U = 10 \text{ kV}$, $I_{el} = 36,7\text{-}37 \mu\text{A}$; (E) YDPAP₆, $1,15 \text{ mg.ml}^{-1}$, BGE II, $U = 10 \text{ kV}$, $I_{el} = 69\text{-}70 \mu\text{A}$; (F) deamino-oxytocin $1,15 \text{ mg.ml}^{-1}$, BGE VIII, $U = 20 \text{ kV}$, $I_{el} = 69\text{-}71 \mu\text{A}$; 1 – hlavní syntetický produkt, x – neidentifikované příměsi, 2 – YDPAP₅, 3 – YDPAP₄, 4 – YDPAP₂, 5 – YD, 6 – elektroosmotický marker.

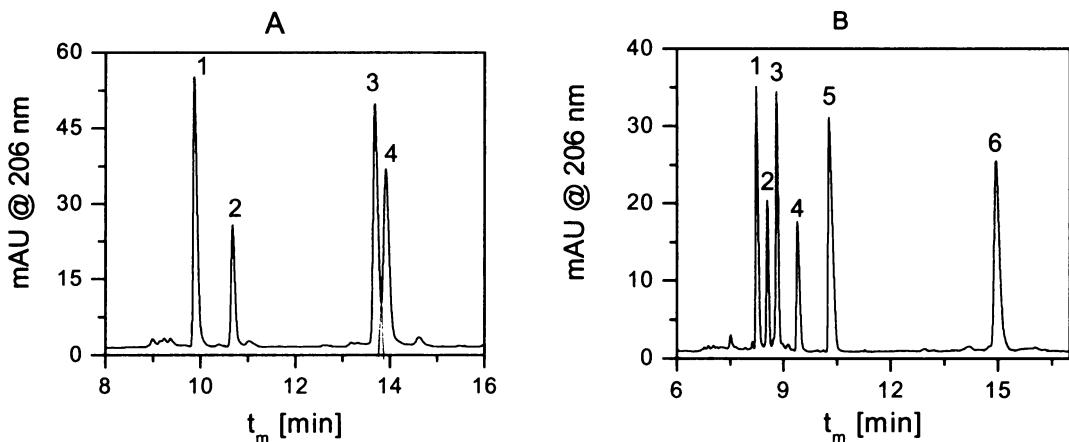
patrné čtyři příměsi, které byly identifikovány jako jeho kratší fragmenty, YDPAP₅, YDPAP₄, YDPAP₂, YD, na základě porovnání jejich efektivních elektroforetických pohyblivostí. Jejich přítomnost byla také potvrzena přídavkem standardů těchto kratších peptidů ke vzorku dekapeptidu YDPAP₆. Stupeň čistoty tohoto dekapeptidu dosahoval 86,7%.

6.1.6 Separace strukturně podobných peptidů

Separace strukturně podobných peptidů má velký význam při jejich čištění po syntéze, ale také při sledování biochemických procesů v organismech, například při monitorování degradačních produktů biologicky aktivní peptidů. Příkladem separace strukturně podobných peptidových hormonů jsou separace skupiny dvou enkefalinů a jejich dvou fragmentů, dalarginu a jeho pěti fragmentů a derivátů (publikace II), dvou skupin oostatických peptidů (publikace III) a tří čtyřčlenných skupin hormonů uvolňující gonadotropin (publikace IV). Vhodné experimentální podmínky byly odvozeny na základě strategie pro racionální výběr podmínek pro separaci peptidů, především ze závislosti specifického náboje peptidů na pH, a ze zkušeností získaných z analýz jednotlivých peptidů v základních elektrolytech.

Na základě výsledků z předešlých analýz pro separaci enkefalinů a dalarginů a jejich fragmentů a derivátů byl vybrán izoelektrický pufr, 100 mM kyselina iminodioctová, BGE III. K získání lepšího rozlišení byly testovány různé koncentrace IDAA a separační napětí. Nakonec jako nejlepší podmínky pro separaci směsi dalarginu, jeho fragmentů a derivátů byly vybrány tyto podmínky – 100 mM IDAA, pH 2,30, separační napětí 10 kV. Dvojnásobná koncentrace IDAA, tedy 200 mM, pH 2,32, BGE IV, byla nalezena jako nejlepší BGE pro separaci ENKs a jejich derivátů při stejném separačním napětí 10 kV. Separace směsi enkefalinů a jejich fragmentů ([Leu⁵]ENK, [Met⁵]ENK, YGG, YG) je na obrázku 4A, separace směsi dalarginu a jeho fragmentů a derivátů (DLR, YA, YaG, DLR-NHEt, I₂DLR, PacDLR) na obrázku 4B. Rozlišení každé dvojice peptidů bylo alespoň 2,1 s výjimkou [Leu⁵]ENK a [Met⁵]ENK, kde rozlišení dosáhlo pouze hodnoty 1,2, tedy rozdelení nebylo úplné. Ve směsi ENKs a jejich fragmentů se nejrychleji pohybovaly fragmenty a poté ENKs (YG, YGG, [Leu⁵]ENK, [Met⁵]ENK). Ve směsi dalarginu, jeho analogů a fragmentů rychleji migrovaly delší peptidy s vyšším efektivním a specifickým nábojem než kratší fragmenty s nižším specifickým nábojem.

CZE separace směsí IOPs lišících se v počtu aminokyselinových zbytků v lineárním řetězci nebo v malé změně složení aminokyselin, jako záměna prolinu za dehydroprolin nebo tyrosinu za homotyrosin, byly provedeny ve třech kyselých

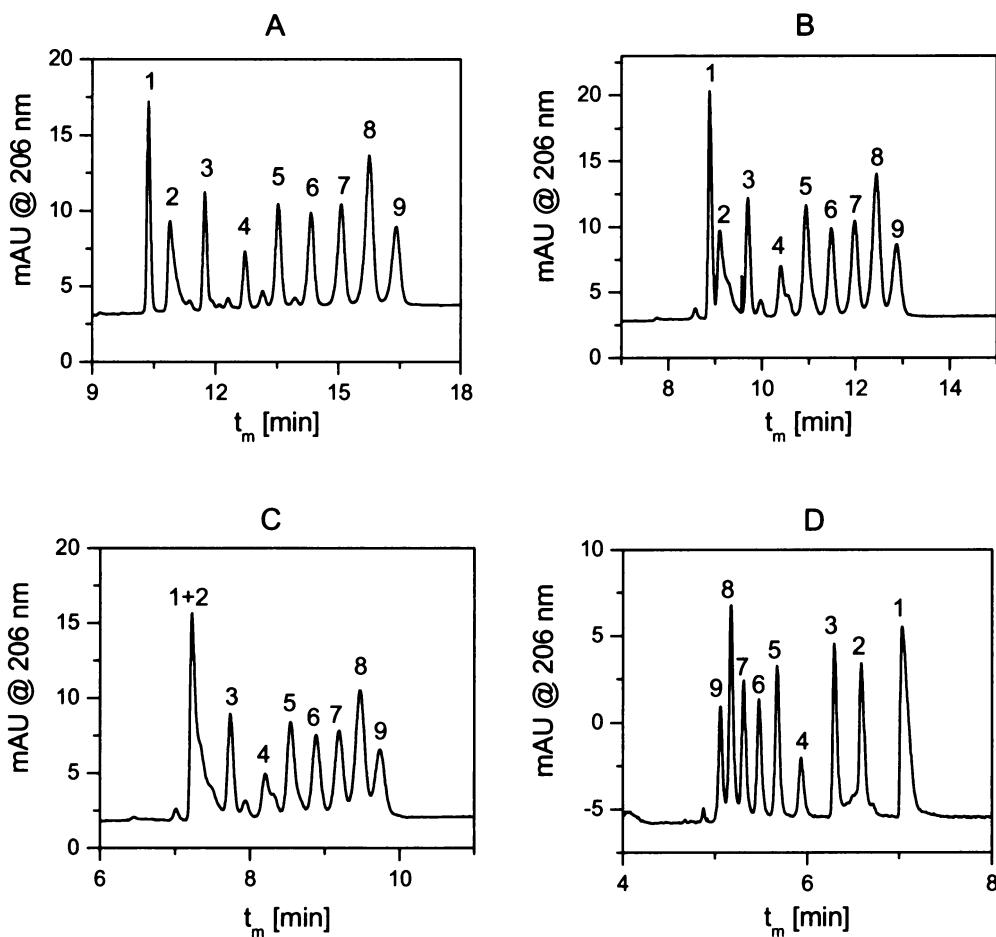


Obrázek 4 CZE separace (A) enkefalinových analogů a fragmentů v BGE IV, pH 2,32, 1 – YG, 2 – YGG, 3 – [Leu⁵]ENK, 4 – [Met⁵]ENK; (B) dalarginové fragmenty a analogy v BGE III, pH 2,30, 1 – DLR-NH₂, 2 – DLR, 3 – I₂DLR, 4 – YA, 5 – YaG, 6 – PacDLR. Experimentální podmínky: křemenná kapilára celk./efekt. délka 394/292 mm, i.d. 75 μ m, o.d. 375 μ m, separační napětí 10 kV, UV absorpční detektor, $\lambda = 206$ nm.

BGEs, BGE II, III, V, a v jednom alkalickém elektrolytu, BGE VII. Elektroforegramy separace peptidů se stejným efektivním nábojem a s klesajícím počtem prolinových zbytků na C-konci řetězce YDPAP_x, x = 6-0, a dipeptidu, YD a tripeptidu, YDP, analyzovaných jako kationty v kyselých základních elektrolytech a jako anionty v alkalickém pufru, jsou na **obrázku 5**. Nejlepší rozlišení separace bylo nalezeno v klasickém tris-fosfátovém pufru, BGE II (**obrázek 5A**). V izoelektrickém pufru BGE III byl rychlejší EOF, čas analýzy byl kratší, ale páky nebyly úplně rozděleny na základní linii (**obrázek 5B**). V BGE V, 50 mM IDAA, dipeptid a tripeptid migrovaly ve směsné zóně (**obrázek 5C**). V alkalickém tris-tricinovém pufru, BGE VII, pomalejší IOPs nebyly rozděleny na základní linii a rychlejší IOPs měly trojúhelníkový tvar páku způsobený elektromigrační disperzí (**obrázek 5D**).

Další strukturně podobné peptidy ze skupiny 20 IOPs byly separovány pouze ve vybraných elektrolytech. Tetrapeptid, YDPA, a jeho analog s homotyrosinem na N-konci, HtyDPA, byly úspěšně separovány v alkalickém BGE VII s rozlišením 1,92. V kyselých pufrech byly rozděleny s rozlišením 1,01-1,5, v izoelektrických pufrech, BGE III a V, nebyly rozděleny na základní linii. Analogy s delším peptidovým řetězcem lišící se prolinem nahrazeným dehydroprolinem dekapetidů, YDPAP₆ a YDPAP₅ΔP, a

pentapeptidů, YDPAP a YDPA Δ P, byly úplně separovány na základní linii ve třech kyselých elektrolytech BGE II, III a V, na rozdíl od kratších analogů, tetrapeptidů, YDPA a YD Δ PA, a tripeptidů YDP a YD Δ P, které byly rozděleny pouze částečně nebo migrovaly ve směsných zónách. Žádný z derivátů s dehydroprolinem se nedělil od analogu s prolinem v alkalickém BGE VII. Cyklické analogy byly separovány pouze v alkalickém BGE VII, neboť v kyselých základních elektrolytech nebyly nabité.



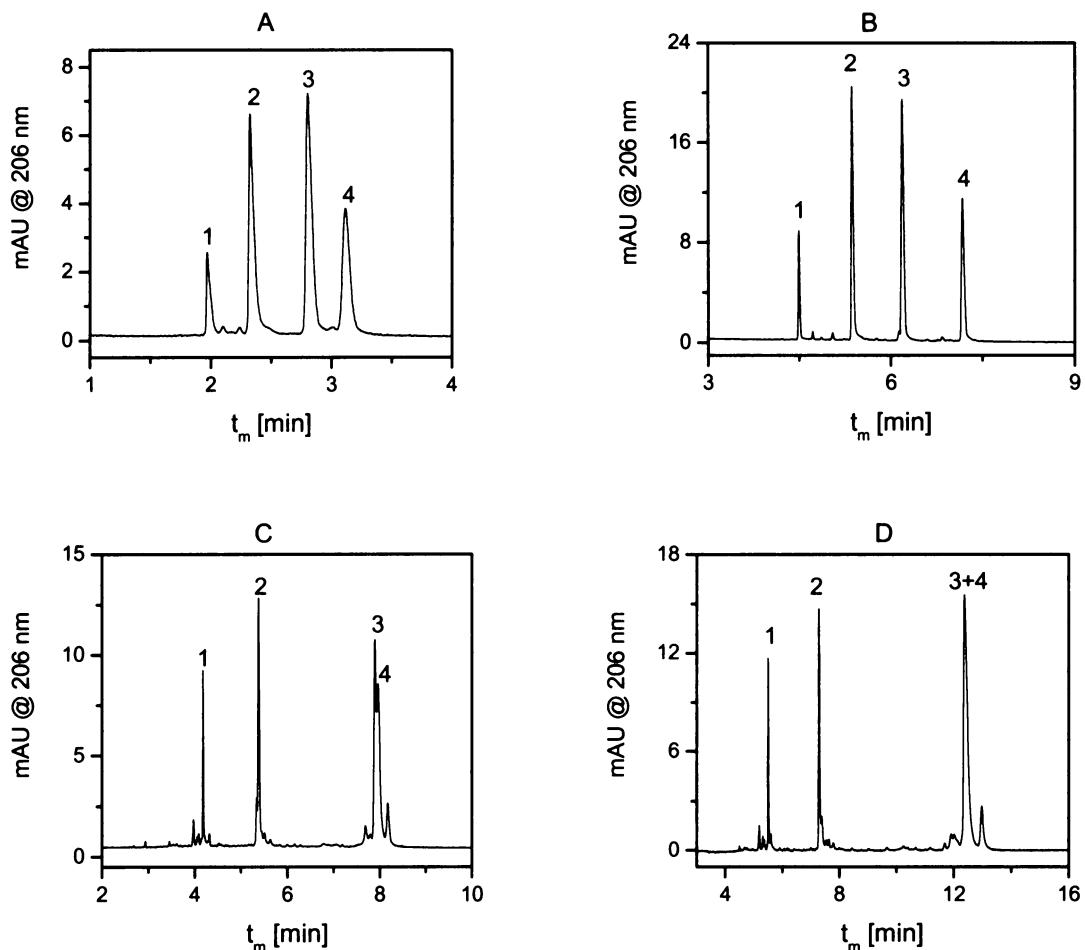
Obrázek 5 CZE separace devíti IOPs s klesajícím počtem aminokyselinových zbytků v lineárním řetězci v (A) BGE II, pH 2,25, (B) BGE III, pH 2,30, (C) BGE V, pH 2,40 a (D) BGE VII, pH 8,1. 1 – YD, 2 – YDP, 3 – YDPA, 4 – YDPAP, 5 – YDPAP₂, 6 – YDPAP₃, 7 – YDPAP₄, 8 – YDPAP₅, 9 – YDPAP₆. Experimentální podmínky: křemenná kapilára celk./efekt. délka 402/300 mm, i.d. 75 μ m, o.d. 375 μ m, separační napětí 10 kV, UV absorpční detektor $\lambda = 206$ nm.

Tři směsi po čtyřech GnRHs byly na základě experimentálních podmínek zvolených dle strategie pro racionální výběr podmínek pro separaci peptidů děleny v kyselém prostředí v úzkém rozsahu pH 2,18-2,50 v pěti různých základních

elektrolytech, BGE I-IV a BGE VI. Směs 1 byla složená z lidského GnRH a jeho fragmentů (pGluHWS, SYGLRPG-NH₂, dAPGNH₂-hGnRH), směs 2 z dekapeptidů tří druhů GnRH ([β Ala⁶-ProNHEt¹⁰]hGnRH, hGnRH, sGnRH, cGnRH I) a směs 3 z [Des-pGlu¹-D-Orn⁶]sGnRH, [Des-Pro-Gly-NH₂]hGnRH, sGnRH a [Des-Gly-NH₂]sGnRH, viz **tabulka 6**. Srovnání účinností a rozlišení separace jednotlivých peptidů ve směsích v klasických BGEs jako kyselině octové, BGE I a BGE VI, a tris-fosfátovém pufru, BGE II, a v izoelektrických BGEs, 0,1-0,2 M kyselině iminodioctové, BGE III a BGE IV, je uvedeno v **tabulce 11**. Nejrychlejší separace peptidů byly provedeny v kyselině octové, ale hodnoty jejich rozlišení a separační účinností dosahovaly nejnižších hodnot. Nejkratší čas analýzy, 3,4-4,5 minut, byl získán v 0,5 M kyselině octové (BGE VI), jak je doloženo na separaci směsi 1 na **obrázku 6A**. Nejvyšší hodnoty separační účinnosti, 0,5-1,6·10⁶ teoretických pater na metr, byly nalezeny pro izoelektrický BGE IV 0,2 M IDAA, pH 2,32. Důkazem úspěšné separace směsi 1 je **obrázek 6B** a směsi 3 **obrázek 6C**. Hodnoty rozlišení peptidů ve směsích 1 a 2 v klasickém tris-fosfátovém (BGE II) a izoelektrickém pufru (BGE IV) byly srovnatelné, ale ve směsi 3 peptidy [Des-Gly-NH₂]sGnRH a sGnRH v BGE II migrovaly ve směsné zóně bez náznaku rozlišení (viz **obrázek 6D**) na rozdíl od separace s rozlišením 1,1 v izoelektrickém BGE IV (viz **obrázek 6C**). Z výsledků vyplývá, že úspěšná separace nezávisí pouze na pH základního elektrolytu, které se v daném případě lišilo o minimální rozdíl 0,05 jednotky, ale také na složení BGE a tedy na interakcích mezi ionty analytu a složkami BGE. Na příkladě separace GnRHs lze ukázat, že izoelektrický BGE, kyselina iminodioctová, může poskytnout lepší dělení než klasické BGEs.

Tabulka 11 Separační účinnost a rozlišení CZE separace tří směsi GnRHs a jejich fragmentů a analogů v přetí kyselých základních elektrolytech, BGE I-IV, BGE VI. Složení BGES je uvedeno v tabulce 7.

Směs	Peptid	Separační účinnost [počet teoretických pater na metr]						Rozlišení		
		BGE I	BGE II	BGE III	BGE IV	BGE VI	BGE I	BGE II	BGE III	BGE IV
1	SYGLRPG-NH ₂	21709	125188	60959	164189	14299	-	-	-	-
	hGnRH	18605	67104	43677	112854	12955	6,3	16,8	11,2	15,7
	pGluHWS	21967	56571	40334	106615	13811	6,7	10,9	9,6	11,5
	dAPGNH ₂ -hGnRH	17631	75473	47091	123868	12119	5,6	14,6	9,2	12,2
2	βANHEt-hGnRH	28602	104971	67695	152187	17714	"	"	"	"
	hGnRH	16941	58881	42961	100696	11929	0,8	2,1	1,8	2,5
	sGnRH	12807	36244	26417	55750	8739	6,9	24,9	17,9	23,6
	cGnRH I	12807	36244	26417	55750	8739	0	0	0	0
3	dPyrDm-sGnRH	27892	211526	74027	148832	14898	-	-	-	-
	dPGNH ₂ -hGnRH	17129	106062	54877	120507	13414	10,7	25,8	18,3	24,7
	sGnRH	12985	36711	18131	59590	6529	13,4	29,3	18,2	29,6
	dGNH ₂ -sGnRH	12985	36711	13857	48050	4681	0	0	0,7	1,1
									0,2	

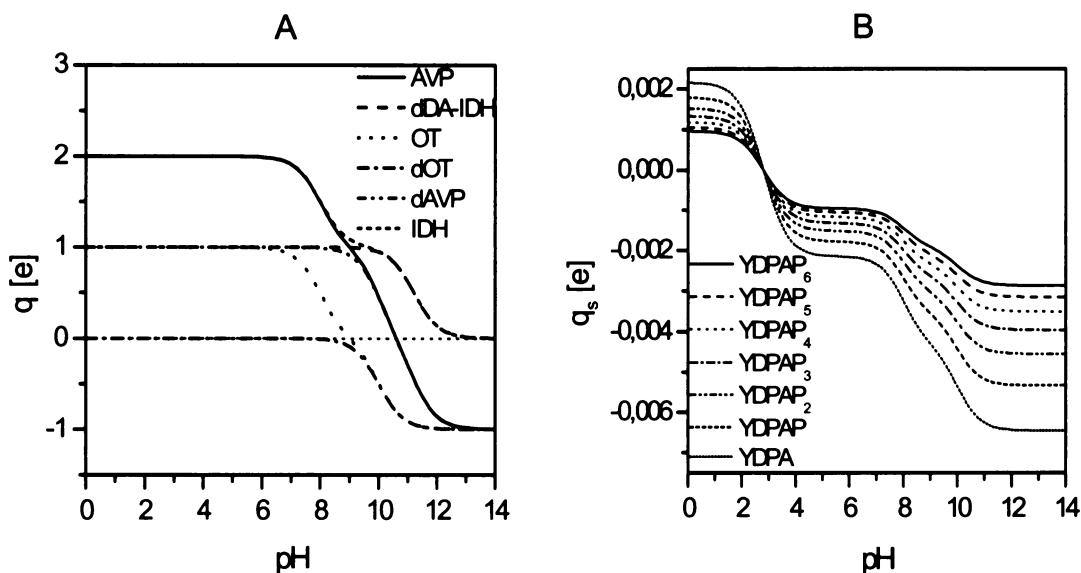


Obrázek 6 CZE separace strukturně podobných GnRHs. Směs 1 v (A) BGE VI, pH 2,50, $U = 25\text{ kV}$; (B) BGE IV, 2,32, $U = 15\text{ kV}$; a směs 3 v (C) BGE IV, pH 2,32, $U = 15\text{ kV}$; (D) BGE II, pH 2,25, $U = 15\text{ kV}$; směs 1, 1 – SYGLRPG-NH₂, 2 – hGnRH, 3 – pGluHWS, 4 – dAPGNH₂-hGnRH; směs 3, 1 – dPyrDOrn-sGnRH, 2 – dPGNH₂-hGnRH, 3 – sGnRH, 4 – dGNH₂-sGnRH. Experimentální podmínky: křemenná kapilára celk./efekt. délka 390/288 mm, i.d. 50 μm , o.d. 375 μm , UV absorpční detektor $\lambda = 206\text{ nm}$.

6.2 Fyzikálně-chemická charakterizace peptidů

6.2.1 Výpočet efektivního a specifického náboje

Efektivní náboj a specifický náboj (efektivní náboj dělený relativní molekulovou hmotností) peptidů byl vypočten pomocí dříve vytvořeného počítačového programu Nabamfo [88]. Při výpočtu jsme vycházeli ze známých primárních struktur peptidů (viz tabulky 3-6 a obrázek 1), tj. z přítomnosti jednotlivých ionogenních skupin v peptidových řetězcích. Vstupními daty pro výpočet byly hodnoty acidobazických disociačních konstant těchto ionogenních skupin, které byly získány jako průměrné hodnoty tabelovaných acidobazických disociačních konstant pro ionogenní skupiny aminokyselin uvedených v tabulce 1, kapitola 2.5.1. Závislosti efektivního náboje NHPs a specifického náboje pro sedm IOPs s klesajícím počtem prolinových zbytků na C-konci řetězce $YDPAP_x$, $x = 6-0$, na pH jsou znázorněny na obrázku 7.



Obrázek 7 (A) Závislost efektivního náboje, q , neurohypofyzárních hormonů na pH; **(B)** Závislost specifického náboje, q_s , sedmi vybraných lineárních hmyzích oostatických peptidů s klesajícím počtem prolinových zbytků na C-konci řetězce, $YDPAP_x$, $x = 6-0$, na pH.

Pro pH optimálních základních elektrolytů byly vypočteny hodnoty efektivního náboje. Pro ionogenní NHPs v kyselém pH 2,25-2,50 byly vypočteny hodnoty 1 e nebo 2 e. Efektivní náboj pro ENKs a jejich fragmenty v kyselém pH 2,25-2,30 nabýval kladných hodnot v rozsahu 0,6-0,7 e, zatímco v alkalickém pH 8,1 byly nabité záporně (kolem hodnoty -0,6 e). DLR, jeho fragmenty a analogy se chovaly jako kationty nejen

v kyselém pH s hodnotou náboje v rozsahu 0,6-2 e, ale i v alkalickém pH 8,1 některé s blokovaným C-koncem peptidového řetězce byly kladně nabité v rozmezí 0,4-1,4 e. Zbylé dalarginy s volným C-koncem peptidového řetězce měly záporný efektivní náboj (kolem hodnoty -0,6 e) jako ENKs. Přesné hodnoty pro jednotlivé ENKs, DLR, jejich fragmenty a analogy jsou uvedeny v publikaci **II** v tabulce 5. Lineární IOPs v kyselých pufrech, pH 2,25-2,40, byly kladně nabité v rozsahu 0,7-0,8 e, a záporně nabité (-1,6 e) v alkalickém BGE VIII, pH 8,1. Pro cyklické IOPs v kyselém prostředí se blížila hodnota efektivního náboje k nule a v alkalickém dosahovala hodnot -2 e nebo -1 e. GnRHs byly analyzovány pouze v kyselé oblasti pH 2,18-2,50, kde v závislosti na počtu ionogenních skupin dosahovaly pozitivního efektivního náboje 0,9-3 e, závislost specifického náboje na pH je znázorněna v publikaci **IV** na obrázku 1 a přesné hodnoty jsou uvedeny tamtéž v tabulce 3.

6.2.2 Stanovení efektivní elektroforetické pohyblivosti při standardní teplotě

Pro výpočet efektivní elektroforetické pohyblivosti peptidů v základních elektrolytech s dostatečně rychlým EOF jsme použili vztah (2.5). V případě, že EOF byl pomalý a stanovení jeho pohyblivosti bylo časově náročné, použili jsme tzv. tlakem urychlené stanovení elektroosmotické pohyblivosti, a efektivní elektroforetickou pohyblivost vypočítali ze vztahu (2.6).

Pohyblivost je závislá na teplotě, vzrůstá přibližně o 2% na 1°C, proto pro přesné stanovení a porovnání pohyblivosti je nezbytné znát teplotu, při které byla pohyblivost změřena. V pracích **II**, **III**, **IV** byl pro stanovení teploty uvnitř kapiláry použit dříve navržený postup [97] založený na měření elektrické vodivosti roztoku standardního roztoku 0,02 M chloridu draselného o známé teplotní závislosti elektrické vodivosti. Pro specifickou vodivost, κ , 0,02 M KCl platí v teplotním rozmezí 15-35°C lineární vztah:

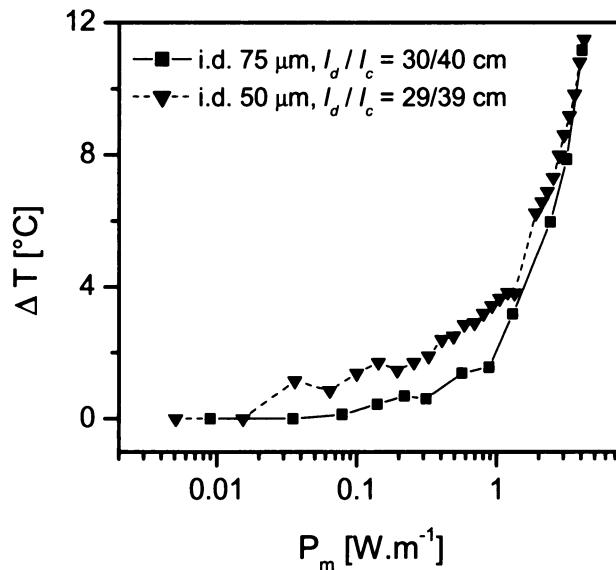
$$\kappa = 0,14304 + 5,36 \cdot 10^{-3} T \quad (5.1)$$

Aktuální specifickou vodivost roztoku KCl lze vypočítat ze vztahu:

$$\kappa = \frac{I_{el}}{U} \frac{l_c}{\pi R_i^2} = \theta \frac{I_{el}}{U} \quad (5.2)$$

kde l_c je celková délka kapiláry, R_i je vnitřní poloměr kapiláry, θ je odporová konstanta nádobky, I_{el} je elektrický proud a U je elektrické napětí na kapiláře. V použité metodě sloužila separační kapilára za konduktometrickou celu a měřeným roztokem byl 20 mM KCl. Konstanta nádobky se stanovila z hodnoty proudu, I_{el} , protékajícího kapilárou při

nízkém napětí, U, nejčastěji 1 kV, kdy bylo možné zanedbat nárůst teploty roztoku uvnitř kapiláry. Poté byla proměnena závislost elektrického proudu na napětí a byl sestrojen kalibrační graf ve tvaru závislosti rozdílu teplot uvnitř a vně kapiláry na elektrickém příkonu vloženém na kapiláru. Kalibrační graf byl poté použit pro kapiláry o stejných délkách a vnitřních průměrech pro daný přístroj.



Obrázek 8 Kalibrační graf náruštu teploty roztoku uvnitř kapiláry vzhledem k okolí, ΔT , v závislosti na elektrickém příkonu vztaženém na jednotku délku kapiláry, P_m . i.d. – vnitřní průměr kapiláry, l_c – celková délka kapiláry, l_d – efektivní délka kapiláry.

V rámci této práce pro přesné stanovení pohyblivostí byl použit komerční přístroj Beckman P/ACE MDQ a kapiláry o dvou různých vnitřních průměrech, 50 μm a 75 μm . Závislost rozdílu teplot uvnitř a vně kapiláry na elektrickém příkonu vloženém na kapiláru pro obě kapiláry je zobrazena na obrázku 8. Teplotní nárušty v křemenné kapiláře s i.d. 50 μm byly 3,6°C pro BGE I a BGE V, 4,8°C pro BGE II, 4,5°C pro BGE III a 3,9°C pro BGE IV a v kapiláře s i.d. 75 μm 4,5°C pro BGE II, 2,1°C pro BGE III, 3,2°C pro BGE IV, 1,5°C pro BGE V a 0,7°C pro BGE VII. Efektivní elektroforetické pohyblivosti korigované na standardní teplotu, $\mu_{ep,25}$, byly spočteny podle vztahu:

$$\mu_{ep,25} = \mu_{ep,t} [1 - 0,020(t - 25)] \quad (5.3)$$

kde $\mu_{ep,25}$ je efektivní pohyblivost při teplotě 25°C a $\mu_{ep,t}$ je efektivní elektroforetická pohyblivost měřená za skutečné teploty, t , uvnitř kapiláry.

Efektivní elektroforetické pohyblivosti korigované na standardní teplotu 25°C byly určeny pro enkefaliny, dalargin, jejich fragmenty a deriváty v práci **II**, tabulka 5, pro IOPs v práci **III**, tabulka 3 a pro GnRHs v práci **IV**, tabulka 3. Elektroforetické pohyblivosti byly určeny jako průměrné hodnoty ze dvou měření, které se nelišily o více než 1%. Tlakem urychlené stanovení elektroosmotické pohyblivosti byla použito u stanovení GnRHs, ENKs a DLR v tris-fosfátovém pufru, BGE II, kde elektroosmotická pohyblivost byla velmi nízká, mezi $0,1 - 0,9 \cdot 10^{-9} \text{ m}^2 \text{V}^{-1} \text{s}^{-1}$.

6.2.3 Testování semiempirických modelů korelujících pohyblivost peptidů a jejich náboj, velikost a prostorové uspořádání

V pracích **III** a **IV** bylo testováno sedm respektive šest semiempirických modelů korelujících pohyblivost IOPs a GnRHs s jejich nábojem, velikostí a tvarem: model pro sféricky tvarované molekuly viz vztah (2.15) [107], model pro klasický lineární polymer (2.16), Offordův model (2.14) [101], Grossmanův model (2.20) [114], Crossův model (2.17) [109], model Cifuentese a Poppeho (2.21) [119] a Kimův model (2.18) [112]. Kromě toho v práci **II** byl Crossův model aplikován na nehomologickou řadu ENKs a DLRs.

Všech sedm modelů bylo testováno na 20 IOPs s relativní molekulovou hmotností v rozsahu 299,3 až 1087,2 obsahujících 2 až 10 aminokyselinových zbytků. Grafy korelací aplikovaných na celou sérii těchto peptidů jsou v publikaci **III**, obrázek 5. V tomto případě žádný z modelů nepopisoval závislost pohyblivostí peptidů na jejich nábojích a velikostech zcela přesně, což se projevilo na hodnotách korelačních koeficientů pohybujících se v rozsahu 0,972-0,888. Hodnoty směrnice a korelačního koeficientu lineární závislosti Crossova modelu $\log(\mu_{ep}/q)$ versus $\log(M_r)$, vztah (2.17), umožňují navrhnut nejpravděpodobnější model pro daný elektrolyt (viz **tabulky 2 a 12**). Model pro sféricky tvarované molekuly nejlépe vyhovoval elektromigraci peptidů v kyselém BGE III s korelačním koeficientem, $r = 0,972$, a v BGE V s $r = 0,958$. Pohyblivost peptidů v kyselém BGE II nejlépe popisoval model pro klasický lineární polymer s $r = 0,981$. Stejně tak model pro klasický lineární polymer se nejlépe hodil pro peptidy v alkalickém BGE VII s $r = 0,972$, alternativně by byl též použitelný Offordův model s $r = 0,963$. Ostatní modely jako Grossmanův model s $r = 0,888-0,936$, model Cifuentese a Poppeho s $r = 0,911-0,965$, stejně tak jako Kimův model s $r = 0,933-0,965$ jsou méně vhodné pro popis chování IOPs v kyselých elektrolytech BGE II, III, V a v alkalickém BGE VII.

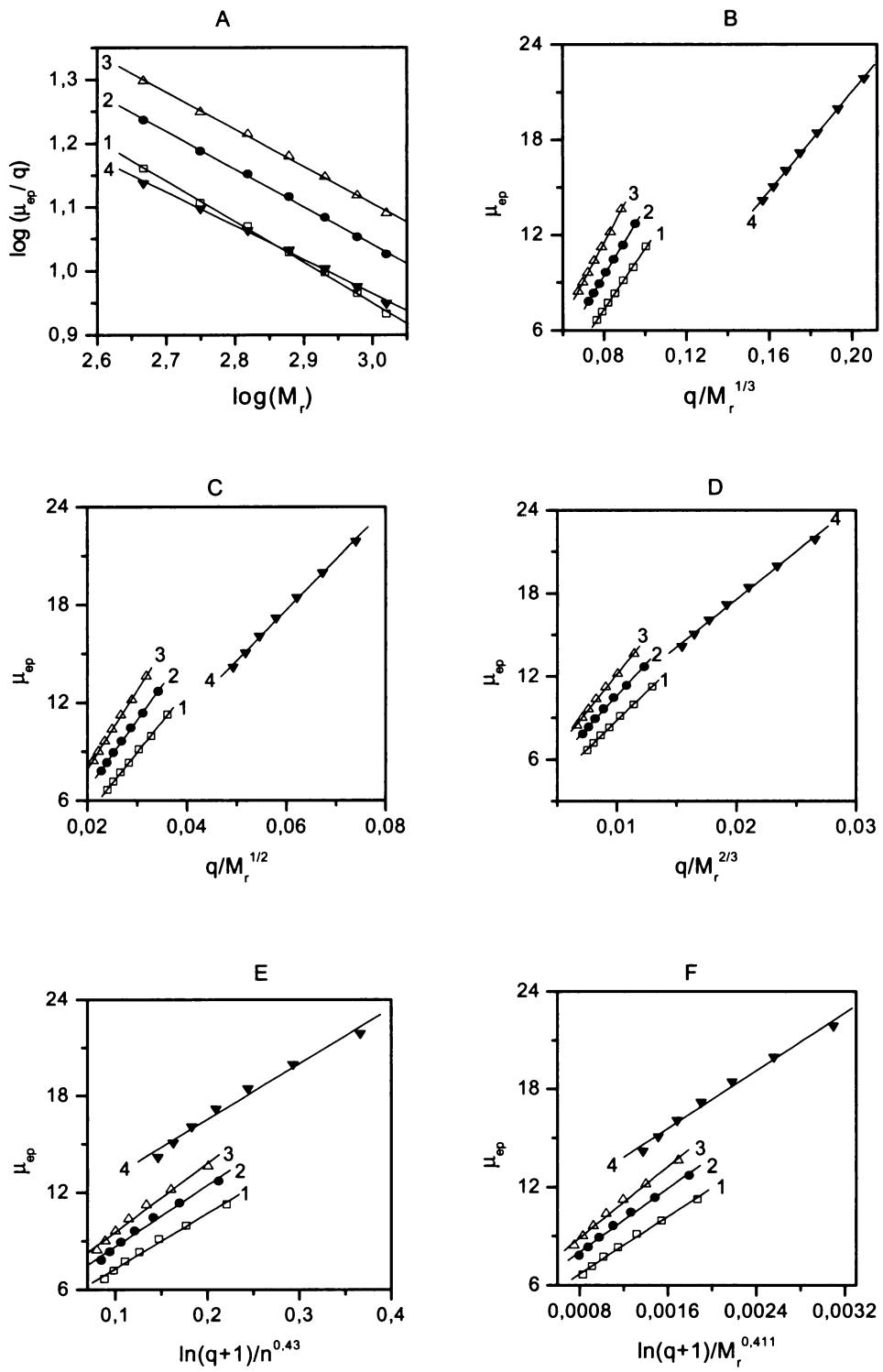
Pro další testy byla vybrána skupina devíti analogů IOPs s klesajícím počtem prolinových zbytků na C-konci řetězce, od dekapeptidu, YDPAP₆, k dipeptidu, YD. Výsledkem byly vyšší hodnoty korelačních koeficientů získaných regresní analýzou. Z grafů bylo také zřejmé, že body odpovídající di- a tripeptidu vybočovaly z lineární části grafu. Kvůli nelineárnímu charakteru korelací mezi pohyblivostí a poměrem náboje a velikosti dvou krátkých peptidů s ohledem na malou pravděpodobnost tvorby organizované sekundární struktury, byla skupina omezena na sedm peptidů s lineárně klesajícím počtem prolinových zbytků na C-konci peptidového řetězce, YDPAP_x, x = 6-0, s konstantním klesajícím rozdílem relativní molekulové hmotnosti 97,1. Grafy šesti empirických modelů aplikovaných na homologickou sadu sedmi IOPs jsou na obrázku 9. Z grafů je patrné, že korelace efektivních elektroforetických pohyblivostí peptidů s jejich efektivním nábojem a velikostí se výrazně zlepšila, $r = 0,999$, (viz tabulka 12), peptidy této série tedy vykazují podobné migrační chování.

Tabulka 12 Parametry lineární regrese Crossova modelu $\log (\mu_{ep}/q)$ versus $\log (M_r)$ pro nehomologickou řadu 20 IOPs a pro homologickou řadu 7 lineárních IOPs s klesajícím počtem prolinových zbytků na C-konci řetězce, YDPAP_x (x = 6-0), ve čtyřech základních elektrolytech. Složení a pH BGEs jsou uvedeny v tabulce 7.

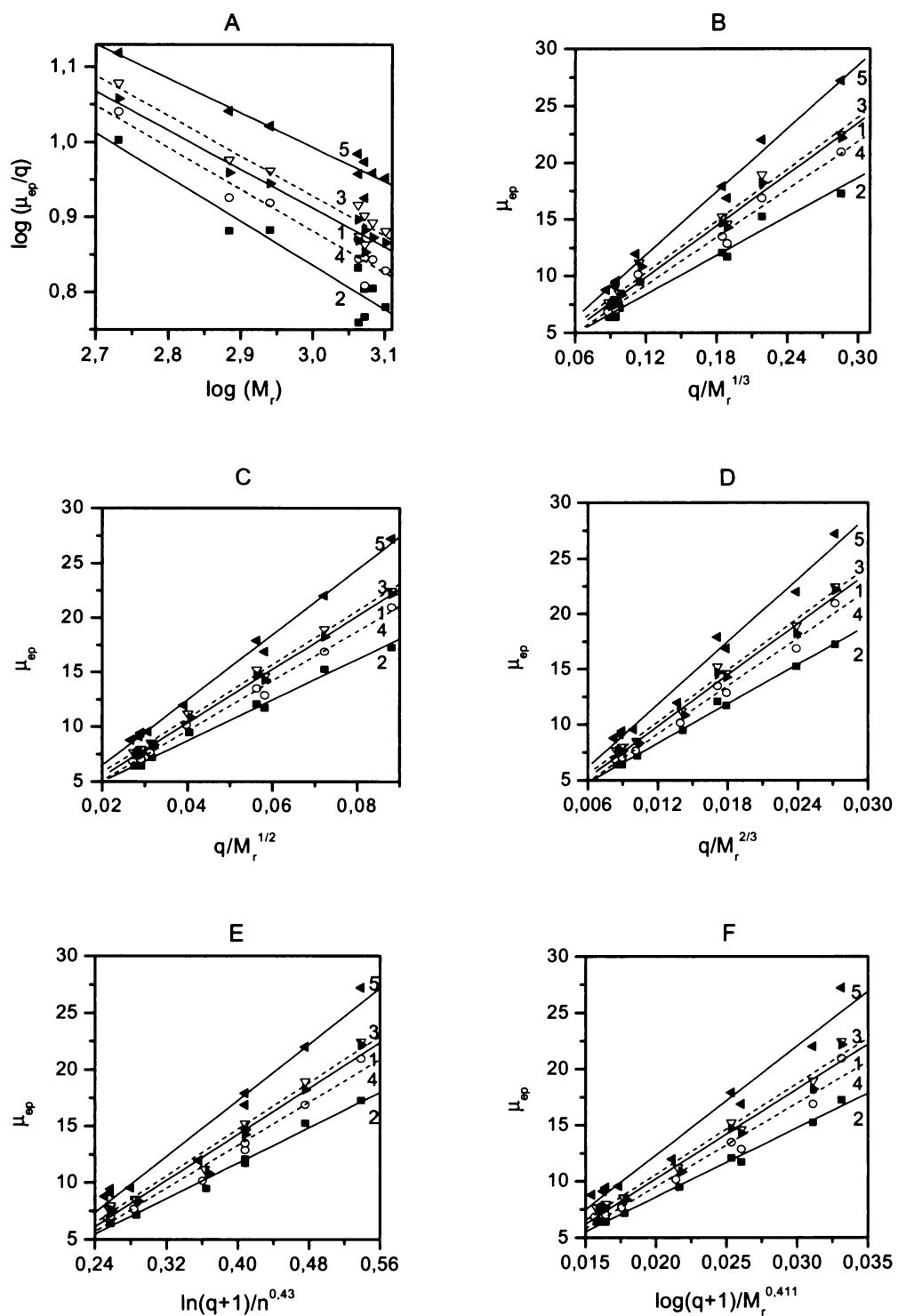
BGE	Nehomologická řada 20 IOPs		Sada lineárních IOPs, YDPAP _x (x = 6-0)	
	k	r	k	r
II	-0,557	0,9821	-0,638	0,9992
III	-0,506	0,9738	-0,594	0,9993
V	-0,481	0,9546	-0,583	0,9993
VII	-0,441	0,9792	-0,531	0,9988

k – směrnice přímky, r – korelační koeficient

Všechny uvedené modely kromě Kimova byly aplikovány na sadu deseti GnRHs v pěti kyselých základních elektrolytech, BGE I-IV a VI, v oblasti pH 2,18-2,50, grafy jejich korelací jsou na obrázku 10. Z lineárních závislostí je zřejmé, že nejvhodnějším modelem je model pro klasický lineární polymer. Toto tvrzení je podpořeno také nejvyšším korelačním koeficientem, jehož hodnoty se pro všech pět základních elektrolytů pohybovaly v rozmezí 0,995-0,999. Výsledek je též v souladu s dříve publikovanou prací podobné série peptidů obsahující též triptorelin a buserelin



Obrázek 9 Semiempirické modely korelace mezi absolutní hodnotou efektivní pohyblivosti peptidů, μ_{ep} , a jejich efektivním nábojem, q , a velikostí (vyjádřenou relativní molekulovou hmotností, M_r , nebo počtem aminokyselinových zbytků v peptidovém řetězci, n) aplikované na sadu sedmi IOPs s klesajícím počtem prolinových zbytků na C-konci, YDPAP₆ – YDPA, ve čtyřech základních elektrolytech, 1 – BGE II, pH 2,25, 2 – BGE III, pH = 2,30, 3 – BGE V, pH = 2,40, 4 – BGE VII, pH 8,10, (A) Crossův model, (2.17), (B) model pro sféricky tvarované molekuly (2.15), (C) model pro klasický lineární polymer (2.16), (D) Offordův model (2.14), (E) Grossmanův model (2.20), (F) model Cifuentesa a Poppeho (2.21).



Obrázek 10 Semiempirické modely korelace mezi absolutní hodnotou efektivní pohyblivosti peptidu, μ_{ep} , a jejich efektivním nábojem, q , a velikostí (vyjádřenou relativní molekulovou hmotností, M_r , nebo počtem aminokyselinových zbytků v peptidovém řetězci, n) aplikované na sadu deseti GnRHs v pěti kyselých základních elektrolytech, 1 – BGE I, pH 2,18, 2 – BGE II, pH 2,25, 3 – BGE III, pH = 2,30, 4 – BGE IV, pH = 2,32, 5 – BGE VI, pH 2,50, (A) Crossův model, (2.17), (B) model pro sféricky tvarované molekuly (2.15), (C) model pro klasický lineární polymer (2.16), (D) Offordův model (2.14), (E) Grossmanův model (2.20), (F) model Cifuentese a Poppeho (2.21).

analyzované v pH 2,85 [187]. Vhodným modelem pro popis peptidů v elektrolytech BGE I-IV je rovněž Offordův model s hodnotami korelačního koeficientu 0,994-0,998. Alternativním modelem pro základní elektrolyt BGE VI je model pro sféricky tvarované molekuly. Model Cifuentese a Poppeho nebo Grossmanův model jsou pro popis elektroforetické migrace této série peptidů v kyselině octové jako základním elektrolytu méně vhodné ($r = 0,990$ a $0,984$). Konkrétní hodnoty korelačních koeficientů jsou uvedeny v publikaci IV, tabulce 5.

Vztahy mezi pohyblivostí peptidů a jejich nábojem a velikostí pro skupinu ENKs, DLR, jejich fragmentů a derivátů ve třech elektrolytech, BGE II, III a VII, byly prostudovány s využitím Crossova modelu. Protože peptidy netvořily homologickou řadu, podobně jako v případě 20 IOPs některé body nezapadalý do lineární části této závislosti, viz obrázek 4 v publikaci II. Nejvyšší koeficient korelace této závislosti byl získán pro kyselý základní elektrolyt BGE II, $r = 0,960$. Pro alkalický elektrolyt BGE VII, se po vyřazení odlehlych bodů korelační koeficient zvýšil na hodnotu 0,957.

6.2.4 Predikce pravděpodobné struktury peptidu v roztoku

Na základě výběru vhodného semiempirického modelu v předchozí kapitole 6.2.3 a především z hodnot směrnice, k , lineární závislosti $\log(\mu_{ep}/q)$ versus $\log(M_r)$ v Crossově modelu, vztah (2.17), bylo možné předpovědět nejpravděpodobnější tvar molekuly peptidu v roztoku. Tvary molekul odvozené ze vztahu mezi směrnicí v Crossově modelu a exponentem relativní molekulové hmotnosti jsou uvedené v tabulce 2, kapitola 2.6.2.

Studiem tvaru molekul peptidů v základních elektrolytech se zabývaly práce II, III a IV. Pro nehomologickou sadu enkefalinů, dalarginu, jejich fragmentů a derivátů je z Crossova grafu zřejmé, že směrnice v závislostech jsou podobné pro dva kyselé elektrolyty, $k = -0,674$ pro BGE II a $k = -0,596$ pro BGE III, zatímco pro alkalický BGE VII nabývá směrnice výrazně odlišné hodnoty $k = -0,323$. Z hodnot směrnice tedy vyplývá, že v kyselých základních elektrolytech se hodnota blíží k $2/3$, a tedy pravděpodobný tvar molekul ENKs, DLR, jejich fragmentů a derivátů v roztoku při pH 2,25-2,30 je tvar zploštělého disku. V alkalickém pufu se blíží hodnotě $1/3$, což odpovídá kulovitému tvaru molekuly. Pro homologickou řadu GnRHs je směrnice k v rozmezí (-0,5) - (-0,6), přesněji od -0,519 do -0,589 pro BGE I-IV, jen pro BGE VI je poněkud nižší, -0,457. Nejpravděpodobnějším tvarem molekul GnRHs v pH 2,18-2,50 je náhodné klubko. Pro skupinu dvaceti různě velkých IOPs nebylo možné určit

stejný pravděpodobný tvar molekuly v roztoku. Z homologické skupiny devíti IOPs s klesajícím počtem aminokyselin v lineárním řetězci, od dekapeptidu, YDPAP₆, k dipeptidu, YD, byly dva nejkratší peptidy vyloučeny. Vzhledem ke krátkosti jejich řetězce netvoří organizované struktury. Z **tabulky 12** pro skupinu sedmi lineárních homogenních IOPs vyplývá, že v Crossově modelu hodnota k v alkalickém elektrolytu BGE VII se blíží 1/2, takže nejpravděpodobnějším tvarem jejich molekul v roztoku je náhodné klubko. Pro klasický kyselý elektrolyt BGE II, pH 2,25, se hodnota k přiblížila k 2/3, což odpovídá tvaru zploštělého disku. V izoelektrických pufrech BGE III a V, lišících se pouze v iontové síle, jsou hodnoty k téměř stejné, -0,583 a -0,593, což odpovídá tvaru náhodného klubka.

7 Závěr

Výsledky dosažené v této dizertační práci prokazují, že kapilární zónová elektroforéza a micelární elektrokinetická chromatografie jsou vysoce účinné a vysoce citlivé metody pro separaci, kvalitativní i kvantitativní analýzu a pro fyzikálně-chemickou charakterizaci peptidových hormonů.

Na základě strategie pro racionální výběr podmínek pro CE analýzu a separaci strukturně podobných peptidů byly ionogenní peptidy analyzovány jako kationty v klasických a izoelektrických pufrech v kyselé oblasti pH (pH = 2-2,5) a jako anionty v amfoterním pufru ve slabě alkalické oblasti pH, pH = 8,1. Neionogenní peptidy byly analyzovány metodou micelární elektrokinetické chromatografie s micelární fází tvořenou anionickým detergentem dodecylsulfátem sodným v alkalickém prostředí, pH 8,8. Tyto podmínky byly zvoleny s cílem minimalizovat adsorpci peptidů na vnitřním povrchu separačního prostoru, čehož bylo dosaženo potlačením disociace silanolových skupin na vnitřní stěně křemenné kapiláry v kyselé oblasti pH a elektrostatickým odpuzováním záporně nabitých peptidů od záporně nabitých disociovaných silanolových skupin na vnitřní stěně kapiláry v alkalické oblasti pH. Hodnota pH základního elektrolytu byla zvolena v oblasti pH, ve které analyzované peptidy vykazovaly největší rozdíly ve spočtených hodnotách svých specifických nábojů, tj. efektivních nábojů vztažených na jednotku relativní molekulové hmotnosti. Účinnost separací peptidových hormonů v klasických pufrech a v izoelektrických pufrech byla ve většině případů srovnatelná, v případě hormonů uvolňujících gonadotropin dosahovala vyšších hodnot v izoelektrických pufrech.

Při CZE analýze lidského insulinu a jeho oktapeptidového fragmentu značených fluorescenční značkou NBD bylo ukázáno, že citlivost fluorescenční detekce s detektorem na bázi argonovým laserem indukované fluorescence (excitace/emise 488/520 nm) je desetkrát až třicetkrát vyšší než citlivost UV-absorpčních detektorů v oblasti vlnových délek kolem 200 nm.

Kapilární zónová elektroforéza byla využita rovněž k fyzikálně-chemické charakterizaci analyzovaných peptidů. Byl vypracován postup pro určení teploty uvnitř kapiláry v průběhu CE analýzy, který umožnil přesné stanovení efektivních elektroforetických pohyblivostí peptidů při standardní teplotě 25°C.

Pomocí semiempirických modelů korelujících efektivní elektroforetickou pohyblivost peptidů s jejich efektivním nábojem, velikostí a prostorovým uspořádáním byly ověřeny vzájemné vztahy těchto parametrů peptidů v roztocích a pro homologické řady peptidových hormonů bylo potvrzeno jejich podobné elektroforetické chování. Na základě těchto studií byly navrženy pravděpodobné tvary peptidových molekul v jednotlivých elektrolytových systémech.

8 Literatura

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10 Dodatek - jiné projekty

Souběžně se studiem peptidových hormonů pomocí kapilární zónové elektroforézy byla tato metoda využita rovněž ke stanovení acidobazických disociačních konstant, pK_a , ionogenních skupin třinácti amino- a guanidinopurinových nukleotidových analogů a jejich příbuzných látek. Tyto nově syntetizované acyklické nukleosidové fosfonáty, acyklické nukleosidové fosfonátové diestery a další analogy představují významnou skupinu látek s cytostatickými a antivirovými účinky. Hodnoty acidobazických disociačních konstant ionogenních skupin, protonované guanidinylové skupiny, kyseliny fosforité disociované do druhého stupně a protonovaného dusíku v pozici (N1) purinové báze, byly stanoveny nelineární regresní analýzou experimentálně změřené pH závislosti jejich efektivních elektroforetických pohyblivosti. Efektivní elektroforetická pohyblivost byla změřena v sérii základních elektrolytů s širokým rozsahem pH (3,50-11,25) za konstantní iontové síly (25 mM) a teploty (25°C). Termodynamické acidobazické disociační konstanty protonované guanidinylové skupiny dosahovaly hodnot v rozmezí 7,75-10,32; pK_a kyseliny fosforité disociované do druhého stupně byly v rozsahu 6,64-7,46; a pK_a protonovaného dusíku v pozici (N1) purinové báze byly v rozmezí 4,13-4,89. Detailní informace o výsledcích jsou uvedeny v přiložené publikaci V, Šolínová V., Kašička V., Koval D., Česnek M., Holý A., Determination of Acid-Base Dissociation Constants of Amino- and Guanidinopurine Nucleotide Analogs and Related Compounds by Capillary Zone Electrophoresis, *Electrophoresis*, 2006, 27, 1006-1019.

Během mého doktorského studia byl sepsán přehledný článek, VI, Šolínová V., Kašička V., Recent Applications of Conductivity Detection in Capillary and Chip Electrophoresis, *J. Sep. Sci.*, 2006, 29, 1743-1762, který shrnuje problematiku, kterou se zabývala moje diplomová práce „Využití bezkontaktního vodivostního detektoru v kapilární zónové elektroforéze“. Publikace se zabývá přehledem aktuálních aplikací kontaktní a hlavně bezkontaktní vodivostní detekce ve spojení s kapilární a čipovou elektroforetickou analýzou širokého spektra látek. Též shrnuje poslední vývoj v oblasti konstrukce vodivostních detektorů.

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Analysis of synthetic derivatives of peptide hormones by capillary zone electrophoresis and micellar electrokinetic chromatography with ultraviolet-absorption and laser-induced fluorescence detection

Veronika Šolínová, Václav Kašička*, Dušan Koval, Tomislav Barth,
Alice Ciencialová, Lenka Žáková

Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Flemingovo 2, 166 10 Prague 6, Czech Republic

Available online 27 February 2004

Abstract

Capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC) were used for the analysis of new synthetic derivatives of hypophysis neurohormones—vasopressin and oxytocin, and pancreatic hormone—human insulin (HI) and its octapeptide fragment, derivatized by fluorescent probe, 4-chloro-7-nitrobenzo[1,2,5]oxadiazol (NBD). The suitable composition of background electrolytes (BGEs) was selected on the basis of calculated pH dependence of effective charge of analyzed peptides. Basic ionogenic peptides were analyzed by CZE in the acidic BGE composed of 100 mM H₃PO₄, 50 mM Tris, pH 2.25. The ionogenic peptides with fluorescent label, NBD, were analyzed in 0.5 M acetic acid, pH 2.5. The best MEKC separation of non-ionogenic peptides was achieved in alkaline BGE, 20 mM Tris, 5 mM H₃PO₄, with micellar pseudophase formed by 50 mM sodium dodecylsulfate (SDS), pH 8.8. Selected characteristics (noise, detectability of substance, sensitivity of detector) of the UV-absorption detectors (single wavelength detector, multiple-wavelength photodiode array detector (PDA), both of them operating at constant wavelength 206 nm) and laser-induced fluorescence (LIF) detector (excitation/emission wavelength 488/520 nm) were determined. The detectability of peptides in the single wavelength detector was 1.3–6.0 μmol dm⁻³ and in the PDA detector 1.6–3.1 μmol dm⁻³. The LIF detection was more sensitive, the applied concentration of NBD derivative of insulin fragment in CZE analysis with LIF detection was three orders lower than in CZE with UV-absorption detector, and the detectability of this peptide was improved to 15.8 nmol dm⁻³.

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Keywords: Derivatization; CE; Derivatization; MEKC; Insulin; Peptides

1. Introduction

High-performance capillary electromigration methods, capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC), providing fast (few minutes) and high-efficient separation (10⁵–10⁶ theoretical plates per meter) of picomole to attomole quantities of analytes in the nanolitre sample volume, have a high application potential in the field of chemistry of peptides: they are broadly utilized for separation, analysis, preparation and characterization of peptides in chemistry, biochemistry, biomedicine, biotechnology, pharmacy and agriculture, as documented in several recent reviews [1–7].

Peptides and their analogs, fragments and derivatives are either isolated from the natural material or synthesized from amino acid building blocks. Sometimes they are prepared semisynthetically; i.e. synthetic peptide is attached to the isolated peptide or its fragment. In all cases it is important to check the success of the whole isolation or synthesis, eventually single steps of the process, because the obtained products can contain various admixtures, e.g. by-products of peptide synthesis. This is a suitable task for capillary electrophoresis (CE), since determination of purity degree is the most common application of CE in peptide chemistry [2].

One group of biopeptides analyzed in this work are represented by synthetic derivatives of neurohormones, oxytocin and vasopressin, produced by the pituitary gland (hypophysis) [8]. Oxytocin is responsible for contraction of the uterus in labor and ejection of milk from mammary glands during breast-feeding. Vasopressin and its analogs

* Corresponding author. Tel.: +420-220-183-239;
fax: +420-220-183-592.

E-mail address: kasicka@uochb.cas.cz (V. Kašička).

are also known as hormones with anti-diuretic activity [9]; they stimulate the retention of water by kidneys [10].

Structurally similar analogues of vasopressin and other biopeptides, enkephalins and dalargin, have been separated by CZE in acidic background electrolyte (BGE), 150 mM phosphoric acid, pH 1.8 [11,12]. Analogues of dalargin were analyzed by CZE in 0.5 M acetic acid, pH 2.5, and then separated in preparative scale by free-flow zone electrophoresis [13]. These peptides were also separated by MEKC in 20 mM tetraborate BGE with micellar pseudophase formed by sodium dodecylsulfate (SDS), pH 9.2 [11,12]. Oxytocin, vasopressin and other neurohypophyseal nonapeptides were determined by MEKC in four different micellar systems—with cationic, anionic, zwitterionic and neutral micellar constituents [14]. Chiral separations of dalargin analogs with D- and L-isomers of phenylalanine in the fourth position in peptide chain were performed in 50 mM phosphate buffer, pH 2.5 with 10 mM β -cyclodextrin [12]. Peptide neurohormones, derivatives of vasopressin, enkephalins and dalargin, and human insulin (HI) were analyzed also by different variants of HPLC, reverse-phase [11,12] and ion-exchange [15,16] HPLC.

Insulin is the peptide hormone produced by pancreatic β -cells that regulates the metabolism of carbohydrates, proteins and fatty acids [17]. Insulin molecule consists of 2 polypeptide chains, A and B, containing 21 and 30 amino acid residues, respectively. The two chains of insulin are connected by two disulfide bonds and the third disulfide bond is intra-chain bond in chain A. Its relative molar mass is $M_r = 5808$, the isoelectric point, pI, is 5.4–5.5.

CZE and HPLC have been frequently used for analysis of human insulin and its derivatives. Stable derivative of insulin with exo-3,6-epoxy-1,2,3,6-tetrahydrophthalic anhydride (ETPA) were identified by ion-exchange HPLC and CE [18]. CZE with UV detection was utilized for analysis of HI in CHES-acetate-acetonitrile BGE, pH 7–8 [19]. CE was also used to validate the HPLC assay of insulin as an analytical method [20]. Human insulin, proinsulin and intermediate forms were simultaneously quantified by CE in TAPS (*N*-[tris(hydroxymethyl)-methyl]-3-aminopropanesulfonic acid), DETA (diethylenetriamine) and methanol [21]. CZE with fluorescence detection was used mainly for immunoassay of insulin. In some cases the native fluorescence of insulin was detected (excitation 275 nm, emission 305 nm) [22]. The analysis was performed in acidic BGE—50 mM citric acid, pH 2.3, or in alkaline BGE 20 mM Tricine, pH 8.5. Insulin with bonded fluorescent dye NN 382 was analyzed in 50 mM phosphate and 25 mM potassium sulfate, pH 7.0 with laser-induced fluorescence (LIF) detection in near infrared range [23].

The aim of this work was to analyze synthetic derivatives and analogs of hypophyseal neurohormones, oxytocin and vasopressin, and of pancreatic hormone, human insulin, and its octapeptide fragment, B23-30-HI, derivatized by fluorescent label, 4-chloro-7-nitrobenzo[1,2,5]oxadiazol (NBD), by CZE and MEKC, in the home-made CE de-

vice with single wavelength UV-absorption detector and in commercial Beckman–Coulter CE-MDQ apparatus with multiple-wavelength UV-absorption detector and LIF detector. The rational approach, based on the calculated pH dependence of effective charge of peptides, is used for selection of suitable separation conditions. The sensitivity of UV and LIF detectors and detectability of non-labeled and labeled peptides as well as other characteristics of detectors are compared and discussed.

2. Experimental

2.1. Chemicals

All chemicals used were of analytical reagent grade. Tris (tris(hydroxymethyl)aminomethane) was obtained from Serva (Heidelberg, Germany), phosphoric acid, acetic acid and dimethyl sulfoxide (DMSO) were supplied by Lachema (Brno, Czech Republic) and sodium dodecylsulfate was from Fluka (Buchs, Switzerland).

2.2. Peptides

The sequences of analyzed peptides and their abbreviations are presented in Table 1. Analogs of oxytocin, vasopressin and octapeptide B23-B30 of the C-terminal part of the B-chain of human insulin (HI) were synthesized in our institute (IOCB) by the solid phase method described elsewhere [24]. 4-Chloro-7-nitrobenzo[1,2,5]oxadiazol (NBD) was used as fluorescent probe for B23-B30 octapeptide of HI. NBD reacted with free ϵ -amino group of lysine B29 in octapeptide of HI. The α -amino group of lysine was protected by the Fmoc protecting group [25]. Fluorescent-labeled human insulin was prepared semisynthetically by the trypsin-catalyzed peptide bond formation between C-terminal arginine B22 of desoctapeptide of insulin and N-terminal glycine residue of NBD-labeled octapeptide B23-B30 of HI [26].

2.3. Instrumentation

The electrophoretic experiments were carried out in two apparatuses. The first one was home made CE device developed in our institute, IOCB, further indicated as CE-IOCB [27]. It is equipped with single wavelength UV-absorption photometric detector operating at 206 nm; the light source is electrodeless, high-frequency excited iodine discharge lamp (LKB-Pharmacia, Uppsala, Sweden), and UV-sensitive Si-photodiode with built-in preamplifier (Hamamatsu Photonics Deutschland, Herrsching, Germany) is used as light detector. The bare fused silica capillary with outer polyimide coating, 50 μm i.d. \times 200 μm O.D., total length 30 cm and effective length 19 cm, was supplied by the Institute of Glass and Ceramics Materials, Czech Academy of Sciences (Prague, Czech Republic). The separations were

Table 1
Sequences of analyzed peptides

Peptide	Abbreviation	Sequence in three-letter code	Sequence in single-letter code
Oxytocin	OT	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Lys-GlyNH ₂ []	CYIQNCPLG-NH ₂ []
Deamino-oxytocin	dOT	Mpa-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-GlyNH ₂ []	MpaYIQNCPLG-NH ₂ []
Arg ⁸ -vasopressin	AVP	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-GlyNH ₂ []	CYFQNCPRG-NH ₂ []
(Deamino-D-Arg ⁸)-vasopressin	dDAVP	Mpa-Tyr-Phe-Gln-Asn-Cys-Pro-D-Arg-GlyNH ₂ []	MpaYFQNCPrG-NH ₂ []
Insect diuretic hormone	IDH	Cys-Leu-Ile-Thr-Asn-Cys-Pro-Arg-GlyNH ₂ []	CLITNCPRG-NH ₂ []
(Deamino-D-Arg ⁸)-IDH	dDA-IDH	Mpa-Leu-Ile-Thr-Asn-Cys-Pro-D-Arg-GlyNH ₂ []	MpaLITNCPrG-NH ₂ []
(D-Arg ⁸)-IDH	DA-IDH	Cys-Leu-Ile-Thr-Asn-Cys-Pro-D-Arg-GlyNH ₂ []	CLITNCPrG-NH ₂ []
(Deamino-Arg ⁸)-vasopressin	dAVP	Mpa-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-GlyNH ₂ []	MpaYFQNCPRG-NH ₂ []
NBD-derivative of B23-B30-human insulin fragment	NBD-B23-30-HI	Gly-Phe-Phe-Tyr-Thr-Pro-Lys-(NBD)-Thr	GFFYTPK(NBD)T

NBD: 4-chloro-7-nitrobenzo[1,2,5]oxadiazol.

performed at ambient temperature 22–24 °C without active cooling of the capillary.

The second apparatus was commercial P/ACE MDQ System (Beckman–Coulter, Fullerton, CA, USA), further indicated as CE-MDQ, with multiwavelength spectrophotometric photodiode array (PDA) detector (190–600 nm) and Ar-ion laser-induced fluorescence (LIF) detector (excitation wavelength 488 nm, emission wavelength 520 nm). The fused silica capillary with outer polyimide coating as well as in IOCB device was internally uncoated, total length was 40 cm and effective length was 30.2 cm, with diameters 75 µm I.D. × 360 µm O.D. The temperature was set at 25 °C with liquid coolant continuously circulating around the capillary.

The new capillary was gradually flushed with water, 0.1 M NaOH, water and BGE, each wash for 5 min. Finally, the capillary was conditioned by a 20 min application of the high voltage to equilibrate the inner surface and to stabilize electroosmotic flow. Between runs under the same conditions, the capillary was rinsed with the BGE for 2 min. Prior to any change of the BGE the capillary was rinsed with 0.1 M NaOH for 5 min and then repeatedly stabilized. The separation voltage was in the range 10–20 kV. The samples were injected with pressure 5–10 mBar for 5–15 s. The samples were dissolved in deionized water or in BGE and their concentrations were in the range 0.005–1.3 mg/ml for UV-absorption detection and 10–1000× lower for CE with

LIF detection. The BGEs were filtered through a 0.45 µm syringe filter (Millipore, Bedford, MA, USA) before use.

3. Results and discussion

3.1. Selection of separation conditions

The strategy of the rational selection of conditions in CE separation of peptides follows the general rules of selection of suitable CE separation conditions [28,29] and takes into account the specific peptide properties. The specific properties of peptides result from their primary structure—number and sequence of the linked amino acid residues. The structure determines the electric charge, size (relative molecular mass), shape (conformation), hydrophobicity and specific binding capability. From the structure of peptides also their ionogenicity is deduced, which determines whether CZE or MEKC can be used for analysis of given peptide sample. CZE is used for analysis of ionogenic peptides and the separation is based on differences in electrophoretic mobilities, which are determined by charge, size and shape of peptide molecules. MEKC is used for analysis of non-ionogenic peptides; the separation is based on their different hydrophobicity.

The selection of the composition of the BGEs includes buffer components type and concentration, pH and takes into

account the request for chemical and temperature stability and biological activity of analyzed peptides [30]. Effective charges of peptides are strongly dependent on pH and pK_a of ionogenic groups of amino acid residues present in peptide chain. Analyzed peptides contain ionogenic groups—all except one $\alpha\text{-NH}_3^+$ group of N-terminal of peptide chain (average pK_a 8.0), four of them guanidium of arginine (average pK_a 11.2) or hydroxy group of tyrosine (average pK_a 10.0) and form cyclic structures by disulfide bridges of cystine. The relative molecular mass of nonapeptides was around 1000. The pH dependence of effective charge was calculated with the earlier developed computer program Nabamfo [31] using the above given average values of pK_a of ionogenic groups. The program is based on mathematical model of acid–base equilibrium of general amphotelyte. The effective charges of ionogenic peptides reached values +1 or +2 elementary charges in the whole acidic pH range. Some peptides behaved as weakly negatively charged in strongly alkaline BGE, which was not suitable for these peptides due to the lability of cystine bond at high pH. In conclusion, ionogenic peptides were analyzed by CZE as cations in acidic BGE. The pH dependence of the effective charges of analyzed nonapeptides is depicted in Fig. 1.

The solubility of peptides is given by their structure. The analyzed peptides were dissolved in deionized water or BGEs. Solubilization in deionized water is advantageous due to the possibility to apply the same sample solution for CE separations in different BGEs and in addition the concentrating effect is utilized. On the other hand using BGE as sample solvent ensures the constant separation conditions (BGE composition) in the whole CE experiment.

Ionogenic peptides were analyzed by CZE as cations in acidic BGEs. The best results were obtained in BGE composed of 100 mM phosphoric acid, 50 mM Tris, pH 2.25, and with the applied voltage 10 kV. The examples of peptide CZE analyses are shown in Fig. 2. The effective electrophoretic mobilities of analyzed peptides in the above tris-phosphate BGE are presented in Table 2. The aver-

Table 2

Effective electrophoretic mobility and differently expressed purity degrees of peptides determined by CZE in home-made CE-IOCB device and in commercial CE-MDQ device with UV-absorption detection (206 nm) in BGE composed of 100 mM H_3PO_4 , 50 mM Tris, pH 2.25

Peptide	m_{ef} ($10^{-9} m^2 V^{-1} s^{-1}$)	P_h (%)	P_A (%)	P_{CA} (%)
Analyses in CE-IOCB device				
OT	11.6	95.6	95.0	94.5
dOT	0	93.3*	89.1*	89.3*
AVP	23.3	94.1	90.1	91.5
dDAVP	12.7	95.1	96.9	96.4
IDH	24.5	42.3	28.6	26.7
dDA-IDH	13.4	66.7	69.3	68.4
dAVP	12.6	92.0	89.9	89.3
DA-IDH	24.4	70.8	52.9	48.2
Analyses in CE-MDQ device				
OT	9.1	93.5	90.6	90.4
AVP	16.8	87.7	83.2	80.4

m_{ef} : Effective electrophoretic mobility; P_h , P_A , P_{CA} : purity degrees expressed on the basis of relative peak height, relative peak area and relative peak corrected area, respectively; (*): purity degrees of dOT obtained from MEKC analyses.

age electroosmotic mobility of the tris-phosphate buffer was $3.46 \times 10^{-9} m^2 V^{-1} s^{-1}$. Peptides with two positive elementary charges migrated the fastest. Peptides containing tyrosine in the second position in peptide chain migrated slower than peptides with other amino acids in this position and the same total positive charge, obviously due to larger size of their hydrated molecules. On the other hand peptide containing arginine residue in position 8 instead of leucine migrated faster than peptides with free N-terminal group, the slowest migrating positively charged peptide had just free N-terminal group. The peptides with fluorescent label NBD were analyzed by CZE in 0.5 M acetic acid, pH 2.5, and with the applied voltage 15 kV. The examples of their electrophoreograms are shown in Fig. 3.

Non-ionogenic peptides with blocked or derivatized ionogenic groups were analyzed by MEKC in alkaline BGE—20 mM Tris, 5 mM H_3PO_4 , pH 8.8, with micellar pseudophase formed by an anionic detergent sodium dodecylsulfate, see Fig. 4. The applied voltage was 20 kV. In all systems symmetrical peaks were obtained without any shoulders and with high separation efficiency in short times. All analyses did not take more than 7 min. The number of theoretical plates/meter was in the range 8×10^5 to 3×10^6 .

3.2. Determination of purity degree

The purity degrees of the crude synthetic or semisynthetic peptide preparations after HPLC purification were determined. Standards of synthesized peptides were not available. Peptide purity and peptide content in the sample can be quantified by several ways: (i) relative peak height, $P_h(i)$ (1), (ii) relative peak area, $P_A(i)$ (2), and (iii) relative peak corrected area, $P_{CA}(i)$ (3), of the UV-positive peaks

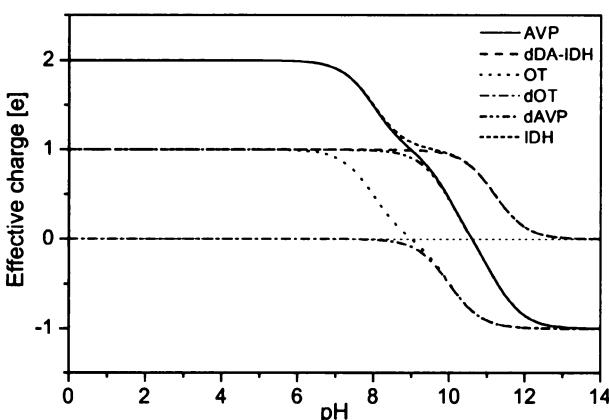


Fig. 1. The pH dependence of the effective charge of analyzed neurohypophyseal peptides. For peptide abbreviations see Table 1.

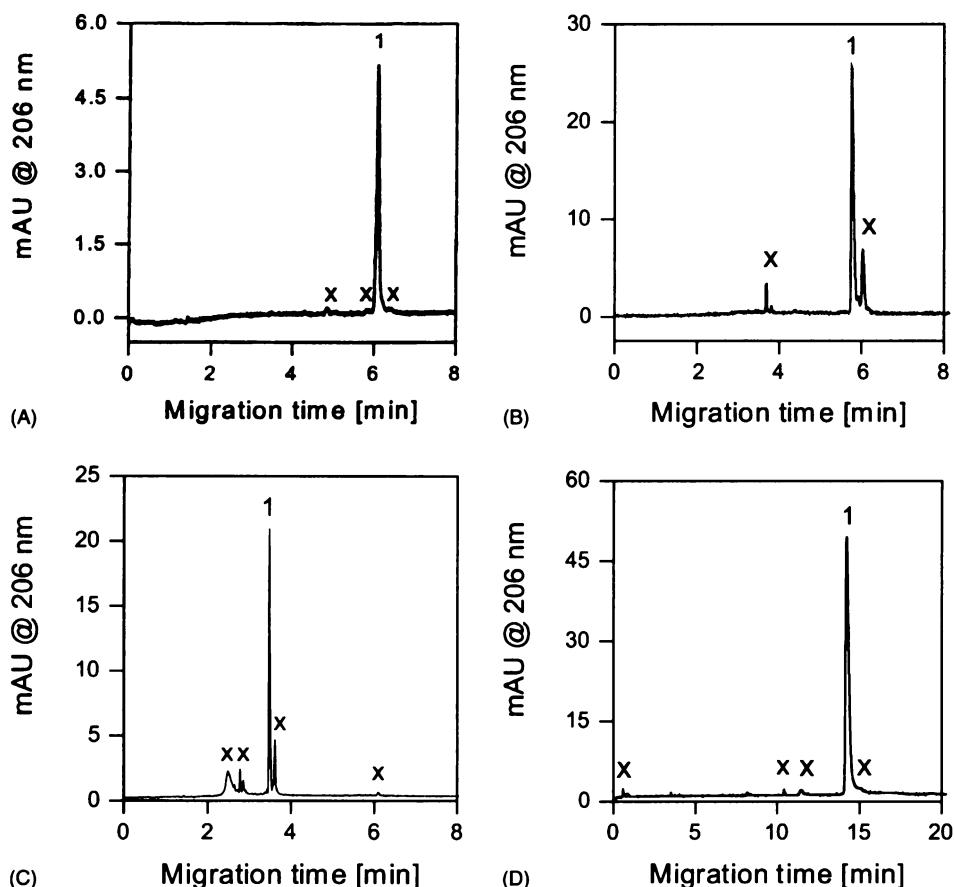


Fig. 2. CZE analyses of derivatives of hypophyseal neurohormones: (A) (deamino-Arg⁸)-vasopressin, 0.40 mg ml⁻¹; (B) (deamino-D-Arg⁸)-IDH, 1.15 mg ml⁻¹; (C) (D-Arg⁸)-IDH, 1.0 mg ml⁻¹; all three analyses performed in CE-IOCB device, current 48–50 μA, injection 10 mBar × 5 s, (D) oxytocin, 0.95 mg ml⁻¹, in CE-MDQ device, 69–70 μA, injection 7 mBar × 5 s. All analyses performed in BGE composed of 100 mM H₃PO₄, 50 mM Tris, pH 2.25; applied voltage 10 kV; (1) main synthetic product; (x) non-identified admixtures.

for *i*-th component:

$$P_h(i) = \frac{h(i)}{\sum h(i)} \quad i = 1 \dots n \quad (1)$$

$$P_A(i) = \frac{A(i)}{\sum A(i)} \quad i = 1 \dots n \quad (2)$$

$$P_{CA}(i) = \frac{A_c(i)}{\sum A_c(i)} \quad i = 1 \dots n \quad (3)$$

where $h(i)$ is height of the *i*-th peak, $A(i)$ is area of the *i*-th peak and $A_c(i)$ is corrected area of the *i*-th peak, corrected peak area is peak area corrected with respect to migration velocity of the given peak, i.e. it is equal to peak area divided by migration time of the given peak, n is the number of sample components.

The values of differently expressed purity degrees are presented in the Table 2. The values of purity degrees were determined as averages of two subsequent analyses, which differed less than 1%. Analyses of peptides with high purity degree are demonstrated by CZE of (deamino-Arg⁸)-vasopressin in Fig. 2A and oxytocin in Fig. 2D. On the other

hand analysis of peptide with low purity degree and with at least three major impurities is shown in electrophoreogram of (deamino-D-Arg⁸)-insect diuretic hormone in Fig. 2B. As follows from the above definitions, the most exact characteristic of peptide purity is the relative corrected peak area. Sometimes other, the simpler ways of purity evaluation, relative peak height or relative peak area, can be used, particularly in the cases when the peaks of sample components are uniformly dispersed and their migration velocities are not too different. The comparison of differently expressed purity degree is demonstrated on (D-Arg⁸)-insect diuretic hormone in Fig. 2C. DA-IDH relative peak height differed in around 20% against relative peak area and relative peak corrected area. As follows from the presented data, the determination of purity degree depends on separation conditions and mostly also on the way of its determination.

3.3. Sensitivity of detection

3.3.1. UV-absorption detection

The most common detection of peptides in CE is UV-absorption detection in the range 200–220 nm, due to

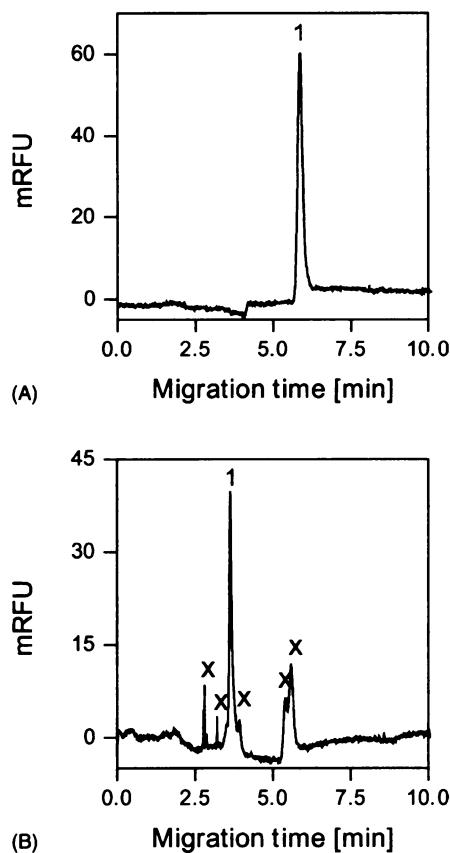


Fig. 3. CZE analyses of fluorescence labeled peptides in CE-MDQ device with LIF-detection: (A) NBD-B23-30-HI, 0.05 mg ml^{-1} ; (B) NBD-HI, $0.5 \mu\text{g ml}^{-1}$. Analyses performed in BGE composed of 0.5 M AcOH , pH 2.5; injection $10 \text{ mBar} \times 5 \text{ s}$; applied voltage 15 kV ; current $20\text{--}21 \mu\text{A}$; (1) main synthetic product; (x) non-identified admixtures.

the absorption of peptide bond CO–NH in this short wavelength UV region. The background electrolytes (BGEs) should not absorb light significantly in this range in order to prevent the decrease of the signal of analytes [7].

In this work two different UV-absorption detectors were tested. The comparison was performed from the analyses on new synthesized nonapeptides by CZE in acidic tris-phosphate buffer, pH 2.25. The first absorption detector used high-frequency excited iodine discharge lamp with emission at 206 nm and UV-sensitive silicon photodiode

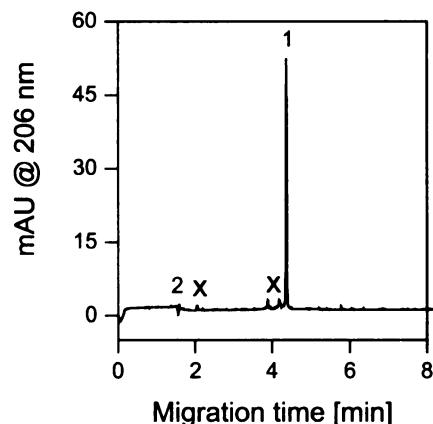


Fig. 4. MEKC analysis of non-ionogenic peptide, deamino-oxytocin, 1.15 mg ml^{-1} , in CE-MDQ device with PDA detector, BGE: 20 mM Tris , $5 \text{ mM H}_3\text{PO}_4$, 50 mM SDS , pH 8.8, injection $5 \text{ mBar} \times 10 \text{ s}$, applied voltage 20 kV , current $69\text{--}71 \mu\text{A}$, (1) main synthetic product, (2) peak of electroosmotic flow marker, (x) non-identified admixtures.

as detector of radiation. The second one was based on multiple-wavelength detection realized by photodiode array and deuterium lamp. Both detectors operated at constant wavelength 206 nm. The characteristics of the detectors, noise, n , response of the detector, R , signal to noise ratio, R/n , detectability of substance, D , and sensitivity of detector, S , were compared. The sensitivity of detector was calculated as the ratio of response of detector and concentration of analyte, $S = R/c$. The detectability of substance was determined as treble of noise divided by sensitivity, $D = 3n/S$. The values of the above characteristics of single wavelength detector (CE-IOCB) measured with oxytocin and Arg⁸-vasopressin are presented in Table 3 and for PDA detector in CE-MDQ device in Table 4. The values were determined as averages of two subsequent analyses with maximal difference 2% in response of the detector. The ratio R/n of the oxytocin and Arg⁸-vasopressin was slightly higher in PDA detector. The detectability of substances copied with small deviations the increase of R/n in PDA detector.

The influence of BGEs absorption on detector sensitivity and detectability of substance was investigated by CZE of oxytocin and Arg⁸-vasopressin, performed in tris-phosphate buffer and acetic acid-based BGEs, respectively.

Table 3

The characteristics of single wavelength (206 nm) UV-absorption detector of CE-IOCB device determined from CZE analyses of peptides in BGE composed of $100 \text{ mM H}_3\text{PO}_4$, 50 mM Tris , pH 2.25

Peptide	$c (\mu\text{mol dm}^{-3})$	$R (\text{mV})$	R/n	$S (\text{V mol}^{-1} \text{dm}^3)$	$D (\mu\text{mol dm}^{-3})$
OT	89.1	64.0	89.0	717.7	3.00
AVP	84.4	141.8	197.3	1680.6	1.28
dDAVP	1036	658.5	916.4	635.4	3.39
IDH	329	181.8	253.0	553.5	3.89
dDA-IDH	818	300.9	418.7	367.7	5.86

c: Peptide concentration; R: detector response; n: noise, average value of noise was 0.72 mV ; S: sensitivity of detector, $S = R/c$; D: detectability of substance, $D = 3n/S$.

Table 4

The characteristics of the UV-absorption PDA detector (operating at 206 nm) of CE-MDQ device determined from CZE analyses of peptides in different BGEs, BGE I: 100 mM H₃PO₄, 50 mM Tris, pH 2.25; BGE II: 0.5 M acetic acid, pH 2.5

Peptide	BGE	c (mmol dm ⁻³)	R (mAU)	R/n	S (AU.mol ⁻¹ dm ³)	D (μmol dm ⁻³)
OT	I	0.85	43.8	1093.2	51.4	2.34
	II	0.91	16.4	859.2	18.0	3.18
AVP	I	0.92	67.2	1675.6	72.9	1.65
	II	0.86	24.6	1285.8	28.4	2.02

c: Peptide concentration; R: detector response; n: noise, average value of noise was 0.04 mAU for BGE I and 0.02 mAU for BGE II; S: sensitivity of detector, $S = R/c$; D: detectability of substance, $D = 3n/S$.

Table 5

The characteristics of the UV-absorption PDA detector set to 206 nm and LIF detector (488 nm/520 nm) of CE-MDQ device determined from CZE analyses of peptides in BGE composed of 0.5 M acetic acid, pH 2.5

Peptide	c (mmol dm ⁻³)	R (mAU)	R/n	S (AU.mol ⁻¹ dm ³)	D (μmol dm ⁻³)
Analyses with UV detector					
NBD-HI	629	2.77	184.7	44.1	1.02
NBD-B23-30-HI	72.9	8.57	571.1	11.7	3.83
Peptide	c (μmol dm ⁻³)	R (mRFU)	R/n	S (RFU.mol ⁻¹ dm ³)	D (μmol dm ⁻³)
Analyses with LIF detector					
NBD-HI	5.09	505.7	136.6	99331	0.112
NBD-B23-30-HI	0.89	629.1	169.9	706590	0.016

c: Peptide concentration; R: detector response; n: noise, average value of noise was 3.70 mRFU for LIF detector and 0.02 mAU for PDA detector; S: sensitivity of detector, $S = R/c$; D: detectability of substance, $D = 3n/S$.

The acetic acid absorbs more light, the peaks were smaller and noise was also lower. The value of the noise in tris-phosphate buffer was 0.0401 mAU and in acetic acid reached 0.0191 mAU in Beckman CE-MDQ with PDA detector. The separation in acetic acid BGE was influenced by electromigration dispersion; the peaks were wide and non-symmetrical. The sensitivity of PDA detector decreased 2.5 times in acetic acid BGE; the detectability was lower in comparison with tris-phosphate buffer in both equipments. On the other hand, the differences were not dramatic (see Table 4) and acetic acid can be also used as BGE in CZE.

3.3.2. LIF detection

Laser-induced fluorescence detection is the most sensitive detection in CE, its sensitivity is two or three orders higher than UV-absorption detection. On the other hand it is necessary to derivatize peptides by fluorogenic labels. Only peptides with aromatic amino acid residues of tryptophane and tyrosine exhibit native fluorescence in the region 200–300 nm. The necessary condition for excitation is UV-laser systems or multiphoton excitation [7].

In this work, the sensitivity of PDA and LIF detectors of CE-MDQ device were compared. Firstly, the derivatives of human insulin and its B23-30 octapeptide fragment with NBD label, NBD-HI and NBD-B23-30-HI, respectively, were detected by PDA detector in CE-MDQ device in 0.5 M acetic acid, pH 2.5. Fluorescent probe NBD was bonded on ε-amino group of lysine B29, the excitation maximum was at 475–478 nm and the emission maximum at

530–534 nm in pH 3–8 [32]. Labeled human insulin is necessary for monitoring of interactions between insulin and insulin-receptor at low concentrations in vivo conditions. Consequently, LIF detector with Ar-ion laser (excitation at 488 nm, emission at 520 nm) was used for analyses of NBD-derivatives of HI and its fragment. The electrophograms of peptide analyses in acetic acid, pH 2.5, with LIF detection are depicted in Fig. 3. Whereas single peak was obtained for NBD-derivative of B23-30 octapeptide fragment of human insulin (see Fig. 3A), several admixtures have been found by CZE analysis of NBD-labeled human insulin, as shown in Fig. 3B. This indicates that the rest of fluorescent probe was not completely removed from the octapeptide fragment and it reacted with non-protected groups of amino acid residues of desoctapeptide of HI. For LIF detection the concentration of human insulin was 10 times lower than for UV-absorption detection, for octapeptide the solution was diluted 1000 times due to too high signal in LIF detector. The main characteristics of detectors as well as the comparison of UV detectors are shown in Table 5. The detectability of substance is one or two orders better for fluorescence detection.

4. Conclusions

CZE and MEKC proved to be powerful and useful tools for fast and sensitive analyses of ionogenic or non-ionogenic derivatives of peptide hormones, oxytocin, vasopressin,

human insulin and its fragment. The purity degrees and effective mobilities of ionogenic peptides were determined by CZE in acidic BGEs. Purity degree of non-ionogenic peptides was evaluated by MEKC in alkaline BGE with SDS micellar pseudophase. Sensitivity of single wavelength UV-absorption detector in home-made CE device was found to be comparable with that of multiple-wavelength PDA detector in commercial Beckman CE-MDQ apparatus. LIF detector was able to detect NBD-labeled octapeptide fragment of human insulin with two orders lower detectability than UV-absorption detector.

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Analysis and separation of enkephalin and dalargin analogues and fragments by capillary zone electrophoresis

Veronika Šolínová^a, Václav Kašička^{a,*}, Tomislav Barth^a, Linda Hauzerová^a, Salvatore Fanali^b

^a Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Flemingovo 2, 16610 Prague 6, Czech Republic

^b Institute of Chemical Methodologies, Consiglio Nazionale delle Ricerche, Area della Ricerca di Roma I, Via Salaria Km 29,300, 00016 Monterotondo Scalo, Rome, Italy

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Abstract

Capillary zone electrophoresis (CZE) has been applied to qualitative and quantitative analysis and separation of synthetic analogues and fragments of enkephalins ($[Leu^5]$ enkephalin, H-Tyr-Gly-Gly-Phe-Leu-OH, $[Met^5]$ enkephalin, H-Tyr-Gly-Gly-Phe-Met-OH), and dalargin (H-Tyr-d-Ala-Gly-Phe-Leu-Arg-OH), biologically active peptides with morphin-like effects acting as ligands for the opiate receptors in the brain. These oligopeptides (dipeptides to hexapeptides) were analyzed as cations in two acidic background electrolytes (BGEs), BGE I (100 mM H_3PO_4 , 50 mM Tris, pH 2.25), BGE II (100 mM iminodiacetic acid, pH 2.30), and both as cations and anions in alkaline BGE IV (40 mM Tris, 40 mM Tricine, pH 8.10). Purity degrees of peptides, expressed in three different ways (relative peak height, relative peak area and relative corrected peak area), were determined by their CZE analyses in the above BGEs, and their values were compared with respect to the peak shapes and migration times of the main synthetic products and their admixtures. Selected analogues and fragments of enkephalins and dalargin were successfully separated by CZE in acidic isoelectric buffers, 100 and 200 mM iminodiacetic acid, pH 2.30 and 2.32, respectively. The effective electrophoretic mobilities at standard temperature 25 °C, and effective and specific charges of all analyzed peptides in the above three BGEs were determined. Correlation between effective electrophoretic mobility of the analyzed peptides and their charge and size (relative molecular mass) was investigated, which revealed different molecular shape of analyzed peptides in acidic and alkaline BGEs. In addition, the selected characteristics of the UV-absorption detector (noise, signal to noise ratio, sensitivity, and limits of detection and quantification) were determined.

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1. Introduction

Capillary zone electrophoresis (CZE), one of the high-performance capillary electromigration methods, providing fast (few minutes) and high-efficient separation (10^5 – 10^6 theoretical plates per meter) of picomole to attomole amounts of analytes in the nanolitre sample volume, possesses a high application potential in the field of separations of peptides: it is broadly utilized for analysis, preparation and physicochemical and biochemical characterization of peptides both in the

research and in practical applications in chemistry, biochemistry, biomedicine, biotechnology, pharmaceutical industry, food and feed industry and fishing farming, as documented in several recent reviews [1–7].

Enkephalins (ENKs), ($[Leu^5]$ enkephalin [Leu^5]ENK), pentapeptide H-Tyr-Gly-Gly-Phe-Leu-OH, and $[Met^5]$ enkephalin ($[Met^5]$ ENK), pentapeptide H-Tyr-Gly-Gly-Phe-Met-OH, and dalargin (DLR), hexapeptide H-Tyr-d-Ala-Gly-Phe-Leu-Arg-OH, are biologically active peptides with morphin-like effects acting as ligands for the opiate receptors in the brain [8]. Enkephalins are fragments of nature opioid hormones, dynorphin A (17 amino acid residues) and dynorphin B (13 amino acid residues).

* Corresponding author. Tel.: +420 220 183 239; fax: +420 220 183 592.
E-mail address: kasicka@uochb.cas.cz (V. Kašička).

Enkephalins and dynorphins originate from the two large precursor proteins, preproenkephalin and preprodynorphin [8]. They play significant roles in mediating stress and abatement of pain and are involved in temperature control, feeding behavior and respiration. These peptides and their analogues are used for the treatment of some mental illnesses (e.g. chronic schizophrenia, senile dementia of Alzheimer type).

CZE has been frequently used for analysis of enkephalins and dalargin, most often to check the purity of synthetic preparations of these peptides but also for their determination in biological fluids (serum, plasma, cerebrospinal fluids). Rational approach to selection and optimization of important experimental parameters (composition and pH of the background electrolyte (BGE), loading limit, capillary diameter and fraction collection) in analytical and preparative CZE of opioid peptides including enkephalin and dynorphin analogues with off-line mass spectrometric (MS) detection was reviewed by Lee and Desiderio [9]. [Leu^5]ENK, [Met^5]ENK and [$\text{desTyr}^1\text{-Leu}^5$]ENK were analyzed with high sensitivity (with limits of detection as low as 3–11 attomole) by CZE in citrate and phosphate BGEs using electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS) [10]. Analogues of enkephalins and dalargin and other biopeptides, such as e.g. vasopressin, desmopressin, insulin-like growth factors, have been separated by CZE in strongly acidic BGE, 150 mM phosphoric acid, pH 1.8 [11]. These analogues were also analyzed and separated by capillary micellar electrokinetic chromatography (CMEKC) with micellar pseudophase formed by anionic detergent sodium dodecylsulfate (SDS) in alkaline BGEs (20 mM tetraborate, pH 9.2, and 20 mM phosphate, pH 8.8) or by cationic detergent cetyltrimethylammonium bromide (CTAB) in acidic BGE (50 mM phosphate, pH 4.1) [11–13]. Analogue of dalargin, [$\text{D-Tle}^{2,5}$]dalargin, was analyzed by CZE in 0.5 M acetic acid, pH 2.5, and then purified in preparative scale by free-flow zone electrophoresis [14]. Six analogues and fragments of enkephalins were separated and determined in biological matrices by multidimensional separation system consisting of on-line coupling of size-exclusion chromatography (SEC), reverse-phase C18 trapping column and CZE [15,16] and by liquid secondary ion mass spectrometry and tandem mass spectrometry (LSI-MS-MS-MS) [17]. Dynorphin peptide analogues were separated by CMEKC employing anionic (SDS), cationic (CTAB) and zwitterionic (CHAPS) surfactants [18]. Enkephalin-related peptides were derivatized by fluorescein isothiocyanate and then separated by CMEKC in borate BGE with SDS micelles and detected with laser-induced fluorescence detector [19]. [$\text{D-Pen}^{2,5}$]ENK (D-Pen is D-penicillamine or D-3-mercaptopvaline) and [$\text{D-Ser}^2\text{,Thr}^6$]DLR were analyzed in rat serum by CZE in phosphate (pH 2.4) and borate (pH 8.3) buffers [20,21]. ENKs and their fragments were electrochemically detected as in-capillary formed copper complexes after their CZE separation [22]. ENK analogues were separated in tris-phosphate and sodium phosphate BGEs, pH 2.5, in capillary noncovalently coated with two layers of oppo-

sitely charged polymers [23]. [Leu^5]ENK, [Met^5]ENK and other peptide hormones were separated in three BGEs, pH 2.61, 2.85 and 10.0, and their pK_a were determined from the pH dependence of their electrophoretic mobilities in the broad pH range, 2–12, [24], and subsequently analyzed with CE-ESI-MS in 50 mM acetic acid and 50 mM formic acid, pH 2.85 [25].

The aim of this work was to perform qualitative and quantitative analysis of synthetic preparations of opioid peptide hormones, enkephalins and dalargin, and their analogs and fragments, by CZE both in acidic and alkaline BGEs. Suitable experimental conditions should be found for CZE separation of the mixtures of these structurally related peptides. In addition to the purity degree also some physicochemical characteristics of the analyzed peptides, such as effective electrophoretic mobilities, effective and specific charges should be determined, and the correlation between effective electrophoretic mobility of analyzed peptides and their charge and size (relative molecular mass) should be investigated.

2. Experimental

2.1. Chemicals

All chemicals used were of analytical reagent grade. Iminodiacetic acid (IDAA) was obtained from Bachem (Bubendorf, Switzerland), Tris (tris(hydroxymethyl)aminomethane) was supplied by Serva (Heidelberg, Germany), phosphoric acid and dimethyl sulfoxide (DMSO) were obtained from Lachema (Brno, Czech Republic) and Tricine ([tris(hydroxymethyl)-methyl]-glycine) was from Merck (Darmstadt, Germany). Isophorone (3,5,5-trimethyl-2-cyclohexen-1-one) was supplied by Fluka (Buchs, Switzerland).

2.2. Peptides

The list of analyzed peptides and their abbreviations, sequences and relative molecular masses, M_r , are presented in Table 1. The oligopeptide fragments of ENKs, Tyr-Gly (YG) and Tyr-Gly-Gly (YGG), were obtained from Bachem (Bubendorf, Switzerland), the di- and tripeptide fragments of DLR, Tyr-Ala (YA) and Tyr-D-Ala-Gly (YaG), were purchased from Sigma (St. Louis, MO, USA). The analogs and derivatives of ENKs and DLR were prepared by solid phase synthesis [26]. The structure of DLR was altered by the substitution of leucine in position 5 by bulky amino acid such as D-tertiary leucine [27], by glycosylation [28] or iodination of tyrosine in position 1, by derivatization of the C-terminal arginine carboxyl group and by combination of these modifications.

2.3. Instrumentation

The capillary electrophoretic experiments were carried out in commercial P/ACE MDQ System (Beckman-Coulter,

Table 1
Sequences of analyzed peptides and their relative molecular masses (M_r)

Peptide	Abbreviation	Sequence in three-letters code	M_r
[Leu ⁵]jenkephalin	[Leu ⁵]ENK	H-Tyr-Gly-Gly-Phe-Leu-OH	555.8
[Met ⁵]jenkephalin	[Met ⁵]ENK	H-Tyr-Gly-Gly-Phe-Met-OH	573.8
ENK dipeptide fragment	YG	H-Tyr-Gly-OH	238.3
ENK tripeptide fragment	YGG	H-Tyr-Gly-Gly-OH	295.4
Dalargin	DLR	H-Tyr-D-Ala-Gly-Phe-Leu-Arg-OH	726.1
DLR dipeptide fragment	YA	H-Tyr-Ala-OH	252.3
DLR tripeptide fragment	YaG	H-Tyr-D-Ala-Gly-OH	309.4
[D-Tle ⁵]dalargin	[D-Tle ⁵]DLR	H-Tyr-D-Ala-Gly-Phe-D-Tle-Arg-OH	726.1
Dalargin ethylamid	DLR-NHEt	H-Tyr-D-Ala-Gly-Phe-Leu-Arg-NH-Et	749.0
[Tyr(I ₂)]dalargin	I ₂ DLR	H-(3,5-di-I)-Tyr-D-Ala-Gly-Phe-Leu-Arg-OH	960.8
[D-Ser ² ,Thr ⁶]dalargin	[D-Ser ² Thr ⁶]DLR	H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH	689.9
N-Phenylacetyl-dalargin	PacDLR	C ₆ H ₅ -Ac-Tyr-D-Ala-Gly-Phe-Leu-Arg-OH	859.1

Fullerton, CA, USA), data acquisition and evaluation were performed using the software P/ACE System MDQ, version 2.3 supplied by Beckman.

The apparatus was equipped with the internally non-coated fused silica capillary with outer polyimide coating, total/effective length 39.4/29.2 cm, I.D./O.D. 75/360 μm (Polymicro Technologies, Phoenix, AR, USA). The analytes were detected by UV-vis absorption spectrophotometric photodiode array detector (190–600 nm) set at constant wavelength 206 nm. The temperature was set at 25 °C with liquid coolant continuously circulating around the capillary.

The new capillary was gradually flushed with water, 0.1 M NaOH, water and BGE, each wash for 5 min. Finally, the capillary was conditioned by a 20 min application of the high voltage to equilibrate the inner surface and to stabilize electroosmotic flow. Between runs under the same conditions, the capillary was rinsed with the BGE for 2 min. Prior to any change of the BGE the capillary was rinsed with 0.1 M NaOH for 5 min and then repeatedly stabilized. The separation voltage was 10 kV. The samples were injected with pressure 5–10 mbar for 5–15 s. The samples were dissolved in deionized water or in BGE and their concentrations were in the range 0.1–1.5 mg/ml. The BGEs were filtered through a 0.45 μm syringe filter (Millipore, Bedford, MA, USA) before use.

3. Results and discussion

3.1. Selection of separation conditions

The strategy for the rational selection of experimental conditions for CZE analysis and separations of ENKs, DLR and their derivatives and fragments followed the general rules of selection of suitable CZE separation conditions [29] and took into account the specific properties of these peptides resulting from their structure—number and sequence of the linked amino acid residues, i.e. their amphoteric character and strong pH dependence of effective electrophoretic mobilities, which are governed by the effective electric charge, size

(relative molecular mass) and shape (conformation) of these peptides.

The selection of the composition of the BGEs includes the type and concentration of buffer components and pH, and it also takes into account the requests for preserving chemical and temperature stability and biological activity of analyzed peptides [30,31]. Effective charges of peptides are strongly dependent on pH and pK_a of ionogenic groups of amino acid residues present in peptide chain. The analyzed peptides, see Table 1, contain several different types of ionogenic groups—all peptides, except Pac-DLR, possess α -amino group of the N-terminus of the peptide chain (average pK_a 8.0), and all peptides, except DLR-NHEt, contain α -carboxyl group of the C-terminus of the peptide chain (average pK_a 2.6), dalargin analogues (except [D-Ser²Thr⁶]DLR) possess guanidium group of arginine (average pK_a 11.3) and all of them hydroxy group of tyrosine (average pK_a 10.4). The relative molecular mass of analyzed peptides was in the range 238.3–1065, see Table 1.

One of the most important parameters for selection of suitable experimental conditions for CZE analysis and separation of peptides is the pH dependence of their effective and specific charges, since the electrophoretic mobility of peptides is directly proportional to their effective charge. For that reason the dependence of effective charge and specific charge (effective charges divided by relative molecular mass) of all peptides to be analyzed has been calculated by the earlier developed computer program Nabamfo [32] using the above given average values of pK_a of ionogenic groups. From the course of the pH dependence of the specific charge of peptides to be analyzed and separated (see Fig. 1) it follows, that these peptides can be analyzed both as cations (preferably in acid pH range) and as anions (preferably in alkaline pH range). Consequently, the two strongly acidic BGEs (pH 2.25–2.30) and one weakly alkaline BGE (pH 8.1) were selected for CZE analyses and separations of the above peptides. Full composition of the used BGEs together with the separation voltage and electric currents are presented in Table 2. Separation conditions are significantly influenced by solubility of peptides. With respect to the relatively hydrophilic character of the analyzed

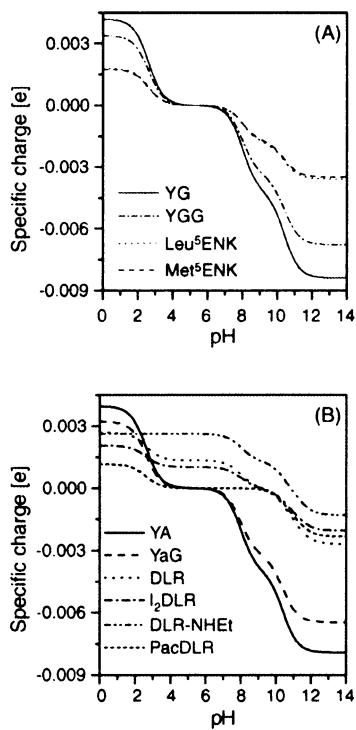


Fig. 1. The pH dependence of specific charge (effective charge divided by relative molecular mass) of selected peptides. (A) Enkephalin analogues and fragments and (B) dalargin analogues and fragments.

peptides most of them could be dissolved in deionized water, which brings an advantage that the same sample solution can be applied to CZE analyses in different BGEs, and in addition the electric-field-enhanced concentrating effect is utilized to concentrate the diluted peptide solutions. On the other hand using BGE as sample solvent ensures the constant separation conditions (movement in the BGE of the same composition) in the whole CZE experiment, which is important when CZE is applied for the physicochemical measurements.

3.2. Qualitative and quantitative analysis

The selected BGEs were used for qualitative and quantitative analysis of synthetic preparations of ENKs, DLR and their new analogues and fragments. For full characterization of these peptides before their application in the biological tests it is necessary to find out the content of their all admixtures, originating from the synthesis of peptides and/or

from their subsequent purification procedures. Consequently, the purity degrees of these peptides, both crude synthetic products and peptides purified by chromatographic methods, size-exclusion chromatography and reverse phase high-performance liquid chromatography (RP-HPLC), were determined. Peptides were analyzed as cations in acidic BGEs and as anions or cations in weakly alkaline BGE. The examples of CZE analyses of some peptides are shown in Fig. 2. Standards of synthesized peptides were not available, therefore only relative degrees of purity could be determined. Peptide purity and peptide content in the sample was quantified by three ways: (i) relative peak height, $P_h(i)$ (1), (ii) relative peak area, $P_A(i)$ (2), and (iii) relative corrected peak area, $P_{CA}(i)$ (3), of the UV-positive peaks for the i th component of the peptide preparation:

$$P_h(i) = \frac{h(i)}{\sum h(i)}, \quad i = 1, \dots, n \quad (1)$$

$$P_A(i) = \frac{A(i)}{\sum A(i)}, \quad i = 1, \dots, n \quad (2)$$

$$P_{CA}(i) = \frac{A_c(i)}{\sum A_c(i)}, \quad i = 1, \dots, n \quad (3)$$

where $h(i)$ is height of the i th peak, $A(i)$ is area of the i th peak and $A_c(i)$ is corrected area of the i th peak, corrected peak area is peak area corrected with respect to migration velocity of the given peak, which is obtained as peak area divided by the migration time of the given peak, n is the number of sample components.

The values of differently expressed purity degrees of analyzed peptides are presented in Table 3. The values of purity degrees were determined as averages of values obtained in two subsequent analyses, which differed less than 1%. The synthetic peptides exhibited rather different degree of purity. Analyses of peptides with high purity degree, [Leu^5]ENK and DLR-NHEt, are demonstrated in Fig. 2A and F, respectively. On the other hand analysis of peptide with low purity degree, with one major and several minor impurities, is shown in electrophoregram of [$D\text{-Tle}^5$]DLR in Fig. 2D. Analysis of peptide with some minor impurities is demonstrated in electrophoregram of $I_2\text{DLR}$ in Fig. 2C. As follows from the above definitions, the most exact characteristic of peptide purity is the relative corrected peak area. Sometimes other, the simpler ways of purity evaluation, relative peak height or relative peak area, can be used, particularly in the cases when the peaks of sample components are uniformly dispersed and their migration times (velocities) are not too different. The great difference in differently expressed purity degree was found, e.g. in the CZE analysis of DLR presented in Fig. 2B. Relative peak height of DLR was about 10–15% lower than its relative peak area and relative peak corrected area in acidic BGEs I and II due to the relatively high height and small area of the sharp peak of the main admixture X. On the other hand relative peak height of tripeptide fragment of ENK, YGG, was about 6–7% higher than relative peak area

Table 2

Composition and pH of the BGEs applied for CZE analyses and separations of ENKs and DLR analogues and fragments, and electric current, I , in these BGEs at constant voltage 10 kV

BGE no.	BGE constituents	pH	I (μA)
I	50 mM Tris, 100 mM H_3PO_4	2.25	58–65
II	100 mM iminodiacetic acid	2.30	36.7–37
III	200 mM iminodiacetic acid	2.32	52.3–52.8
IV	40 mM Tris, 40 mM Tricine	8.10	13.2–14.2

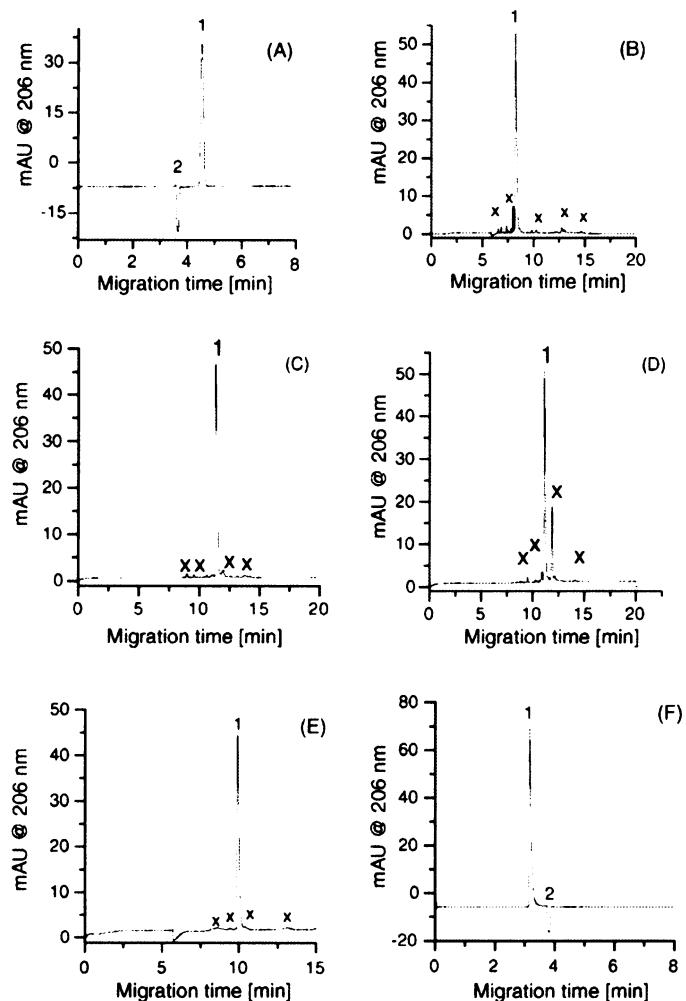


Fig. 2. CZE analyses of selected synthetic peptides. Experiments were carried out in uncoated fused-silica capillary: total/effective length 394/292 mm, I.D. 75 μ m, O.D. 360 μ m, constant voltage $U = 10$ kV, UV-absorption detection at 206 nm. (A) [Leu^5]ENK, 1.25 mg ml $^{-1}$, in BGE IV; (B) DLR, 1.25 mg ml $^{-1}$, in BGE II; (C) I₂DLR, 1.00 mg ml $^{-1}$, in BGE II; (D) [D-Tle^5]DLR, 1.00 mg ml $^{-1}$, in BGE I; (E) YGG, 1.10 mg ml $^{-1}$, in BGE II; (F) DLR-NHEt, 1.15 mg ml $^{-1}$, in BGE IV. (1) Main synthetic product; (2) neutral electroosmotic flow marker; and (X) non-identified impurities.

Table 3

Purity degrees of analyzed peptides, expressed alternatively as relative peak height, P_h , relative peak area, P_A , and relative peak corrected area, P_{CA} , determined by CZE in three BGEs

Peptide	BGE I, pH 2.25			BGE II, pH 2.3			BGE IV, pH 8.1		
	P_h (%)	P_A (%)	P_{CA} (%)	P_h (%)	P_A (%)	P_{CA} (%)	P_h (%)	P_A (%)	P_{CA} (%)
[Leu^5]ENK	94.5	92.7	92.7	93.3	89.4	89.4	95.8	93.1	93.0
[Met^5]ENK	94.0	92.6	92.6	91.3	95.7	95.6	97.5	97.8	97.8
YG	98.3	98.8	98.9	97.4	98.5	98.7	98.5	99.0	99.0
YGG	92.5	86.0	85.7	95.8	89.6	89.6	96.2	91.0	91.8
DLR	75.4	84.6	84.6	65.8	80.7	81.9	91.5	89.2	90.3
YA	99.2	99.2	99.3	99.7	99.8	99.9	99.3	99.4	98.2
YaG	92.7	93.1	93.4	96.3	96.3	94.4	98.5	98.7	98.7
[D-Tle^5]DLR	66.6	69.8	70.9	50.8	58.8	58.8	91.3	92.1	92.1
DLR-NHEt	94.5	96.8	96.9	95.0	94.2	94.2	94.7	94.3	94.2
I ₂ DLR	91.5	90.5	90.6	87.5	82.8	82.8	90.2	89.9	90.5
[$\text{D-Ser}^2\text{Thr}^6$]DLR	87.7	88.3	87.3	92.5	94.2	94.2	97.9	98.7	98.8
PacDLR	81.0	78.1	81.8	94.9	95.0	89.7	n.d.	n.d.	n.d.

n.d.: not determined.

or relative corrected peak area in acidic BGEs I and II due to the relatively small height and broad width of the peaks of its admixtures (see Fig. 2E). Similar values of purity degrees expressed by relative peak height, relative peak area and corrected peak area were obtained in analysis of highly purified peptides, as e.g. in analysis of DLR-NH₂ both in acidic and alkaline BGEs, see Table 3 and Fig. 2F. As follows from the presented data, the purity degree of given peptide depends on BGE used and on the way of purity degree evaluation. Consequently, purity degree of peptides should be tested in more than one BGE and only the purity degrees obtained under the same conditions and evaluated in the same way can be compared.

As can be seen from the analyses presented in Fig. 1A–F, in all BGEs used symmetrical peaks were obtained with high separation efficiency in short times, with migration times of the main component between 3 and 12 min. The best results were obtained in BGE II (100 mM iminodiacetic acid, pH 2.30) using the separation voltage 10 kV. The separation efficiency in BGE II was in range $(0.5\text{--}1.0) \times 10^6$ theoretical plates per meter, concentration limit of detection (LOD = three times noise) was $(0.60\text{--}2.26) \times 10^{-6}$ mol dm⁻³, concentration limit of quantification (LOQ = 10 times noise) was $(2.01\text{--}7.54) \times 10^{-6}$ mol dm⁻³, sensitivity of the UV-absorption detector was 46.5–114.4 AU mol⁻¹ dm³ and the noise of UV-detector signal was 0.023 mA.U. These parameters for BGE I were almost the same as for BGE II, however the time of analysis was longer because of the very low electroosmotic flow (EOF) in this BGE. BGE IV was found less suitable for analysis of these series of peptides due to the basic character of some of the analyzed peptides, DLR and its derivatives, which are positively charged even at pH 8.1 and due to the fast EOF at this pH, which means that the electrophoretically migrated trajectory of these peptides is relatively short and they could not be well separated from their admixtures and other structurally related peptides. The characteristics of the UV-absorption detector for all BGEs are presented in Table 4.

3.3. Separation of structurally related peptides

In addition to qualitative and quantitative analysis of individual synthetic peptide preparations, the separation of closely related structures of ENKs and their fragments, and DLR and its analogues and fragments was tested. These separations are important from the point of view of separations of the whole molecules of ENKs and DLR from their degradation products. The suitable conditions for the separations were derived from the course of the pH dependence of specific charge of these peptides (see Fig. 1), and from the experience obtained in the analysis of individual peptides by CZE in BGEs I, II, and IV. As it was already mentioned above from the BGEs tested the BGE II, composed of isoelectric buffer, 100 mM iminodiacetic acid (IDAA), provided the best results. To achieve the best

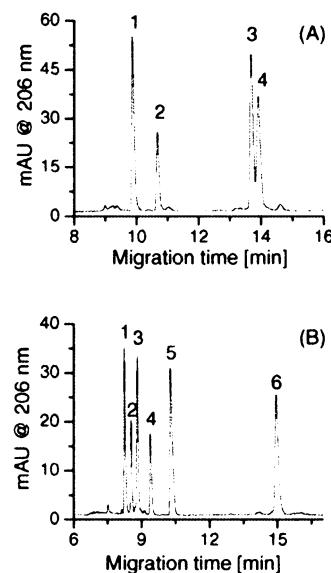


Fig. 3. CZE separations of structurally related peptides. Experiments were carried out in uncoated fused-silica capillary: total/effective length 394/292 mm, I.D. 75 μm , O.D. 360 μm , constant voltage $U = 10$ kV, UV-absorption detection at 206 nm. (A) Enkephalin analogues and fragments in BGE III: 1, YG; 2, YGG; 3, [Leu⁵]ENK; 4, [Met⁵]ENK. (B) Dalarin fragments and analogues in BGE II: 1, DLR-NH₂; 2, DLR; 3, I₂DLR; 4, YA; 5, YaG; and 6, PacDLR.

resolution different concentrations of IDAA and separation voltages were tested. Finally, 100 mM iminodiacetic acid, pH 2.30 and voltage 10 kV were selected as the best conditions for separation of DLR and its analogues and fragments. The doubled concentration of IDAA, i.e. 200 mM, pH 2.32, was found to be most suitable for separation of ENKs and their fragments, providing the best resolution at the same separation voltage, 10 kV. Separations of these two mixtures are shown in Fig. 3, mixture of [Leu⁵]ENK, [Met⁵]ENK and their fragments (YG, YGG) in Fig. 3A, and the mixture of DLR and its derivatives (DLR-NH₂, I₂DLR, PacDLR) and fragments (YA, YaG) in Fig. 3B. The resolution of any pair of peptides was higher than 2.1 with the exception of [Leu⁵]ENK and [Met⁵]ENK, which were separated with resolution 1.11. In the mixture of ENKs and their fragments the latter migrated faster, with migration times in the order YG < YGG < [Leu⁵]ENK < [Met⁵]ENK. In the mixture of DLR and their analogues and fragments the longer peptides with higher values of effective and specific charges migrated faster than the short fragments with lower specific charges.

3.4. Determination of effective mobility

From CZE analyses of ENKs, DLR and their analogues and fragments the effective electrophoretic mobilities of these peptides were determined. The effective electrophoretic mobility, m_{eff} , of peptide in the given BGE was calculated from the migration time of the peptide, $t_{\text{ mig}}$, in this BGE, and from the migration time of the electroneutral marker of

Table 4

The characteristics of the UV-absorption photodiode array detector set at 206 nm

Peptide	BGE	<i>c</i> (mmol dm ⁻³)	<i>R/n</i> × 10 ⁻³	<i>S</i> (AU mol ⁻¹ dm ³)	LOD (μmol dm ⁻³)	LOQ (μmol dm ⁻³)
[Leu ⁵]ENK	I	2.08	4.02	42.6	1.55	5.17
	II	2.01	4.06	46.5	1.48	4.95
	IV	2.16	1.04	32.9	6.20	20.7
[Met ⁵]ENK	I	1.61	5.46	74.7	0.88	2.95
	II	1.49	7.41	114.4	0.60	2.01
	IV	1.70	1.49	59.7	3.42	11.4
YG	I	4.56	4.75	22.9	2.88	9.60
	II	5.38	7.14	30.5	2.26	7.54
	IV	5.61	1.20	14.5	14.1	46.9
YGG	I	3.19	2.55	17.6	3.75	12.5
	II	3.34	1.85	12.8	5.41	18.0
	IV	3.58	0.44	8.35	24.4	81.4
DLR	I	1.46	4.50	67.8	0.97	3.24
	II	1.41	5.74	93.6	0.74	2.46
	IV	1.55	1.07	46.7	4.37	14.6
YA	I	4.32	2.63	13.4	4.93	14.4
	II	4.35	1.73	9.13	7.56	25.2
	IV	4.33	0.49	7.67	26.6	88.7
YaG	I	3.46	5.23	33.2	1.99	6.62
	II	3.58	4.02	25.8	2.70	8.90
	IV	3.66	1.54	28.7	7.12	23.7
[D-Tle ⁵]DLR	I	0.98	3.76	84.4	0.78	2.61
	II	0.81	3.00	85.3	0.81	2.70
	IV	1.11	0.83	50.9	4.01	13.4
DLR-NHEt	I	1.48	7.57	112.6	0.59	1.95
	II	1.45	5.21	82.7	0.83	2.78
	IV	1.45	2.25	105.3	1.94	6.46
I ₂ DLR	I	0.94	4.16	97.4	0.68	2.26
	II	1.16	4.52	89.6	0.77	2.57
	IV	0.94	1.16	83.6	2.44	8.14
[D-Ser ² Thr ⁶]DLR	I	1.33	2.74	45.4	1.45	4.85
	II	1.44	2.96	47.3	1.46	4.86
	IV	1.51	0.86	38.6	5.29	17.6
PacDLR	I	0.91	2.56	62.0	1.07	3.55
	II	1.38	4.02	67.0	1.03	3.43
	IV	n.d.	n.d.	n.d.	n.d.	n.d.

R: detector response, *n*: noise, *c*: concentration, *S*: sensitivity of detector $S = R/c$, LOD: limit of detection $LOD = 3n/S$, LOQ: limit of quantification $LOQ = 10n/S$, n.d.: not determined. BGE I: 100 mM H₃PO₄, 50 mM Tris, pH 2.25, *n* = 0.022 mAU; BGE II: 100 mM iminodiacetic acid, pH 2.30, *n* = 0.023 mAU; BGE IV: 40 mM Tris, 40 mM Tricine, pH 8.1, *n* = 0.068 mAU.

EOF (DMSO or isophorone), *t_{eo}*, according to the following relation:

$$m_{\text{eff}} = \frac{l_t l_{\text{ef}}}{U} \left(\frac{1}{t_{\text{mig}}} - \frac{1}{t_{\text{eo}}} \right) \quad (4)$$

where *l_t* is total capillary length, *l_{ef}* is effective capillary length, *U* is the separation voltage.

In CZE analyses in highly acidic BGEs with very low EOF due to the suppressed dissociation of silanol groups the effective electrophoretic mobility was determined using the pressure accelerated measurement of EOF as described in [33]. Briefly the sample was injected along with zone A of the neutral EOF marker into the capillary filled with BGE. Then the separation voltage was applied, the analytes were

separated and the neutral marker zone A migrated with the velocity of EOF. Analysis was stopped (separation voltage switched-off) at a specific time, *t_u*, just after the peptide zone passed the detector. Then the second zone (B) of the (same or different) EOF marker was injected and the injection pressure (15 mbar) was applied onto the pure BGE vessel. Both zones of the EOF marker, A and B, reached the detector at times, *t_A* and *t_B*, respectively, which were recorded in the usual manner. Then the electrophoretic mobility can be calculated from the following relation:

$$m_{\text{eff}} = \frac{l_t l_{\text{ef}}}{U} \left(\frac{1}{t_{\text{mig}}} - \frac{1}{t_u} + \frac{t_A}{t_u t_B} \right) \quad (5)$$

where the symbols have the same meanings as in Eq. (4). The values of effective electrophoretic mobility were determined as averages of two subsequent measurements, which differed less than 1%.

The real temperature inside the capillary was higher than the temperature of the coolant due to Joule heating. The real temperature in the capillary for each BGE was obtained from the dependence of temperature increase in fused silica capillary on the input power per unit length of the capillary, which was determined from the measurement of specific electric conductivity of standard solution of 0.01 mol dm^{-3} KCl in the capillary at different values of input power as described in [34]. The temperature increase in fused silica capillary was 4.5°C for BGE I, 2.0°C for BGE II, 3.2°C for BGE III and 0.7°C for BGE IV. The effective electrophoretic mobility corrected to standard temperature (25°C), $m_{\text{eff},25}$, was calculated according to the equation:

$$m_{\text{eff},25} = m_{\text{eff},t}[1 - 0.022(t - 25)] \quad (6)$$

where $m_{\text{eff},25}$ is effective electrophoretic mobility at 25°C , $m_{\text{eff},t}$ is effective electrophoretic mobility measured at real temperature, t , inside the capillary.

The values of effective electrophoretic mobilities of peptides in different BGEs corrected to standard temperature, 25°C , are presented in Table 5. The effective electrophoretic mobilities measured at real temperature inside the capillary differed up to 10% in the most conductive BGE I in comparison with standard effective electrophoretic mobilities at 25°C .

Generally the effective mobilities of analyzed peptides were in agreement with the values predicted from the course of the dependence of the specific charge on pH, i.e. peptides with higher specific charge exhibited higher effective mobilities than those with lower specific charge. Peptides containing guanidium group of arginine, particularly DLR and its analogues with the exception of [$\text{D-Ser}^2\text{Thr}^6$]DLR and PacDLR, migrated faster than the enkephalins, and due to their strongly basic character they migrated as cations even in the weak alkaline BGE IV, pH 8.10. Enkephalin dipeptide

and tripeptide fragments (YG, YGG) migrated faster than enkephalins, all these peptides had almost the same charge (see Table 5). DLR analogs, [$\text{D-Ser}^2\text{Thr}^6$]DLR and PacDLR, lacking positive charged group of arginine behaved similarly as ENKs, however their mobilities were lower than those of ENKs due to their larger molecules.

3.5. Relationship between mobility of peptides and their charge and size

Several models correlating effective electrophoretic mobilities of peptides, m_{eff} , with their effective charge, q , and molecular size expressed as relative molecular mass, M_r , or number of amino acids in polypeptide chain, n , respectively, have been developed [35–39]. The models are based on Stoke's law, describing the motion of a particle in liquid medium, and on the action of electric field force on charged molecule. The semiempirical models describe the electrophoretic behavior of various conformations of molecules, which can be utilized to predict secondary or tertiary structure of peptides and proteins in solution [40].

In the current work we have used Cross's model [41], due to its suitability for prediction of the most probable secondary structure of peptides:

$$\log \left(\frac{m_{\text{eff}}}{q} \right) = k \log M_r \quad (7)$$

where the constant of proportionality, k , is the exponent of M_r in the non-logarithmic expression of this relation, which can be determined as a slope of this dependence and which is related to the shape of the charged molecule. The graph of these dependences in three BGEs is shown in Fig. 4. Because not all of the analyzed peptides formed a homologous series, some points did not fit well in the linear part of this dependence. This concerns especially DLR, and its analogs [$\text{D-Tle}^{2,5}$]DLR and DLR-NHEt, analyzed at alkaline pH in BGE IV, where their effective mobilities were rather low with respect to their estimated effective charges (see Table 5). The best coefficient of the correlation of the dependence expressed by Eq. (7)

Table 5
Calculated effective charges, q , and CZE determined effective electrophoretic mobilities, m_{eff} , corrected to standard temperature, 25°C , of analyzed peptides in three different BGEs

Peptide	q (e)			$m_{\text{eff}} (\times 10^{-9} \text{ m}^2 \text{ V}^{-1} \text{ s}^{-1})$		
	BGE I, pH 2.25	BGE II, pH 2.30	BGE IV, pH 8.1	BGE I, pH 2.25	BGE II, pH 2.30	BGE IV, pH 8.1
[Leu ⁵]ENK	0.72	0.62	-0.57	9.44	11.4	-9.99
[Met ⁵]ENK	0.71	0.61	-0.56	9.12	11.3	-10.0
YG	0.69	0.69	-0.57	14.3	17.6	-13.9
YGG	0.69	0.69	-0.56	12.8	15.3	-12.8
DLR	1.71	1.63	0.42	15.7	19.0	1.91
YA	0.69	0.69	-0.57	14.5	16.7	-13.3
YaG	0.69	0.69	-0.55	12.3	15.2	-12.7
[D-Tle ^{2,5}]DLR	1.63	1.55	0.42	15.7	18.7	2.13
DLR-NHEt	2.00	2.00	1.42	15.9	20.0	10.3
I ₂ DLR	1.75	1.59	0.45	15.4	18.1	7.58
[D-Ser ² Thr ⁶]DLR	0.68	0.64	-0.56	7.83	9.78	-8.91
PacDLR	0.71	0.68	0.00	7.49	9.32	0.00

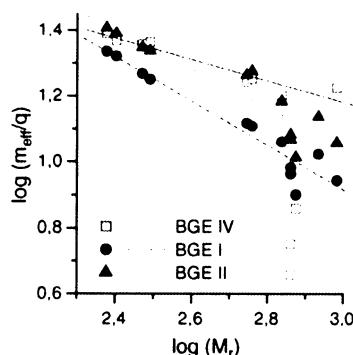


Fig. 4. Dependence of the decadic logarithm of the ratio of the effective electrophoretic mobility and effective charge on the decadic logarithm of relative molecular mass of analyzed enkephalin and dalargin analogues and fragments.

was obtained in BGE I, pH 2.25, $R = 0.9602$. When “outlier” points of DLR and its derivatives in BGE IV were omitted, the high coefficient of correlation was obtained also for BGE IV, reaching the value 0.9566. From the graph in Fig. 4 it is obvious, that the slopes of these dependences, k , are similar for two acidic BGEs, $k = -0.67386$ for BGE I, and $k = -0.5958$ for BGE II, whereas the slope of this dependence in alkaline BGE IV, is significantly different, $k = -0.3233$. The values of k in acidic BGEs are close to the value $2/3$, which indicates that at this pH the peptides molecules have the shape of a wide thin disk, whereas the value of k in alkaline BGE is close to $1/3$, which corresponds to the spherical shape of the molecule [40].

4. Conclusions

CZE proved to be powerful and useful tool for the fast and sensitive analyses and highly efficient separations of opioid peptide hormones, enkephalins, dalargin, and their analogues and fragments, in picomole to femtomole level in the nanolitre applied sample volume. The purity degree of analyzed peptides was best evaluated by relative corrected peak area. From the CZE analyses of peptides also their important physicochemical characteristics, particularly effective electrophoretic mobilities at both acidic and alkaline pH, could be determined. From the investigated correlation between effective electrophoretic mobility of analyzed peptides and their charge and size (relative molecular mass) the probable secondary structure of peptides could be predicted.

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Veronika Šolínová
Václav Kašička
Dušan Koval
Jan Hlaváček

Institute of Organic Chemistry
and Biochemistry,
Academy of Sciences of the
Czech Republic,
Prague, Czech Republic

Separation and investigation of structure-mobility relationships of insect oostatic peptides by capillary zone electrophoresis

Capillary zone electrophoresis (CZE) has been applied to qualitative analysis, separation, and physicochemical characterization of synthetic insect oostatic peptides (IOPs) and their derivatives and fragments. Series of homologous IOPs were separated in three acidic background electrolytes (BGEs; pH 2.25, 2.30, 2.40) and an alkaline BGE (pH 8.1). Best separation was achieved in acid BGE composed of 100 mM H_3PO_4 , 50 mM Tris, pH 2.25. The effective electrophoretic mobilities, μ_{ep} , of all IOPs in four BGEs were determined and several semiempirical models correlating effective mobility with charge-to-size ratio (μ_{ep} versus q/M_r^k) were tested to describe the migration behavior of IOP in CZE. None of models was found to be unambiguously applicable for the whole set of 20 IOPs differing in size (dipeptide – decapeptide) and charge (−2 to +0.77 elementary charges). However, a high coefficient of correlation, 0.9993, was found for the subset of homologous series of IOPs with decreasing number of proline residues at C-terminus, H-Tyr-Asp-Pro-Ala-Pro_x-OH, $x = 6 - 0$, for the dependence of μ_{ep} on q/M_r^k with $k = 0.5$ for IOPs as anions in alkaline BGE and with $k = 2/3$ for IOPs as cations in optimized acidic Tris-phosphate BGE. From these dependences the probable structure of IOPs in solution could be predicted.

Keywords: Capillary zone electrophoresis / Insect oostatic peptides / Structure-mobility relationships
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1 Introduction

1.1 General aspects

Capillary zone electrophoresis (CZE) has developed into a fast, high-efficient, and high-sensitive separation technique which is now frequently used for analysis, micro-preparation, and physicochemical characterization of peptides [1–7]. CZE can provide not only the information on qualitative and quantitative composition of peptide samples but also important physicochemical characteristics of separated peptides such as effective and absolute mobilities, charges, and parameters of molecular size and conformation. Using capillary electrophoresis (CE) methods for physicochemical characterization is advantageous, since only small amounts of analyzed compounds are sufficient, and, in addition, the admixtures separable by CZE need not to be removed in advance from the sample. Several models of the correlation between electro-

phoretic mobility, and the effective charge and size of polypeptide molecules, expressed as relative molecular mass or number of amino acid residues, respectively, have been developed [8–21]. The models are based on Stoke's law, describing the motion of a particle in liquid medium, and on the action of electric field force on the charged molecule. The semiempirical models describe the electrophoretic behavior of various conformation of molecules, which can be utilized to predict the secondary or tertiary structure of peptides and proteins in solution [8, 22–25].

Insect oostatic peptides (IOPs) are antigenadotropic insect hormones. Borovsky *et al.* [26] have firstly described the oostatic factor Aed-TMOF (trypsin modulating oostatic factor) as native decapeptide H-Tyr-Asp-Pro-Ala-Pro-Pro-Pro-Pro-OH, which was isolated from the mosquito *Aedes aegypti*. IOPs have specific effects on the reproduction of the flies, they cause the changes in the follicular epithelium, proliferation of nuclei, and cell division toward the inner part of the egg chamber. Consequently, IOPs are considered as a potential new type of biologically degradable insecticides. Borovsky *et al.* [27] also suggested the probable structure of the decapeptide as left-handed helix. Later, analogs with shortened C-terminal sequences, cyclic structure, and analogs with proline-substituted dehydroproline were synthesized

Correspondence: Dr. Václav Kašička, Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Flemingovo 2, CZ-166 10 Prague 6, Czech Republic
E-mail: kasicka@uochb.cas.cz
Fax: +420-220-183-592

Abbreviations: IDAA, iminodiacetic acid; IOP, insect oostatic peptide

[28, 29], and characterized by NMR and circular dichroism spectra [29, 30]. Their biological activities were tested in the fly *Neobellieria bullata* (Diptera). The peptides with the C-terminus shortened linear chain-affected processes of egg development in 20–80% of ovarioles [28]. Weak oostatic activity was observed with the cyclic analogs due to their changed conformation in comparison with linear peptides possessing the ability in receptor binding [29]. Determination of secondary structure of IOPs is of great importance for estimation of their interactions with receptors.

In the current work, the first aim was to analyze qualitatively the synthetic IOPs, to determine their purity degree before they are used for biological tests, and to develop a BGE suitable for separation of closely related IOPs. The second aim was to determine effective electrophoretic mobilities of IOP at standard temperature (25°C), to test different models of the dependence of mobility of IOPs on their charge and size, and, consequently, to predict the structure of IOPs in solution.

1.2 Theory

1.2.1 Electrophoretic mobility

Electrophoretic mobility of a peptide is a complex function of the properties of the peptide itself (charge, size, and shape), properties of a medium in which the peptide is moving (composition, pH, ionic strength, viscosity, permittivity, and temperature) and interactions of the peptide with medium components (solvatation, dissociation, complex formation). Consequently, the experimental conditions must be strictly defined for determination of peptide's effective electrophoretic mobility, which has to be related to given composition, pH, and temperature of the BGE. The effective electrophoretic mobility, μ_{ep} , of a charged particle moving in liquid medium in an electric field, can be described [8] by the following equation:

$$\mu_{\text{ep}} = \frac{q}{6\pi\eta r} \quad (1)$$

where q is the effective charge of the ion, η is the viscosity of the solution, and r is the hydrodynamic radius of the ion.

1.2.2 Calculation of effective charge

Peptides are amphoteric electrolytes containing different types of ionogenic groups. The effective charge of peptides is given by the sum of charges (including their sign) of all ionogenic groups present in the polypeptide chain. The relationships usually used for the calculation of the effective charge are based on the Henderson-Hassel-

balch equation [31]. The effective charge of peptide is a function of pH of liquid medium and pK_a values of the ionogenic groups of amino acid residues in the peptide [31]:

$$q = \sum_{i=1}^m \frac{1}{1 + 10^{pH - pK_a(i)}} - \sum_{j=1}^p \frac{1}{1 + 10^{-pK_a(j) - pH}} \quad (2)$$

where m and p are the integral number of cationic and anionic groups, and $pK_a(i)$ and $pK_a(j)$ are negative decadic logarithms of acid dissociation constants of the i -th and j -th ionogenic group of the analyte, respectively.

1.2.3 Models of correlations between mobility of peptides and their charge and size

Several models correlating effective electrophoretic mobilities of peptides, μ_{ep} , with their effective charge, q , and molecular size expressed as relative molecular mass, M_r , or number of amino acids in polypeptide chain, n , respectively, have been developed [8–21, 32].

Offord [12] has firstly quantitatively described this relationship for the series of oligo- and polypeptides separated by paper electrophoresis:

$$\mu_{\text{ep}} = \frac{k q}{M_r^{2/3}} \quad (3)$$

where k is a constant of proportionality. This relationship was found also by some other authors, Issaq et al. [13], Rickard et al. [14], and Basak and Ladisch [15] for peptides separated in free solution by CZE.

For rigid spherically shaped molecules in low-ionic-strength buffers the following relationship was derived [8] from Eq. (1):

$$\mu_{\text{ep}} = \frac{k q}{M_r^{1/3}} \quad (4)$$

Another semiempirical model has been proposed from the study of the synthetic polymers with cylindrical or rod-shaped molecules [33]:

$$\mu_{\text{ep}} = \frac{k q}{M_r^{1/2}} \quad (5)$$

Cross and Garnham [16] modified Offord's relation by expressing it in logarithmic form:

$$\log\left(\frac{\mu_{\text{ep}}}{q}\right) = k \log M_r \quad (6)$$

where k is the exponent of M_r in nonlogarithmic relation, which can be determined as a slope of this dependence.

Kim *et al.* [20] suggested another formula, which fitted well to his set of peptides originating from enzyme digests of proteins. It is similar to the semiempirical model for polymers:

$$\mu_{\text{ep}} = \frac{k q}{M_r^{0.56}} \quad (7)$$

Compton [21] combined two relationships for small (Eq. 4) and large (Eq. 3) molecules and obtained the relation:

$$\mu_{\text{ep}} = \frac{k q}{k_1 M_r^{1/3} + k_2 M_r^{2/3}} \quad (8)$$

where the first term in the denominator, $M_r^{1/3}$, represents smaller molecules, and the second term, $M_r^{2/3}$, larger molecules, both in low-ionic-strength buffers.

In all these cases the relative molecular mass was used as size parameter. Another possibility is to express the molecular size by the number of amino acid residues in the peptide chain, n . This approach was used by Grossman *et al.* [17], who derived the following relation for dependence of effective mobility on charge and size:

$$\mu_{\text{ep}} = \frac{k \ln(q+1)}{n^{0.43}} \quad (9)$$

Cifuentes and Poppe [18] reported the modification of Grossman's classical linear model, where the logarithmic dependence of mobility on the charge expressed electrostatic charge suppression in highly charged peptides:

$$\mu_{\text{ep}} = \frac{k_1 \log(1+k_2 q)}{M_r^{0.411}} \quad (10)$$

The same equation, only with different exponent of M_r , 0.435 instead of 0.411, was derived by Adamson and Reynolds [9].

Sanz-Nebot *et al.* [19] tested relationships (Eq. 10) with q compensated for electrostatic charge suppression in highly charged peptides and the relationships of classical semiempirical models (Eqs. 3–5), compared their results, and found a better correlation for the formula:

$$\mu_{\text{ep}} = \frac{k_1 \ln(k_2 q + 1)}{M_r^k} \quad (11)$$

where $k = 1/3$ for small molecules, $k = 1/2$ for medium-sized polymers, $k = 2/3$ for large molecules, respectively.

These models can be used for prediction of the possible structure of the peptides and proteins in free solution. The structure of the molecule is derived from the relationship between frictional coefficient or electrophoretic mobility, respectively, and the exponent of the relative molecular mass or alternatively exponent of the number of amino acid residues in the peptide [8]. Each type of correlation corresponds to the specific shape of a molecule in free solution. For determination of molec-

ular conformations it is important to find the best way in which electrophoretic mobility in free solution is related to molecular size. Usually at the low-molecular-mass range the short oligopeptides, such as di- and tripeptide fragments of IOPs studied in this paper, have no secondary structure and behave as simple organic ions. Peptides with more amino acids in their molecules form more organized secondary structures, which are strongly dependent on the medium [34]. α -Helical or β -sheet conformation features are preferable to random coil structures under CZE experimental conditions. At the high-molecular-mass range as long-chain polymers, polypeptides may form different types of ordered structures [11]. The structure is dependent on solvent, ionic strength, and pH of the BGE [22, 25]. The structure of synthetic peptides has been studied by CZE with MS detection [23]. The peptide structures help to predict shapes of proteins in free solution [24] and also in peptide mapping of proteins [10]. The data on secondary peptide structures derived from the relation between electrophoretic mobility and charge-to-size ratio are advantageously utilized in the structure-activity studies of biologically active peptides.

2 Materials and methods

2.1 Chemicals

All chemicals used were of analytical reagent grade. Imidodiacetic acid (IDAA) was obtained from Sigma (St. Louis, MO, USA), Tris was purchased from Serva (Heidelberg, Germany), phosphoric acid and dimethyl sulfoxide (DMSO) were supplied by Lachema (Brno, Czech Republic), and Tricine was from Merck (Darmstadt, Germany).

2.2 Peptide synthesis

All samples were synthesized in the group of Dr. Hlaváček in our institute by a procedure described in [28, 29]. Linear di-, tri-, and tetrapeptides were prepared by stepwise synthesis in solution [35]. Larger peptides were prepared by solid-phase approach using a polymer with a 2-chlorotriptylchloride linker [28]. 9-Fluorenylmethoxycarbonyl/tertiary-butyl (Fmoc/tBu) protection of amino acids was utilized. The synthesized peptides were purified by preparative HPLC and characterized by amino acid analysis, mass spectrometry, and analytical HPLC. The synthesis of cyclic peptides was carried out by a cyclization of their linear, tBu side-chain protected, tetra- and pentapeptide precursors followed by side-chain deprotection [29].

2.3 Instrumentation

All experiments were carried out on a capillary electrophoretic analyzer P/ACE MDQ System (Beckman-Coulter, Fullerton, CA, USA). Data were recorded on a personal computer with software supplied by Beckman. The analyses were performed in an uncoated fused-silica capillary 402 mm long with 75 μm ID, 360 μm OD, with outer polyimide coating (Polymicro Technologies, Phoenix, AZ, USA), the detection window was located 300 mm from the injection end. The new capillary was flushed with water, 0.1 M NaOH, again water followed by the BGE, each for 5 min, and then the capillary was conditioned by a 20 min application of high voltage to equilibrate the inner surface and to stabilize the electroosmotic flow. Between runs at the same pH, the capillary was rinsed with BGE for 2 min. Prior to any change of the BGE, the capillary was rinsed with 0.1 M NaOH for 5 min and then repeatedly stabilized by the above-described procedure. The samples were injected with pressure (5–10 mbar for 5–15 s). The samples were dissolved in deionized water, their concentrations ranging from 0.05–0.5 mg \cdot mL $^{-1}$. The BGEs were filtered through a 0.45 μm syringe filter (Millipore, Bedford, MA, USA) before use. The analytes were detected by a spectrophotometric photodiode array detector (190–600 nm) at constant wavelength (206 nm). The separation voltage was 10 kV, with the anode at the inlet side of the capillary. The temperature of the coolant continuously circulating around the capillary was set to 25°C.

3 Results and discussion

3.1 Determination of peptide charge

Effective charges of IOPs were calculated in all BGEs on the basis of Eq. (2) and utilizing the computer program Nabamfo [36] earlier developed to calculate the pH dependence of the effective and specific charges of peptides. The analyzed IOPs contain the following ionogenic groups – all of them, except of cyclic ones, possess an $\alpha\text{-NH}_3^+$ group of the *N*-terminal of the peptide chain and an $\alpha\text{-COOH}$ group of the C-terminal of the peptide chain, most of the peptides contain the hydroxy group of tyrosine and the $\beta\text{-COOH}$ group of aspartic acid. The average values of pK_a of amino acid residues in peptides [2] were used: C-terminal of peptide chain 2.6, hydroxy group of tyrosine 10.0, *N*-terminal of peptide chain 8.0, β -carboxy group of aspartic acid 4.0. The pH dependence of the specific charge (effective charge divided by relative molecular mass) of seven linear IOPs with decreasing number of proline residues at the C-terminus, YDPAP_x, where $x = 6 - 0$, is depicted in Fig. 1. The calculated pH depend-

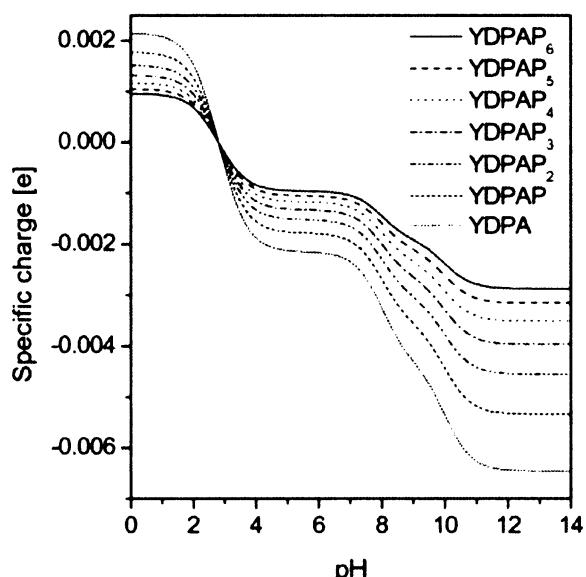


Figure 1. pH dependence of the specific charge (effective charge divided by relative molecular mass) of seven selected IOPs with decreasing number of proline residues at the C-terminus of the peptide chain.

ence of the effective or specific charge is important for finding a pH region of BGE suitable for the separation of the given analytes. The experimental conditions, particularly composition and pH of BGE, used for IOPs separation were selected on the basis of this dependence. They are presented together with other parameters, voltage, currents, and electroosmotic mobilities, in Table 1.

The calculated values of effective charge at four different pH of the BGEs used and relative molecular mass of the whole set of 20 analyzed IOPs are given in Table 2. In acidic BGEs the linear IOPs had positive charges in the

Table 1. CZE conditions

BGE No.	BGE constituents	pH	I ^{a)} (μA)	$\mu_{\text{eo}}^{\text{b)}$ ($10^{-9}\text{m}^2\text{V}^{-1}\text{s}^{-1}$)
I	50 mM Tris, 100 mM H_3PO_4	2.25	69–70	4.98 (0.13) ^{c)}
II	100 mM iminodiacetic acid	2.30	38.5	7.90 (0.09) ^{c)}
III	50 mM iminodiacetic acid	2.40	28.5	11.92 (0.08) ^{c)}
IV	40 mM Tris, 40 mM Tricine	8.1	13–14	57.91 (0.81) ^{c)}

a) Constant voltage, 10 kV

b) Average value from 20 experiments

c) Value in parentheses is the standard deviation.

Table 2. Relative molecular mass and calculated effective charge of 20 IOPs in four different BGEs

Peptide sequence ^{a)}	M_r	Effective charge (e)			
		BGE I	BGE II	BGE III	BGE IV
YDPAP ₆	1044.1	0.778	0.737	0.686	-1.594
YDPAP ₅	950.0	0.778	0.737	0.686	-1.594
YDPAP ₄	852.9	0.778	0.737	0.686	-1.594
YDPAP ₃	755.8	0.778	0.737	0.686	-1.594
YDPAP ₂	658.7	0.778	0.737	0.686	-1.594
YDPAP	561.6	0.778	0.737	0.686	-1.594
YDPA	464.47	0.778	0.737	0.686	-1.594
YDP	393.4	0.778	0.737	0.686	-1.594
YD	296.3	0.778	0.737	0.686	-1.594
c(YDPAP) ₂	1087.2	-0.019	-0.023	-0.029	-2.000
c(YDPAP)	543.6	-0.014	-0.019	-0.029	-1.000
c(YDPA)	446.5	-0.014	-0.019	-0.029	-1.000
YDPAP ₅ ΔP	1044.5	0.778	0.737	0.686	-1.594
YDPAΔP	559.6	0.778	0.737	0.686	-1.594
YDΔPA	462.5	0.778	0.737	0.686	-1.594
YDΔP	391.4	0.778	0.737	0.686	-1.594
DPAP	399.0	0.778	0.737	0.686	-1.594
DPA	301.3	0.778	0.737	0.686	-1.594
DΔPA	299.3	0.778	0.737	0.686	-1.594
HtyDPA	476.6	0.778	0.737	0.686	-1.594

a) Peptide sequences are given in single letter code: Y, tyrosine; D, aspartic acid; P, proline; A, alanine; c, with peptide sequence in parentheses indicates cyclic peptide, ΔP, dehydroproline; Hty, homotyrosine

range of 0.686 to 0.778 of elementary charge and in alkaline BGE they had a negative charge (-1.594 e). The pK_a values of ionogenic groups presented in all linear IOPs were considered to be identical, consequently, the effective charges of all linear IOPs were also identical at given pH of the appropriate BGE. The cyclic derivatives behaved differently in comparison with linear IOPs, in acid BGEs their charge was close to zero, in alkaline BGE their charge was -2.000 or -1.000 e.

3.2 Qualitative analysis of IOPs and determination of purity degree

As follows from the course of pH dependence of specific charge (see Fig. 1), either a strong acid region, pH < 2.5, or alkaline pH region, pH > 8, are suitable for separation of the set of linear IOPs. Consequently, three acidic BGEs (pH 2.25, 2.30, 2.40) and an alkaline BGE (pH 8.1) were tested for peptide analysis. The cyclic analogs were analyzed only in alkaline BGE, in acidic BGE they migrated with an electroosmotic marker as neutral components. The best separation of linear IOPs was achieved in BGE I, composed of 100 mM H_3PO_4 , 50 mM Tris, pH 2.25.

Other BGEs were also applicable for analyses, the obtained peaks of analytes were symmetrical but the resolution was lower.

For full characterization of peptide preparations, especially pharmaceuticals and peptides used in biological tests, it is important to know the content of admixtures, originating from peptide synthesis and purification procedures. The purity degrees of IOPs were determined as the ratio of the corrected peak area of the peptide itself to the sum of corrected areas of all peaks presented in electropherograms; the correction is done with respect to migration velocity of the given peak. The analyzed peptides were mostly well purified by HPLC, the values of purity degrees reached 95–99%. The minor impurities of longer IOPs were sometimes identified as their shorter fragments. The CZE analysis of a highly pure tripeptide, DPA, with a purity degree 98.7% is depicted in Fig. 2A. CZE analysis of a decapeptide, YDPAP₆, (see Fig. 2B) demonstrates an example of a peptide with four admixtures, which were identified as shorter fragments of this decapeptide by their effective mobilities and by addition of the standards of these shorter peptides to the decapeptide sample. The purity degree in this case was only 86.7%.

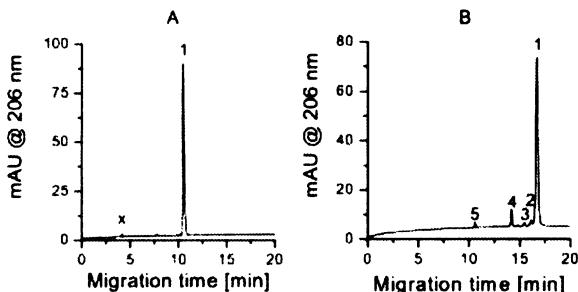


Figure 2. CZE analyses of IOPs. (A) Tripeptide DPA, (B) Decapeptide YDPAP₆. Experiments were carried out in BGE I, 100 mM H_3PO_4 , 50 mM Tris, pH 2.25, at constant voltage $U = 10$ kV; fused-silica capillary, total/effective length 402/300 mm, ID 75 μ m, OD 360 μ m; UV-absorption detection at 206 nm. Peak 1, main synthetic product DPA (A) and YDPAP₆ (B); x, nonidentified admixture; 2–5, identified admixtures (2 = YDPAP₅, 3 = YDPAP₄, 4 = YDPAP₂, 5 = YD).

3.3 Separation of peptide mixtures and determination of peptide mobilities

In addition to qualitative analysis of individual synthetic peptide preparations, the separation of closely related structures of several IOPs was tested. CZE separations of mixtures of IOPs differing in the number of amino acid residues in linear or cyclic chain or in small changes of amino acid composition, e.g., peptides with dehydropro-

line instead of proline, or peptides with homotyrosine instead of tyrosine, were performed in the same four BGEs, as those used for CZE analysis of individual IOPs. The electropherograms of the separation of peptides with decreasing number of proline residues at the C-terminus sequences YDPAP_x, $x = 6 - 0$, and di- and tripeptide, YD, YDP, in four different BGEs are depicted in Fig. 3. The best resolution was obtained in BGE I (pH 2.25, 100 mM H₃PO₄, 50 mM Tris; Fig. 3A). In isolectric buffer, BGE II (100 mM IDAA, pH 2.30), the electroosmotic flow was faster and the peaks were closer to each other and have not been baseline-separated (Fig. 3B). In BGE III (50 mM IDAA, pH 2.40) peptides YD (1) and YDP (2) comigrated (Fig. 3C). And finally, in alkaline BGE IV (see Fig. 3D) the slower IOP were not baseline-separated and faster IOPs had a triangular peak shape because of electromigration dispersion.

Separation of some other closely related peptides from the set of 20 IOPs was achieved at least in some of the above BGEs. The tetrapeptide, YDPA, and its analog with homotyrosine at the N-terminus, HtyDPA, were well separated in alkaline BGEs, the resolution was 1.92. Furthermore, the resolution was in the range of 1.01–1.51 in acid BGEs and the samples were not baseline-separated in BGE II and BGE III. The analogs with a longer peptide chain, decapeptides, YDPAP₆ and YDPAP₅ΔP, and pentapeptides, YDPAP and YDPAPΔP, were completely baseline-separated in all acid BGEs. On the other

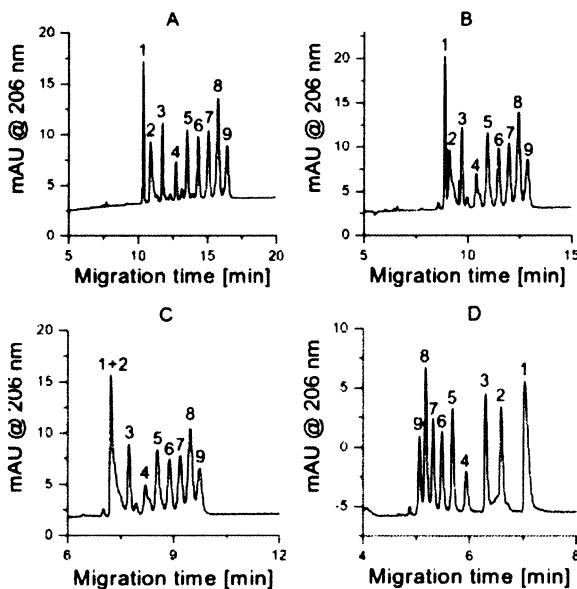


Figure 3. Electropherograms of nine IOPs in (A) BGE I, pH 2.25, (B) BGE II, pH 2.30, (C) BGE III, pH 2.40, and (D) BGE IV, pH 8.1. Other conditions as in Fig. 2. Peak 1, YD; 2, YDP; 3, YDPA; 4, YDPAP; 5, YDPAP₂; 6, YDPAP₃; 7, YDPAP₄; 8, YDPAP₅; 9, YDPAP₆.

hand, shorter analogs, tetrapeptides, YDPA and YDΔPA, and tripeptides, YDP and YDΔP, were separated only partly or comigrated. No derivatives with dehydroproline were separated from their analogs with proline in alkaline BGE. The cyclic IOPs were separated in alkaline BGE.

From CZE analyses of IOPs their effective electrophoretic mobilities were determined. The effective electrophoretic mobility, μ_{ep} , of peptides in BGE was calculated from the migration time of the peptide, t_{mig} , and the migration time of the neutral electroosmotic marker, t_{eo} , according to Eq. (12) with upper signs of the terms in parenthesis for cations and with lower signs of these terms for anions (due to logarithmic plots of mobility, the mobilities of both cations and anions were considered as positive values):

$$\mu_{\text{ep}} = \frac{I_c l_d}{U} \left(\pm \frac{1}{t_{\text{mig}}} \mp \frac{1}{t_{\text{eo}}} \right) \quad (12)$$

where I_c is total capillary length, l_d is the effective capillary length, and U is the applied voltage. The values of effective electrophoretic mobility were determined as averages of two subsequent measurements, which differed by less than 1%. DMSO was used as electroosmotic flow marker.

The real temperature inside the capillary was higher than the temperature of the coolant due to Joule heating. The real temperature in the capillary for each BGE was obtained from the dependence of temperature increase in fused-silica capillary on the input power per unit length of the capillary (see Fig. 4), which was determined by the measurement of the specific electric conductivity of the standard solution of 0.01 mol·dm⁻³ KCl in the capillary at different values of input power as described in [37]. The temperature increase in the fused-silica capillary was 4.5°C for BGE I, 2.1°C for BGE II, 1.5°C for BGE III,

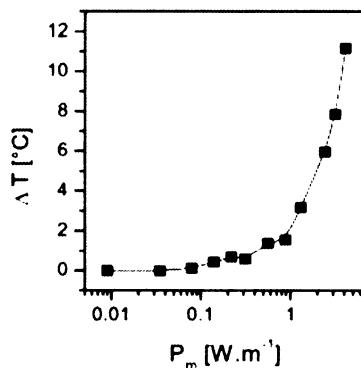


Figure 4. Dependence of temperature increase, ΔT , inside the fused-silica capillary (ID/OD 75/360 μm , effective/total length 30/40.2 cm, thermostated at outer surface to 25°C) on the input power, per unit length of the capillary, P_m .

and 0.7°C for BGE IV. The effective electrophoretic mobility corrected to standard temperature (25°C), $\mu_{\text{ep},25}$, was calculated according to the equation:

$$\mu_{\text{ep},25} = \mu_t [1 - 0.022(t - 25)] \quad (13)$$

where $\mu_{\text{ep},25}$ is the effective electrophoretic mobility at 25°C and μ_t is the effective electrophoretic mobility measured at real temperature t inside the capillary.

The values of effective electrophoretic mobilities of IOPs in different BGEs corrected to standard temperature, 25°C, are presented in Table 3. The effective electrophoretic mobilities measured at real temperature inside the capillary differed by up to 10% in the most conductive BGE (BGE I) in comparison with standard effective electrophoretic mobilities, at 25°C. The increasing values of effective mobilities of IOPs from BGE I (pH 2.25) to BGE III (pH 2.40) in spite of increasing pH of these BGEs in the same order can be explained by the fact that the increase of pH is rather small (0.05 – 0.15 pH unit) and the mobilities are not corrected to the same ionic strength. A significantly higher ionic strength of the BGE I than that of BGEs II and III is the cause of the lower effective mobilities in BGE I than in BGEs II and III, respectively. In addition, the different capabilities of the BGE counterions, phosphate in BGE I and iminodiacetate in BGEs II and III, to form

Table 3. Effective electrophoretic mobilities, μ_{ep} , corrected to standard temperature, 25°C, of 20 analyzed IOPs in four BGEs

Peptide sequence ^{a)}	$\mu_{\text{ep}} (10^{-9} \text{ m}^2 \text{V}^{-1} \text{s}^{-1})$			
	BGE I	BGE II	BGE III	BGE IV
YDPAP ₆	6.66	7.83	8.44	14.19
YDPAP ₅	7.16	8.34	9.00	15.07
YDPAP ₄	7.73	8.94	9.63	16.07
YDPAP ₃	8.31	9.64	10.37	17.19
YDPAP ₂	9.13	10.46	11.24	18.45
YDPAP	9.95	11.36	12.17	19.97
YDPA	11.26	12.70	13.62	21.88
YDP	12.33	14.01	15.10	23.28
YD	13.38	14.52	15.40	25.18
c(YDPAP) ₂	0	0	0	18.50
c(YDPAP)	0	0	0	14.01
c(YDPA)	0	0	0	15.21
YDPAP ₅ ΔP	6.18	7.40	7.80	14.43
YDPAΔP	9.34	10.55	11.05	20.17
YDΔPA	11.38	13.00	13.83	22.18
YDΔP	11.79	13.89	14.84	23.82
DPAP	10.95	12.19	12.70	20.76
DPA	12.98	14.17	14.82	23.83
DΔPA	12.86	14.17	14.82	25.71
HtYDPA	10.89	12.38	13.11	20.15

a) See comment in Table 2.

ionic pairs with cationic peptides may also contribute to the different effective mobilities of peptides. The difference of effective mobilities of IOPs in BGE II and III is smaller than that in BGE I and II and it can be explained by the lower ionic strength of BGE III than BGE II.

3.4 Testing semiempirical models

Several various models of the correlations between the effective electrophoretic mobility of peptides and their effective charge and size parameters were suggested (see Section 1.2.3) but none of them was found to be generally applicable to the unambiguous description of migration behavior of different peptide sets and for prediction of peptide structure. In the current work, we have tested and compared seven semiempirical models of the correlation between effective electrophoretic mobility of IOPs and their charge/size ratio: the rigid spherical model, Eq. (4) [8], the classical linear polymer model, Eq. (5) [33], Offord's model, Eq. (3) [12], Grossman's model, Eq. (9) [17], Cifuentes's and Poppe's model, Eq. (10) [18], Kim's model, Eq. (7) [20], and Cross's model, Eq. (6) [16]. The set of 20 IOPs was tested in the range of relative molecular masses from 299.3 to 1087.2 and sizes from 2 to 10 amino acid residues. The graphs of the correlations of the above semiempirical models applied to all 20 IOPs are depicted in Fig. 5. As expected, none of the models gave the full agreement between theory and experimental data for the whole set of IOPs. The values of the slope and correlation coefficient of the linear plot $\log(\mu_{\text{ep}}/q)$ versus $\log M_r$ (Fig. 5A) suggested us the most suitable semiempirical model for each electrolyte; parameters of this linear regression are given in Table 4. The rigid spherical model (Fig. 5B) fitted best in acidic BGE II (curve 2) with $R = 0.972$ and BGE III (curve 3) with $R = 0.958$. However, the classical linear polymer model ($R = 0.972$) was found best in alkaline BGE IV (Fig. 5C, curve 4), alternatively the Offord's model (Fig. 5D, curve 1), where $R = 0.963$, the classical polymer model with $R = 0.981$ (Fig. 5C, curve 1) suited to acidic BGE I. On the other hand, Grossman's model (Fig. 5E) with $R = 0.888$ – 0.936, Cifuentes's and Poppe's model (Fig. 5F) with $R = 0.911$ – 0.965 as well as Kim's model (Fig. 5G) with $R = 0.933$ – 0.965 were found less suitable for our set of IOPs. As follows from the data presented in Fig. 5, none of the models was able to describe the correlation between mobility and charge-to-size ratio with high precision for the whole set of IOPs. Therefore, the peptides were divided in smaller groups with more related structures.

The number of peptides in the set was reduced and just nine analogs with decreasing number of proline residues at C-terminal sequences, from decapeptide, YDPAP₆, to

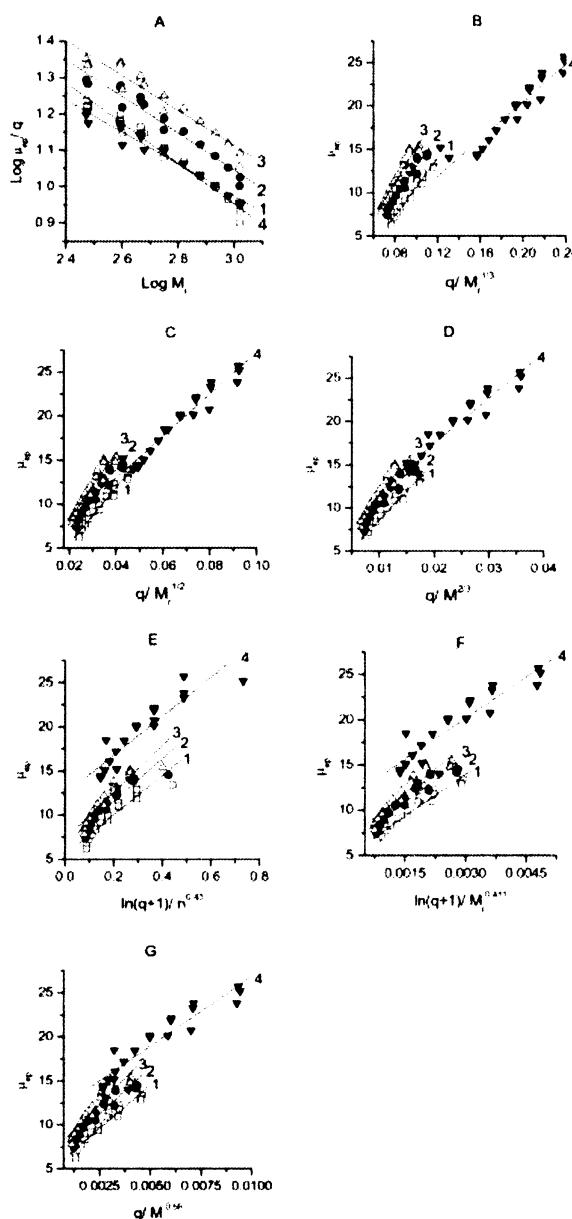


Figure 5. Semiempirical models of the correlation between effective mobility, μ_{ep} , and peptide charge, q , and size (expressed by relative molecular mass, M_r , or by number of amino acid residues in peptide chain, n) applied to all IOPs and their fragments in four different BGEs (1, BGE I 50 mM Tris, 100 mM H_3PO_4 , pH 2.25; 2, BGE II, 100 mM iminodiacetic acid, pH 2.30; 3, BGE III, 50 mM iminodiacetic acid, pH 2.40; 4, BGE IV, 40 mM Tris, 40 mM Tricine, pH 8.1). (A) Cross's model, Eq. (6), (B) rigid spherical model, Eq. (4), (C) classical linear polymer model, Eq. (5), (D) Offord's model, Eq. (3), (E) Grossman's model, Eq. (9), (F) Cifuentes's and Poppe's model, Eq. (10), (G) Kim's model, Eq. (7). The mobilities were taken from Table 3, effective charges and relative molecular masses from Table 2. Other conditions as in Fig. 2.

Table 4. Parameters of linear regression $\log \mu_{\text{ep}}/q$ versus $\log M_r$ for the whole set of twenty analyzed IOPs and for the subset of seven linear IOPs with decreasing number of proline residues at the C-terminus, YDPAP_x ($x = 6 - 0$), in four different BGEs

BGE No.	Set of 20 IOPs			Subset of linear IOPs, YDPAP_x ($x = 6 - 0$)		
	<i>k</i>	<i>b</i>	<i>R</i>	<i>k</i>	<i>b</i>	<i>R</i>
I	-0.5567	2.6217	0.9821	-0.6376	2.8626	0.9992
II	-0.5062	2.5641	0.9738	-0.5935	2.8213	0.9993
III	-0.4813	2.5568	0.9546	-0.5831	2.8547	0.9993
IV	-0.4410	2.2970	0.9792	-0.5309	2.5573	0.9988

k, slope of curve; *b*, intercept; *R*, coefficient of correlation

dipeptide, YD, were selected. Better values of correlation coefficients were obtained but from the regression analysis it was evident, that di- and tripeptide did not fit well in this linear part of the graphs. Due to nonlinearity of the correlations between effective mobility and charge-to-size ratio of these two short peptides and with respect to their low potential to form more organized secondary structures, the set of IOPs was further reduced to the series of seven peptides with linearly decreasing number of proline residues at the C-terminal of the peptide chain, from decapeptide, YDPAP_6 , to tetrapeptide, YDPA, with constant decrement of the relative molecular mass, 97.1. The graphs of semiempirical models of correlations of electrophoretic mobility and effective charge and size parameters applied to this series of seven linear IOPs are depicted in Fig. 6. As evident from the plots, the correlation has improved significantly – the experimental data fitted well in the linear parts of the graphs, indicating similar migration behavior and structure of this homologous series of IOP.

3.5 Predicted probable structure of peptides

Fitting the experimental data into the graphs of different semiempirical models helped us to solve the question with the secondary structure of IOPs. As expected, the group of peptides in the whole range of relative molecular mass and with different number of amino acid residues tested, i.e., from dipeptides to decapeptides, did not exhibit the same structure. From the series of the homologous nine IOPs with decreasing number of proline residues at the C-terminus, YDPAP_6 – YD, the smallest two peptides, YD and YDP, behaved differently, and did not fit to the linear part of regression plot of any model.

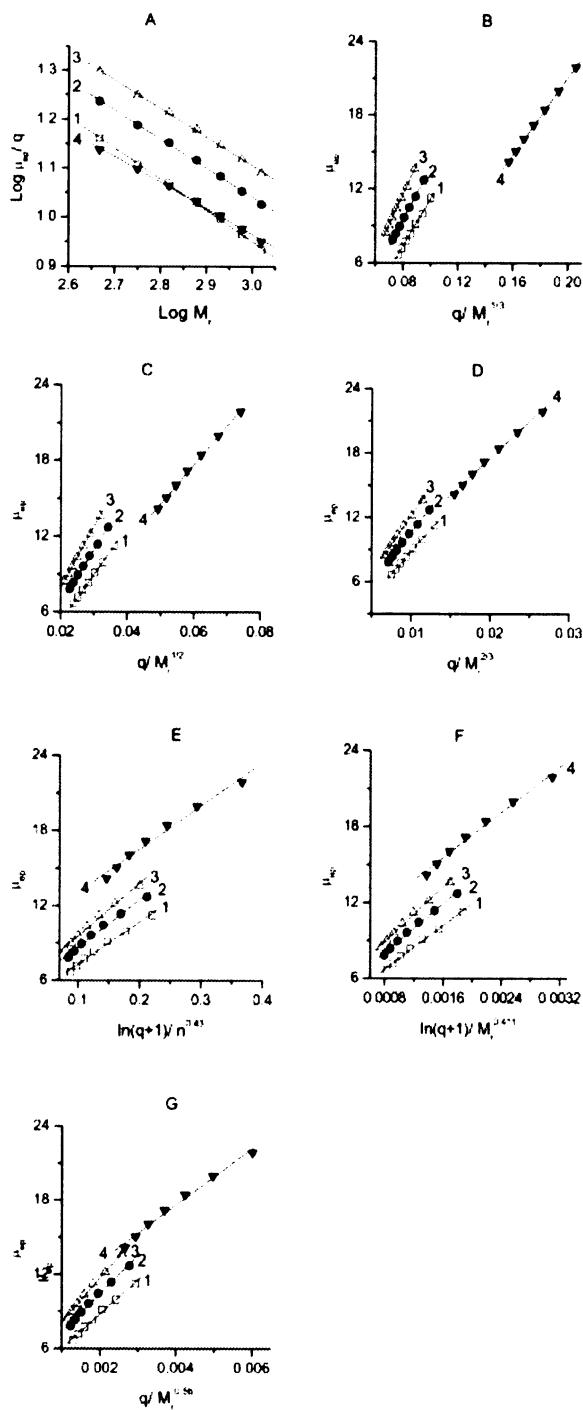


Figure 6. Semiempirical models of the correlation between effective mobility, μ_{ep} , and peptide charge, q , and size (expressed by relative molecular mass, M_r , or by number of amino acid residues in peptide chain, n) applied to seven IOPs with decreasing number of proline residues at the C-terminus, YDPAP₆–YDPA, in four different BGEs. BGEs, models, and other conditions as in Fig. 5.

Cross's plot seems to be the most suitable for predicting the peptide structure, because it directly provides the value of exponent k in the μ_{ep} versus q/M_r^k plot as the slope of the line expressed by Eq. (6). Parameters of linear regression (slope of curve, intercept, coefficient of correlation) of the Cross's plot, $\log(\mu_{\text{ep}}/q)$ versus $\log M_r$, for the whole set of 20 IOP and for the subset of seven linear IOPs are presented in Table 4. As follows from the data presented in this table for the subset of seven linear IOPs, where also high values of correlation coefficients were obtained, the exponent k of the above dependence in alkaline BGE was close to 1/2, i.e., migration of this set of peptides can be best described by the classical linear polymer model. Consequently, the most probable structure of these peptides in alkaline solution is random coil. In acid BGE I the exponent k was close to 2/3, which means that the migration of these peptides fits best to Offord's model and their molecules have the shape of wide thin discs [8]. In the two isoelectric buffers, BGE II and BGE III, differing only in ionic strength, the exponents k were almost identical, with the values ranging from 0.5–0.6, which indicate the shape of random coils. The difference of exponent k for BGE I ($k = 0.64$) and for BGE II ($k = 0.58$) may reflect some differences in the peptide structure caused rather by the change of the ionic strength and quality of the peptide counterions (iminodiacetates instead of phosphates) than by the relatively very small change of pH. However, with respect to small differences in correlation coefficients of different models of correlation between electrophoretic mobility and charge and size presented in Fig. 6, the predicted structures should be considered as the most probable but not determined quite unambiguously.

4 Concluding remarks

CZE proved to be a suitable technique for analysis and physicochemical characterization of IOPs. From the CZE analyses not only the purity degree of synthetic IOPs could be obtained but also their effective electrophoretic mobilities. The probable secondary structure of IOPs in solutions of different composition could be predicted as the result of testing various models of the correlations between electrophoretic mobility of peptides and their effective charge and size.

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IV

Separation and investigation of structure-mobility relationship of gonadotropin-releasing hormones by capillary zone electrophoresis in conventional and isoelectric acidic background electrolytes

Veronika Šolínová ^a, Václav Kašička ^{a,*}, Petra Sázelová ^a, Tomislav Barth ^a, Ivan Mikšík ^b

^a Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic,
Flemingovo 2, 166 10 Prague 6, Czech Republic

^b Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083,
142 20 Prague 4, Czech Republic

*Corresponding author

Dr. Václav Kašička

Institute of Organic Chemistry and Biochemistry

Academy of Sciences of the Czech Republic

Flemingovo 2

166 10 Prague 6

Czech Republic

Phone: +420-220-183-239

Fax: +420-220-183-592

e-mail: kasicka@uochb.cas.cz

Abstract

Capillary zone electrophoresis (CZE) has been applied to qualitative and quantitative analysis, separation and physicochemical characterization of synthetic gonadotropin-releasing hormones (GnRHs) and their analogs and fragments. Structurally related peptides were separated in conventional and isoelectric acidic background electrolytes (BGEs), pH 2.18-2.50. Best separation was achieved in isoelectric BGE composed of 200 mM iminodiacetic acid, pH 2.32. The effective electrophoretic mobilities, m_{ep} , of GnRHs in five BGEs were determined and several semiempirical models correlating effective mobility with charge, q , and relative molecular mass, M_r , (m_{ep} versus q/M_r^k) were tested to describe the migration behavior of GnRHs in CZE. None of the models was found to be definitively applicable for the set of ten GnRHs differing in size (tetrapeptide – decapeptide) and positive charge (0.91 – 3.00 elementary charges). However, high coefficient of correlation, 0.995-0.999, was found for the GnRHs, for the dependence of m_{ep} on q/M_r^k with k (related to molecular shape) close to 0.5 in all five acidic BGEs. From these dependences the probable structure of GnRHs in solution was predicted as random coil.

Keywords:

Capillary electrophoresis; GnRH; LHRH; Peptides; Effective electrophoretic mobility; Structure-mobility relationship

1. Introduction

Gonadotropin-releasing hormone (GnRH) is present in different biological species such as human, pig, lamb, chicken, sea bream and salmon with small modifications in its peptide sequence. Human GnRH, hGnRH, alternatively termed luteinizing hormone-releasing hormone (LHRH), is physiologically important decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂), which is synthesized in neurosecretory cells within the hypothalamus and regulates reproductive functions and maintenance of secondary sex characteristics in males and females. LHRH agonists are used for the treatment of hormone-dependent breast and prostate cancers [1]. In reproductive medicine, they serve to prevent a premature LH surge prior to stimulation of ovulation. Synthetic hGnRH is produced under the name gonadorelin. Several commercially available analogs are used as drugs. Buserelin, [Des-Gly¹⁰,D-Ser(tBu)⁶,Pro-NHEt⁹]hGnRH, and triptorelin, [D-Trp⁶]hGnRH, are used in treatment of prostate cancer, deslorelin, [Des-Gly¹⁰,D-Trp⁶,Pro-NHEt⁹]hGnRH, for treatment of true precocious puberty, goserelin, [D-Ser(tBu)⁶,Azagly¹⁰]hGnRH for treatment of advanced breast cancer and nafarelin, [D-2-Nal⁶]hGnRH, in treatment of endometriosis.

Synthetic GnRHs and their analogs and fragments are frequently used as model analytes, usually in mixtures with other peptides, to demonstrate new methodology and/or instrumentation developments and optimization of separation conditions for analysis and separation of peptides by capillary electromigration methods [2]. Successful combination of capillary electrophoresis (CE) with mass spectrometry (MS) detection without make-up flow or nebulizing gas was demonstrated by analysis of gonadorelin with sensitivity on the level of immunoassay [3]. Home-made on-line preconcentration CE was optimized for the purity control of synthetic biologically active peptides including GnRH in BGE composed of 25 mM K₂HPO₄, pH 3.5 [4]. Membrane preconcentration CE-MS/MS was appropriately constructed for sequencing biologically active peptides at the sub-100 femtomole level [5]. The analysis of nine model peptides was performed in 2 mM ammonium acetate and 1 % v/v acetic acid. Successful application of fused silica (FS) capillary modified by positively charged alkylaminosilyl monomer to capillary zone electrophoresis (CZE) and capillary electrochromatographic (CEC) separations of the mixture of peptides and proteins was demonstrated using UV-absorption and MS detection [6]. Rapid separation of five peptides including GnRH was achieved in 5 mM acetic acid in 50% v/v MeCN. A new CE-nanoflow electrospray ionization (ESI) interface, where separation column, an electrical porous junction and spray tip were integrated on single FS capillary, was found as a suitable device for analysis and separation of nine peptides including GnRH and proteins in 1 M acetic acid, pH 2.4 [7]. Human GnRH and its four analogs, [D-Ala⁶]GnRH, [D-Lys⁶]GnRH, [D-Phe²,D-Ala⁶]GnRH and [Gly-OH¹⁰]GnRH were analyzed in

capillary derivatized with 3-(aminopropyl)trimethoxysilane in 0.01 M acetic acid, pH 3.5, to demonstrate suitability of CE-ESI-MS using high performance magnetic sector MS for identification of toxins in snake venom [8]. For determination of side products of buserelin synthesis, CE with field-amplified sample injection was used [9]. Optimal separation conditions were found in acidic BGEs with pH less than 3.5. An off-line coupling of CE and MALDI-MS was successfully applied for the analysis of four standard peptides, proteins and real tear fluid in 50 mM ammonium acetate buffer, pH 7.4 [10]. According to the known or established characteristics, such as dissociation constants of ionogenic groups of therapeutic peptides optimal separation conditions were determined for set of seven peptides including buserelin and triptorelin for CE [11]. The conditions were tested in a wide pH range 2-12; the best separation was achieved at pH 2.85, in BGE composed of 50 mM acetic acid and 50 mM formic acid, pH adjusted by NH₄OH. The same set of peptides was successfully applied for separation and characterization in hydroorganic mixture of formic acid, acetic acid and 2-propanol by CE-ESI-MS with commercial [12] and home-made graphite coated sheath-flow interface [13] and as well for comparison of predicted and experimentally obtained resolution, electrophoretic mobility and retention factor in CE and HPLC [14]. Complete separation of gonadorelin and its five analogs was provided by RP-HPLC in mobile phase consisting of MeCN and phosphate buffer, pH 2.5, by CE in phosphate or borate buffer and as well by micellar electrokinetic chromatography (MEKC) with the same BGE with addition of CTAB (cetyltrimethylammonium bromide), CHAPS (3-[(3-choloamidopropyl)dimethylammonio]-1-propanesulfonate) and Triton X-100 as micellar constituents [15]. Alternative detection technique to UV-absorption, contactless conductivity detection was employed for the analysis of mixture of nine peptides including GnRH in phosphate buffer, pH 2.5 [16]. Detection limits at the µM level in combination with good resolution were shown. Native GnRHs are generally used to study biological regulatory processes such as kinetics and mechanism of their action. A rapid CE assay for measuring the stability of human and salmon GnRH in the presence of intestinal enzymes was developed and validated [17]. The analysis was performed in acetic acid-based BGE, pH 4.0, and applied to the stability of GnRH analogs in salmon intestinal digest. Determination of gonadorelin in plasma by on-capillary preconcentration CE system was demonstrated [18]. For suppressing adsorption on the wall, the capillary had cationic coating and for increasing sensitivity and selectivity MS detection was used. CE was also applied for determination of proteins during the investigation of influence of GnRH in human ovarian fluid and sera in pre-treatment for in vitro fertilization [19]. The aim of this work was to perform qualitative and quantitative analysis of synthetic preparations of human, salmon and chicken GnRH, and their analogs and fragments, by CZE in acidic conventional and isoelectric BGEs. Suitable experimental conditions should be developed

for CZE separation of the mixtures of these structurally related peptides. In addition to the purity degree also some physicochemical characteristics of the analyzed peptides, such as effective electrophoretic mobilities of GnRHs at standard temperature, 25°C, should be determined, and different models of the dependence of mobility of GnRHs on their charge and size should be tested in order to predict the probable structure of GnRHs in solution.

2. Theory

Models of correlations between peptide mobility and their charge and size

Several semiempirical models correlating effective electrophoretic mobilities of peptides, m_{ep} , with their effective charge, q , and molecular size expressed as relative molecular mass, M_r , or number of amino acids in polypeptide chain, n , respectively, have been developed. The models are based on Stokes law, describing the motion of a particle in liquid medium, and on the action of electric field force on charged molecule.

Offord [20] has firstly quantitatively described this relationship for the series of oligo- and polypeptides separated by paper electrophoresis:

$$m_{ep} = \frac{k_1 \cdot q}{M_r^{2/3}} \quad (1)$$

where k_1 is a constant of proportionality. This relationship was applied by some other authors, Issaq et al. [21], Rickard et al. [22], Basak and Ladisch [23], Tessier et al. [24] and Jalali-Heravi et al. [25] for peptides separated in free solution by CZE.

For rigid spherically shaped molecules in low ionic strength buffers the following relationship was gained by Grossman [26]:

$$m_{ep} = \frac{k_1 \cdot q}{M_r^{1/3}} \quad (2)$$

Another semiempirical model was suggested for the synthetic polymers with cylindrical or rod-shaped molecules [27]:

$$m_{ep} = \frac{k_1 \cdot q}{M_r^{1/2}} \quad (3)$$

Cross [28] adapted Offord's relation to logarithmic form:

$$\log\left(\frac{m_{ep}}{q}\right) = k \cdot \log M_r \quad (4)$$

where k is the exponent of M_r in non-logarithmic relation, which can be determined as a slope of this dependence.

Another formula has been proposed by Kim [29] in which set of peptides originating from enzyme digests of proteins fitted well. The value of exponent was close to that used in semiempirical model for polymers:

$$m_{ep} = \frac{k_1 \cdot q}{M_r^{0.56}} \quad (5)$$

Compton [30] combined two relationships for small (2) and large (1) molecules and obtained the relation:

$$m_{ep} = \frac{k_1 \cdot q}{k_2 \cdot M_r^{1/3} + k_3 \cdot M_r^{2/3}} \quad (6)$$

where the first term in the denominator, $M_r^{1/3}$, represents smaller molecules, and the second term, $M_r^{2/3}$, larger molecules, both in low ionic strength buffers.

In all these cases relative molecular mass was used as size parameter. Other possibility is to express the molecular size by the number of amino acid residues in peptide chain, n . This approach was used by Grossman et al. [31], who derived the following relation for dependence of effective mobility on charge and size:

$$m_{ep} = \frac{k_2 \cdot \ln(q+1)}{n^{0.43}} \quad (7)$$

Cifuentes and Poppe [32] firstly published the modification of the Grossman's classical linear model, where logarithmic dependence of mobility on charge expressed electrostatic charge suppression in highly charged peptides:

$$m_{ep} = \frac{k_2 \cdot \log(1+k_1 q)}{M_r^{0.411}} \quad (8)$$

The same equation, only with different exponent of M_r , 0.435 instead of 0.411, was derived by Adamson [33].

Sanz-Nebot [34] tested relationships (9) with q compensated for electrostatic charge suppression with highly charged peptides and the relationships of classical semiempirical models (1), (2) and (3), compared their results, and found better correlation for the formula:

$$m_{ep} = \frac{k_2 \cdot \ln(k_1 q + 1)}{M_r^k} \quad (9)$$

where $k = 1/3$ for small molecules, $k = 1/2$ for medium size polymers, $k = 2/3$ for large molecules, respectively.

These semiempirical models can be used for prediction of possible structure of the peptides and proteins in a free solution. The structure of the molecule is derived from the relationship between frictional coefficient or electrophoretic mobility, respectively, and the exponent of the relative molecular mass or alternatively exponent of the number of amino acid residues in peptide [26].

Each type of correlation corresponds to the specific shape of molecule in a free solution. For determination of molecular conformations it is very important to find the best way in which electrophoretic mobility in a free solution is related to molecular size. Peptides form more organized secondary structures, which are strongly dependent on the medium [35]. The structure is dependent on solvent, ionic strength and pH of BGE [36, 37]. At the high molecular mass range as long-chain polymers, polypeptides may form different types of ordered structures such as α -helical or β -sheet conformation [32, 38]. The peptide structures help to predict shapes of proteins in free solution [39] and also in peptide mapping of proteins [26]. The data on secondary peptide structures derived from relation between electrophoretic mobility and charge to size ratio, are advantageously utilized in the structure-activity studies of peptide hormones.

3. Experimental

3.1 Chemicals

All chemicals used were of analytical reagent grade. Iminodiacetic acid (IDAA) was obtained from Bachem (Bubendorf, Switzerland), phosphoric and acetic acids were obtained from Lachema (Brno, Czech Republic) and Tris (tris(hydroxymethyl)aminomethane) was supplied by Serva (Heidelberg, Germany). Isophorone (3,5,5-trimethyl-2-cyclohexen-1-one) was supplied by Fluka (Buchs, Switzerland).

3.2 Peptides

The list of analyzed peptides and their abbreviations, sequences and relative molecular masses, M_r , are presented in Table 1. The oligopeptide fragments of human GnRH, YGLRPG-NH₂, LRPG-NH₂, (pGlu)HWSTGLRPG-NH₂ and (pGlu)HWS, were purchased from Sigma (St. Louis, MO, USA). The other analogs and fragments of GnRHs were prepared by solid phase synthesis in the Institute of Organic Chemistry and Biochemistry.

3.3 Instrumentation

The capillary electrophoretic experiments were carried out in commercial P/ACE MDQ System (Beckman-Coulter, Fullerton, CA, USA), data acquisition and evaluation were performed using the software P/ACE System MDQ, version Karat supplied by Beckman. The apparatus was equipped with the internally non-coated FS capillary with outer polyimide coating, total/effective length 39.0/28.8 cm, I.D./O.D. 50/375 μm (Polymicro Technologies, Phoenix, AR, USA). The analytes were detected by UV-Vis absorption spectrophotometric

photodiode array detector (190-600 nm) set at constant wavelength 206 nm. The temperature of capillary liquid coolant was set at 25°C.

The new capillary was gradually flushed with water, 1 M NaOH, water and BGE, each wash for 10 min. Finally, the capillary was conditioned by a 20 min application of the high voltage to equilibrate the inner surface with BGE and to stabilize electroosmotic flow. Between runs under the same conditions, the capillary was rinsed with the BGE for 2 minutes. Before a change of the BGE the capillary was rinsed with 0.1 M NaOH for 5 min and then repeatedly stabilized. The samples were injected with pressure 6.9-13.8 mbar for 5-15 s. The samples were dissolved in deionized water and their concentrations were in the range 0.6-1.3 mg/ml. The BGEs were filtered through a 0.45- μ m syringe filter (Millipore, Bedford, MA, USA) before use. The list of composition and pH of BGEs, separation voltage and electric current are presented in Table 2.

4. Results and discussion

4.1 Selection of separation conditions and determination of peptide charge

The strategy for the rational selection of experimental conditions for CZE analysis and separations of GnRHs and their analogs and fragments followed the general rules of selection of suitable CZE separation conditions [40] and took into account the specific properties of these peptides resulting from their structure. The selection of the composition of the BGEs includes the type and concentration of buffer components and pH, and it also takes into account the requests for solubility and chemical stability of analyzed peptides [41, 42]. Effective charges of peptides are strongly dependent on pH and pK_a of ionogenic groups of amino acid residues present in peptide chain. The analyzed peptides, see Table 1, contain several types of ionogenic groups; all peptides, except fragments of hGnRH 4-10 and 7-10, possess imidazolyl group of the histidine (average pK_a 6.3), and all peptides, except fragments of hGnRH 1-4 and 7-10, contain phenol group of tyrosine (average pK_a 10.4), all hGnRHs except [Des-Arg-Pro-Gly-NH₂]hGnRH and fragment of hGnRH 1-4, possess guanidinyl group of arginine (average pK_a 11.3), three peptides (fragments of hGnRH 4-10 and 7-10 and [Des-pGlu¹-D-Orn⁶]sGnRH) possess α -amino group of the N-terminus of the peptide chain (average pK_a 8.1), five peptides ([Des-Arg-Pro-Gly-NH₂]hGnRH, [Des-Pro-Gly-NH₂] hGnRH, fragment of hGnRH 1-4, [Des-Gly-NH₂]sGnRH and [Des-Gly-NH₂]cGnRH II) contain α -carboxyl group of the C-terminus of the peptide chain (average pK_a 3.5) and salmon fragment of its analog [Des-pGlu¹-D-Orn⁶]sGnRH contains amino group of ornithine (average pK_a 9.9). The relative molecular mass of analyzed peptides was in the range 440.6 – 1262.5, see Table 1.

One of the most important parameters for selection of suitable experimental conditions for CZE analysis and separation of peptides is the pH dependence of their effective and specific charges, since the electrophoretic mobility of peptides is directly proportional to their effective charge. For that reason the dependence of effective charge and specific charge (effective charges divided by relative molecular mass) of all peptides to be analyzed has been calculated by the earlier developed computer program Nabamfo [43] using the above given average values of pK_a of ionogenic groups. From the course of the pH dependence of the specific charge of peptides to be analyzed and separated (see Fig. 1) it follows, that these peptides can be analyzed as cations at $\text{pH} < 7$ and as anions mostly at $\text{pH} > 10$. Consequently, the strongly acidic conventional and isoelectric BGEs (pH 2.18-2.50) were selected for CZE analyses and separations of the above peptides. Full composition and pH of the used BGEs together with the separation voltage and electric currents are presented in Table 2. In all used BGEs, the GnRHs had positive charges in the range from 0.91 to 3.00 of elementary charge. The calculated values of effective charge at five selected pHs of the BGEs used are given in Table 3. With respect to their relatively hydrophilic character all analyzed peptides were dissolved in deionized water, which brought an advantage that the same sample solution could be applied to CZE analyses in different BGEs, and in addition the electric-field-enhanced concentrating effect was utilized to concentrate the diluted peptide solutions.

4.2 Qualitative analysis of GnRHs and determination of purity degree

For full characterization of peptide preparations, especially pharmaceuticals and peptides used in biological tests it is important to know the content of admixtures, originating from peptide synthesis and purification procedures. The purity degrees of GnRHs were determined as the ratio of the corrected peak area of peptide itself to the sum of corrected areas of all peaks presented on electrophoregrams, corrected peak area is peak area corrected with respect to migration velocity of the given peak. The qualitative analyses of GnRHs were carried out in BGE II, 100 mM H_3PO_4 and 50 mM Tris, pH 2.25, peptides with purity degree lower than 75 % were not used for further experiments. The analyzed peptides were mostly well purified by HPLC, the values of purity degrees reached 88-99 %. The CZE analysis of highly pure tetrapeptide, fragment of hGnRH 7-10, with purity degree 96.2 % is depicted in Fig. 2A. CZE analysis of nonapeptide, [Des-Gly-NH₂]cGnRH II, (see Fig. 2B) demonstrates example of impure peptide with four major and a lot of minor admixtures, which could not be used for further experiments. The purity degree in this case was only 55.1 %.

4.3 Separation of structurally related peptides and determination of peptide mobilities

In addition to qualitative analysis of individual synthetic peptide preparations, the separation of closely related GnRHs and their analogs and fragments was tested. These separations are important from the point of view of simultaneous analysis of the whole molecules of GnRHs and their degradation products. The suitable separation conditions for the separations were derived from the course of the pH dependence of specific charge of these peptides (see Fig. 1), and from the experience obtained in the analysis of individual peptides by CZE in different BGEs. Three mixtures of four GnRHs were separated in five BGEs in pH range 2.18-2.50. The mixture I contained hGnRH and its fragments, mixture II contained GnRH decapeptides of three species and in mixture III sGnRH, its two fragments and hGnRH fragment were present. The efficiency and resolution of separation in conventional and isoelectric BGEs as well as composition of mixtures are given in Table 4. 0.5 M acetic acid, pH 2.5, was the best BGE for fast separation, but the efficiency and the resolution were the worst in comparison with other BGEs. The shortest time of analyses in range 3.4-4.5 minutes were achieved in 500 mM acetic acid, pH 2.5, the electrophoregrams are depicted in Fig. 3C for mixture I and Fig. 3F for mixture III. The best efficiency of separation was achieved in isoelectric BGE, BGE IV, 200 mM IDAA, pH 2.32, with theoretical plates number in range $0.5\text{--}1.6 \cdot 10^6/\text{m}$, CZE separations of mixture I are shown in Fig. 3B and separations of mixture III in Fig. 3E. The resolution of separations in BGE IV and BGE II (100 mM H₃PO₄, 50 mM Tris, pH 2.25) was comparable as can be seen from CZE separations of mixture I in Figs. 3B and 3A. [Des-Gly-NH₂]sGnRH and sGnRH in mixture III comigrated in a single peak in BGE II (see Fig. 3D), but in BGE IV and BGE V the partial resolution of these two peptides was achieved, as shown in Fig. 3E and 3F. As follows from the above results it is clear that in some cases better separation can be achieved with isoelectric buffer iminodiacetic acid than with classical phosphate buffer or acetic acid-based BGEs.

From CZE separations of GnRHs their effective electrophoretic mobilities were determined. The effective electrophoretic mobility, m_{ep} , of peptide in BGE was calculated from migration time of the peptide, t_{mig} , and migration time of neutral electroosmotic marker, t_{eo} , according to equation:

$$m_{ep} = \frac{l_l l_{ef}}{U} \left(\frac{1}{t_{mig}} - \frac{1}{t_{eo}} \right) \quad (11)$$

where l_l is total capillary length, l_{ef} is effective capillary length, U is the applied separation voltage. In CZE analyses in highly acidic BGEs with very low electroosmotic flow (EOF) due to the suppressed dissociation of silanol groups of FS capillary the effective electrophoretic mobility was determined using the pressure accelerated measurement of EOF according to [44]. The effective electrophoretic mobilities were determined as averages of two subsequent measurements, the values of which differed less than 1%.

The real average temperature inside the capillary was higher than the temperature of the capillary coolant due to Joule heating. The real temperature in the capillary for each BGE was obtained from the experimentally determined dependence of temperature increase inside the FS capillary capillary on the input power per unit length of the capillary as described in [45]. The average temperature increase inside the capillary was 3.6°C for BGEs I and V, 4.8°C for BGE II, 4.5°C for BGE III and 3.9°C for BGE IV. The values of effective electrophoretic mobilities of GnRHs in different BGEs corrected to standard temperature, 25°C, are presented in Table 3. The effective electrophoretic mobilities determined at real temperature inside the capillary differed about 8-11 % in all BGEs in comparison with standard effective electrophoretic mobilities, at 25°C. Relatively large differences of effective mobilities of GnRHs in different BGEs in spite narrow pH range (0.07 – 0.32 pH unit) are caused by different ionic strength of individual BGEs. For that reason the effective mobilities of GnRHs are minimal in BGE II, the ionic strength of which is maximal, and the mobilities are maximal in BGE V, the ionic strength of which is minimal. In addition, the different capabilities of the BGE counterions, acetate in BGE I and V, phosphate in BGE II and iminodiacetate in BGEs III and IV, to form even very weak ionic pairs with cationic peptides may also contribute to the different effective mobilities of peptides.

4.4 Semiempirical models

In the current work we have tested and compared six semiempirical models of the correlation between effective electrophoretic mobility of GnRHs and their charge/size ratio – rigid spherical model, Eq. (2) [26], classical linear polymer model, Eq. (3) [27], Offord's model, Eq. (1) [20], Grossman's model, Eq. (7) [31], Cifuentes's and Poppe's model, Eq. (8) [32] and Cross's model, Eq. (4) [28], which were utilized for prediction of secondary or tertiary structure of peptides and proteins in solution [26]. The graphs of the correlations of the above semiempirical models applied to ten GnRHs are depicted in Fig. 4. According to the figures it is evident that the best model was classical linear polymer model, Eq. (3), the correlation coefficients, R, reached values 0.995-0.999 in all five BGEs. Offord's model was also suitable for description of peptides in solution for four BGE, BGE I-IV ($R = 0.994\text{-}0.998$), and rigid spherical model could be alternative model for BGE V. Grossman's model and Cifuentes's and Poppe's model did not fit for acetic acid as BGE ($R = 0.990$ and 0.984). The most suitable model, classical linear polymer model, is in agreement with model predicted for set of similar peptides containing triptorelin and buserelin in pH 2.85 [46] and our former work concerning on insect oostatic peptides in pH 2.30 and 2.40 [47].

4.5 Estimation of probable structure of peptide

Fitting the experimental data into the graphs of different semiempirical models helped us to predict the secondary structure of GnRHs. Cross's plot seems to be the most suitable for predicting the peptide structure, because it directly provides the value of exponent k in the m_{ep} versus q/M_r^k plot as the slope of the line expressed by equation (4). Parameters of linear regression (slope of curve, intercept, and coefficient of correlation) of the Cross's plot, $\log(m_{ep}/q)$ versus $\log M_r$, and coefficients of correlation of other five models for the GnRHs are presented in Table 5.

As follows from the data of Cross's model the exponent k in all five BGEs was close to 0.5, i.e. migration of this set of GnRHs can be best described by classical linear polymer model (C). Behavior of peptides in BGE II and BGE III can be also described by Cifuentes's and Poppe's (F) or Offord's (D) model. Consequently, the most probable structure of these peptides is random coil. In BGE V the exponent k was between 1/3 and 1/2, which means that the migration of these peptides fits also to rigid spherical model (B) and their molecules could possibly have the shape of solid sphere. The selection of the most probable model was supported by the high value of correlation coefficient $R = 0.995\text{--}0.999$. The predicted structures should be considered as the most probable, but not determined definitely.

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Legends to Figures

Figure 1

The pH dependence of specific charge (effective charge divided by relative molecular mass) of analyzed peptides, 1 – fragment of hGnRH 7-10, 2 – fragment of hGnRH 4-10, 3 – [Des-pGlu¹-D-Orn⁶]sGnRH, 4 – hGnRH, 5 – sGnRH, 6 – cGnRH, 7 – [β Ala⁶-ProNHEt¹⁰]hGnRH, 8 – [Des-Gly-NH₂]sGnRH, 9 – [Des-Gly-NH₂]cGnRH, 10 – [Des-Pro-Gly-NH₂]hGnRH, 11 – [Des-Arg-Pro-Gly-NH₂]hGnRH, 12 – fragment of hGnRH 1-4.

Figure 2

CZE analyses of (A) HPLC purified fragment of hGnRH 7-10, 1.40 mg.ml⁻¹, and (B) crude synthetic product of [Des-Gly-NH₂]cGnRH II, 1.10 mg.ml⁻¹, 1 – main synthetic product, x – non-identified impurities. Analyses were performed in BGE II (see Table 2). Other experimental conditions are given in the text (see section 3.3).

Figure 3

CZE separations of structurally related GnRHs and their analogs and fragments. Mixture I in (A) BGE II, (B) BGE IV, (C) BGE V, and mixture III in (D) BGE II, (E) BGE IV, (F) BGE V. For composition of BGEs see Table 2. Mixture I, 1 – fragment of hGnRH 4-10, 2 – hGnRH, 3 – fragment of hGnRH 1-4, 4 – [Des-Arg-Pro-Gly-NH₂]hGnRH; mixture III, 1 – [Des-pGlu¹-D-Orn⁶]sGnRH, 2 – [Des-Pro-Gly-NH₂]hGnRH, 3 – sGnRH, 4 – [Des-Gly-NH₂]sGnRH.

Figure 4

Semiempirical models of the correlation between effective mobility, m_{ep} , and peptide charge, q , and size (expressed by relative molecular mass, M_r , or by number of amino acid residues in peptide chain, n) applied to ten peptides in five BGEs, 1 – BGE I, 2 – BGE II, 3 – BGE III, 4 – BGE IV, 5 – BGE V, for BGEs composition see Table 2. (A) Cross's model, Eq. (4), (B) rigid spherical model, Eq. (2), (C) classical linear polymer model, Eq. (3), (D) Offord's model, Eq. (1), (E) Grossman's model, Eq. (7), (F) Cifuentes's and Poppe's model, Eq. (8).

Table 1Sequences of analyzed peptides and their relative molecular masses (M_r)

Peptide	Sequence in three-letter code	M_r
Human GnRH (hGnRH)	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH ₂	1181.5
[β Ala ⁶ -ProNHEt ¹⁰]hGnRH	pGlu-His-Trp-Ser-Tyr- β -Ala-Leu-Arg-Pro-Pro-NHEt	1262.5
[Des-Pro-Gly-NH ₂]hGnRH	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-OH	1028.3
[Des-Arg-Pro-Gly-NH ₂]hGnRH	pGlu-His-Trp-Ser-Tyr-Gly-Leu-OH	872.1
Fragment of hGnRH 1-4	pGlu-His-Trp-Ser-OH	538.6
Fragment of hGnRH 4-10	H-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH ₂	766.0
Fragment of hGnRH 7-10	H-Leu-Arg-Pro-Gly-NH ₂	440.6
Salmon GnRH (sGnRH)	pGlu-His-Trp-Ser-Tyr-Gly-Trp-Leu-Pro-Gly-NH ₂	1211.5
[Des-Gly-NH ₂]sGnRH	pGlu-His-Trp-Ser-Tyr-Gly-Trp-Leu-Pro-OH	1155.4
[Des-pGlu ¹ -D-Orn ⁶]sGnRH	H-His-Trp-Ser-Tyr-D-Orn-Trp-Leu-Pro-Gly-NH ₂	1058.3
Chicken GnRH I (cGnRH)	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Gln-Pro-Gly-NH ₂	1180.4
[Des-Gly-NH ₂]cGnRH II	pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-OH	1179.4

Table 2

Composition and pH of the BGEs applied for CZE analyses and separations of GnRHs and their analogs and fragments, separation voltage, U , and electric current, I .

BGE no.	BGE constituents	pH	U (kV)	I (μ A)
I	2 M acetic acid	2.18	20	19.9
II	100 mM H_3PO_4 , 50 mM Tris	2.25	15	43.2
III	100 mM iminodiacetic acid	2.30	20	33.0
IV	200 mM iminodiacetic acid	2.32	15	35.0
V	500 mM acetic acid	2.50	25	15.6

Table 3

Calculated effective charges, q , and CZE determined effective electrophoretic mobilities, m_{ep} , corrected to standard temperature, 25°C, of analyzed peptides in five different BGEs. Full composition of BGEs is given in Table 2.

Peptide	q [e]					m_{ep} ($10^{-9} \text{ m}^2 \text{V}^{-1} \text{s}^{-1}$)				
	BGE I	BGE II	BGE III	BGE IV	BGE V	BGE I	BGE II	BGE III	BGE IV	BGE V
Human GnRH (hGnRH)	2.00	2.00	2.00	2.00	2.00	14.26	11.69	14.60	12.87	16.85
[β Ala ⁶ -ProNHEt ¹⁰]hGnRH	2.00	2.00	2.00	2.00	2.00	14.68	12.05	15.22	13.47	17.90
[Des-Pro-Gly-NH ₂]hGnRH	2.00	2.00	2.00	2.00	2.00	14.97	12.27	15.37	13.52	17.29
[Des-Arg-Pro-Gly-NH ₂]hGnRH	0.95	0.94	0.93	0.92	0.91	8.33	7.15	8.54	7.66	9.55
Fragment of hGnRH 1-4	0.95	0.94	0.93	0.92	0.91	10.81	9.44	11.18	10.13	11.94
Fragment of hGnRH 4-10	2.00	2.00	2.00	2.00	2.00	18.21	15.22	18.96	16.86	21.99
Salmon GnRH (sGnRH)	1.00	1.00	1.00	1.00	1.00	7.63	6.38	7.98	7.01	9.42
[Des-Gly-NH ₂]sGnRH	0.95	0.94	0.93	0.92	0.91	7.43	6.37	7.68	6.85	8.78
[Des-pGlu ¹ -D-Om ⁶]sGnRH	3.00	3.00	3.00	3.00	22.16	17.24	22.47	20.95	27.20	
Chicken GnRH I (cGnRH)	1.00	1.00	1.00	1.00	7.63	6.38	7.98	7.01	9.42	

Table 4

Separation efficiency (number of theoretical plates) and resolution of CZE separation of three mixtures of GnRHs and their fragments and analogs in five BGEs. Composition of BGEs is given in Table 2.

Mixture no.	Peptide	Efficiency (number of theoretical plates)					Resolution				
		BGE I	BGE II	BGE III	BGE IV	BGE V	BGE I	BGE II	BGE III	BGE IV	BGE V
I	Fragment of hGnRH 4-10	21709	125188	60959	164189	14299	-	-	-	-	-
	hGnRH	18605	67104	43677	112854	12955	6.3	16.8	11.2	15.7	4.8
	Fragment of hGnRH 1-4	21967	56571	40334	106615	13811	6.7	10.9	9.6	11.5	5.4
	[Des-Arg-Pro-Gly-NH ₂]hGnRH	17631	75473	47091	123868	12119	5.6	14.6	9.2	12.2	3.0
II	[βAla ⁶ -ProNHEt ¹⁰]hGnRH	28602	104971	67695	152187	17714	-	-	-	-	-
	hGnRH	16941	58881	42961	100696	11929	0.8	2.1	1.8	2.5	1.2
	sGnRH	12807	36244	26417	55750	8739	6.9	24.9	17.9	23.6	8.0
	cGnRH I	12807	36244	26417	55750	8739	0	0	0	0	0
III	[Des-pGlu ¹ -D-Orn ⁶]sGnRH	27892	211526	74027	148832	14898	-	-	-	-	-
	[Des-Pro-Gly-NH ₂]hGnRH	17129	106062	54877	120507	13414	10.7	25.8	18.3	24.7	9.2
	sGnRH	12985	36711	18131	59590	6529	13.4	29.3	18.2	29.6	10.6
	[Des-Gly-NH ₂]sGnRH	12985	36711	13857	48050	4681	0	0	0.7	1.1	0.2

Table 5

Parameters of linear regression of Cross's (k_A , b_A , R_A), rigid spherical (R_B), classical linear polymer (R_C), Offord's (R_D), Grossman's (R_E), Cifuentes's and Poppe's (R_F) model for analyzed GnRHs, in five BGEs. For BGEs composition see Table 2.

BGE no.	k_A	b_A	R_A	R_B	R_C	R_D	R_E	R_F
I	-0.519	2.468	0.9827	0.9926	0.9989	0.9943	0.9895	0.9897
II	-0.589	2.604	0.9520	0.9826	0.9952	0.9977	0.9948	0.9965
III	-0.534	2.531	0.9725	0.9902	0.9981	0.9956	0.9911	0.9921
IV	-0.557	2.552	0.9723	0.9893	0.9967	0.9940	0.9898	0.9863
V	-0.457	2.468	0.9654	0.9945	0.9975	0.9901	0.9836	0.9839

k - slope of curve, b - intercept, R - coefficient of correlation

Figure 1

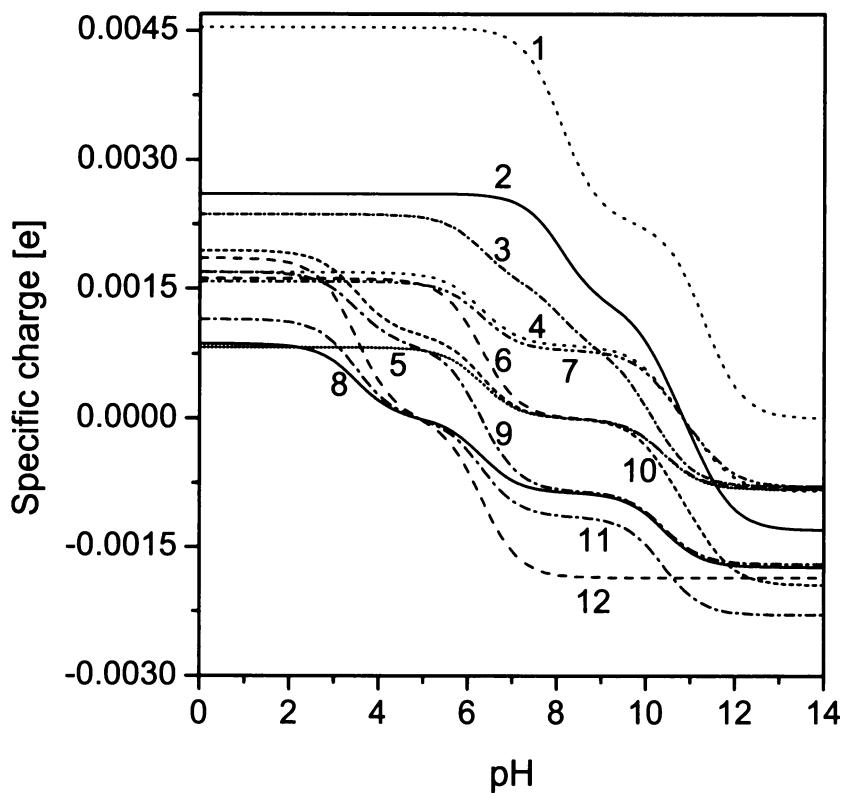


Figure 2

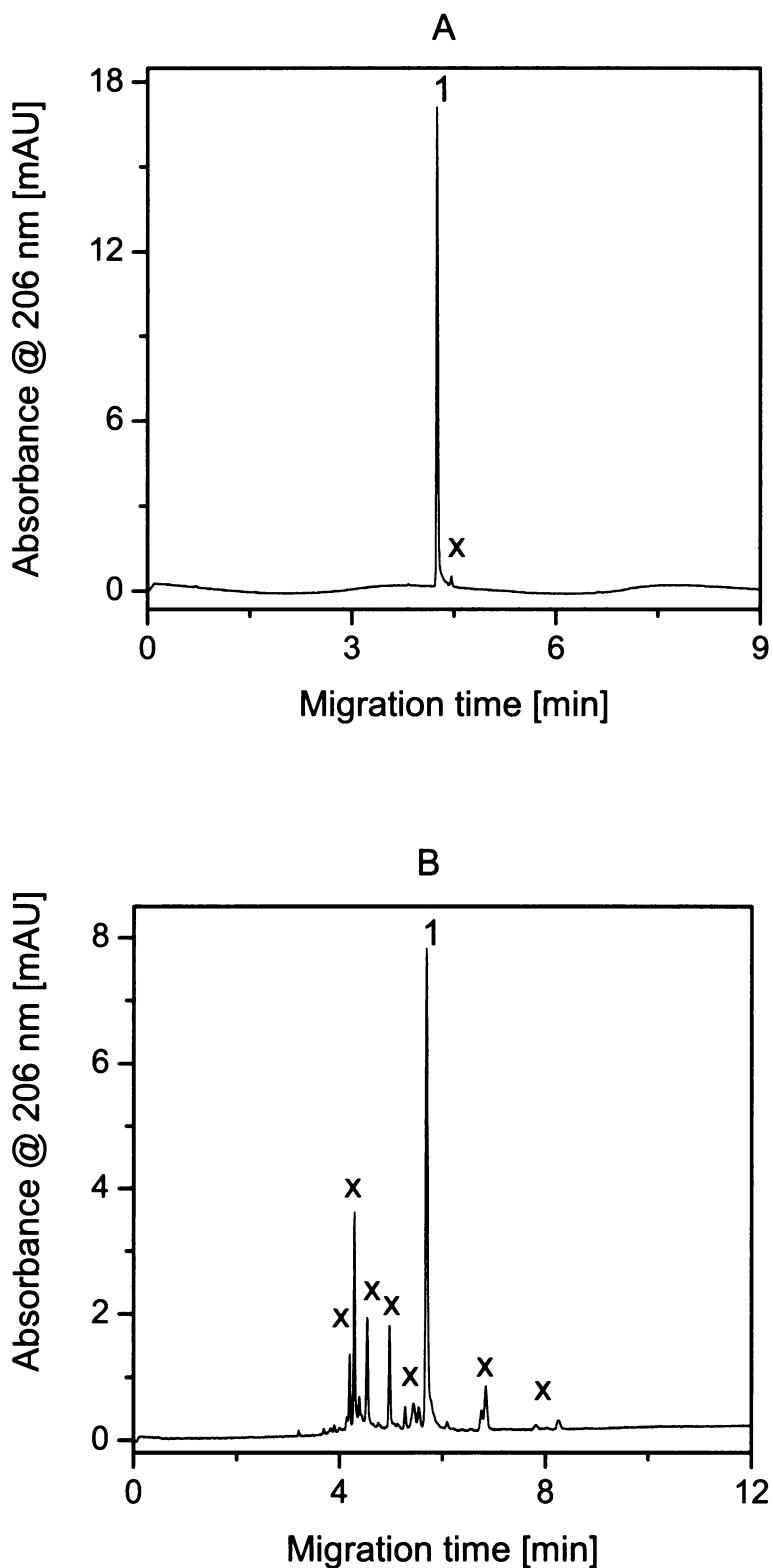


Figure 3

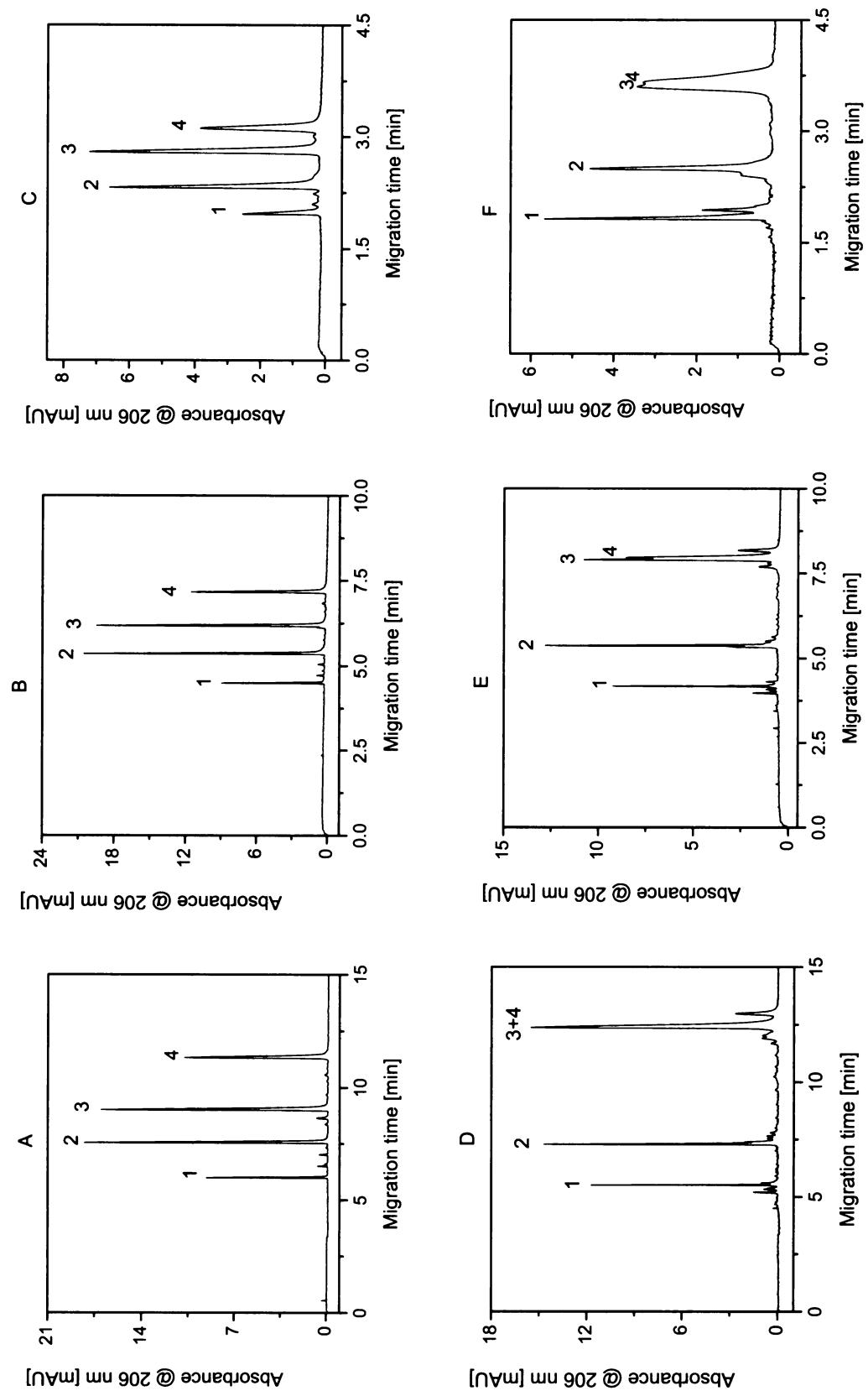
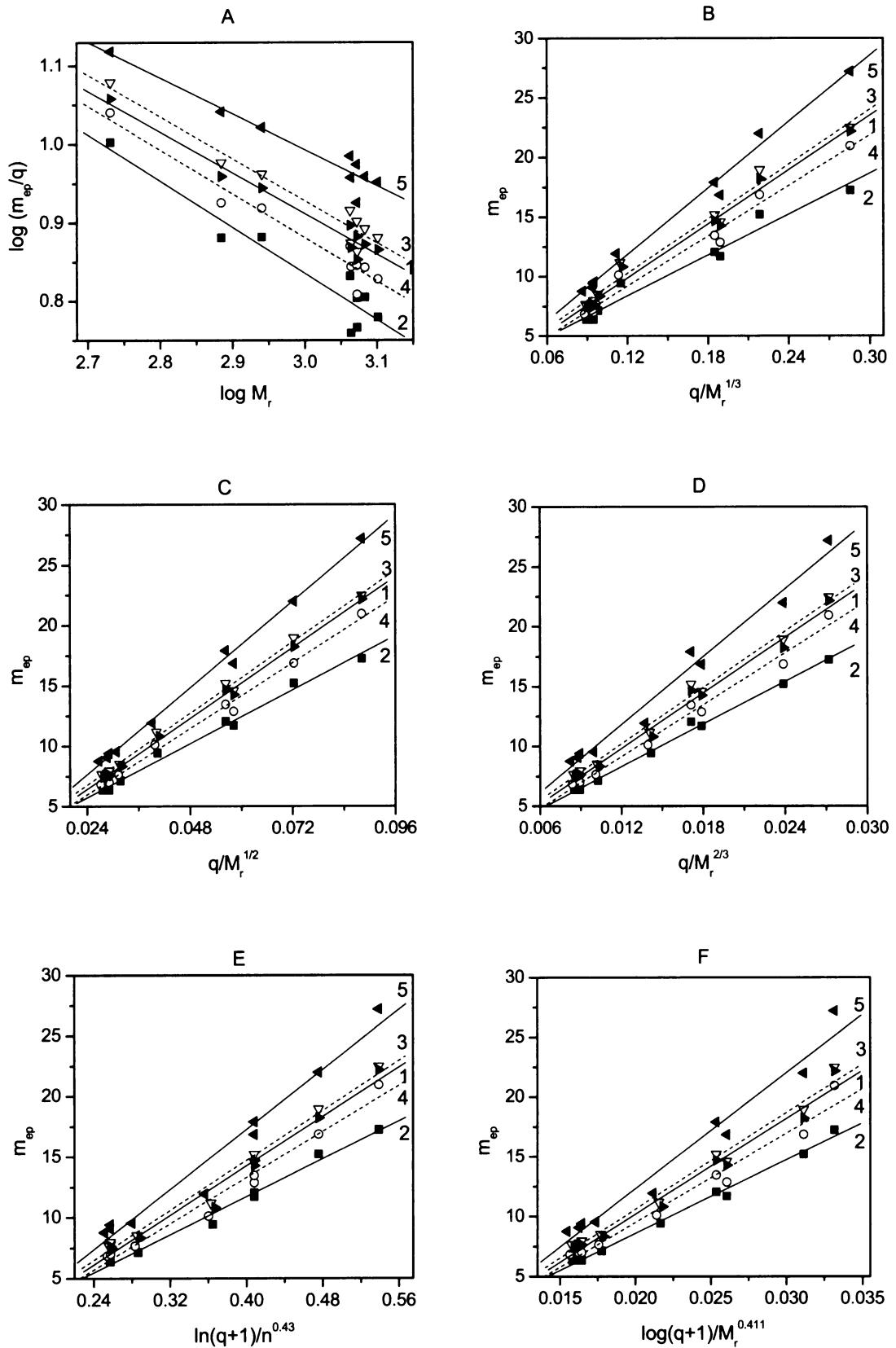


Figure 4



V

Veronika Šolínová
Václav Kašička
Dušan Koval
Michal Česnek
Antonín Holý

Institute of Organic Chemistry
and Biochemistry,
Academy of Sciences
of the Czech Republic,
Prague, Czech Republic

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Research Article

Determination of acid–base dissociation constants of amino- and guanidinopurine nucleotide analogs and related compounds by capillary zone electrophoresis

CZE has been applied for determination of acid–base dissociation constants (pK_a) of ionogenic groups of newly synthesized amino- and (amino)guanidinopurine nucleotide analogs, such as acyclic nucleoside phosphonate, acyclic nucleoside phosphonate diesters and other related compounds. These compounds bear characteristic pharmacophores contained in various important biologically active substances, such as cytostatics and antivirals. The pK_a values of ionogenic groups of the above compounds were determined by nonlinear regression analysis of the experimentally measured pH dependence of their effective electrophoretic mobilities. The effective mobilities were measured by CZE performed in series of BGEs in a broad pH range (3.50–11.25), at constant ionic strength (25 mM) and temperature (25°C). pK_a values were determined for the protonated guanidinyl group in (amino)guanidino 9-alkylpurines and in (amino)guanidinopurine nucleotide analogs, such as acyclic nucleoside phosphonates and acyclic nucleoside phosphonate diesters, for phosphonic acid to the second dissociation degree (–2) in acyclic nucleoside phosphonates of amino and (amino)guanidino 9-alkylpurines, and for protonated nitrogen in position 1 (N1) of purine moiety in acyclic nucleoside phosphonates of amino 9-alkylpurines. Thermodynamic pK_a of protonated guanidinyl group was estimated to be in the range of 7.75–10.32, pK_a of phosphonic acid to the second dissociation degree achieved values of 6.64–7.46, and pK_a of protonated nitrogen in position 1 of purine was in the range of 4.13–4.89, depending on the structure of the analyzed compounds.

Keywords: Acyclic nucleoside phosphonates / Aminopurine nucleosides / Capillary electrophoresis / Dissociation constant (pK_a) / Guanidinopurine nucleosides

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1 Introduction

The knowledge of physicochemical characteristics of new synthetic compounds, such as water solubility, lipophilicity, and acid–base properties, is necessary when these compounds start to be tested as potential new drug candidates. The determination of these characteristics

plays an important role in the optimization stage of a new drug development project. Particularly, the acid–base dissociation constant (pK_a) is a key parameter for compounds containing at least one ionogenic (acidic or basic) functional group for understanding the passage of drugs into and across cell membranes, for estimation of the concentration of the individual ionic forms of the drug in blood, for assessment of reaction rate and for investigation of the biological uptake, and metabolism mechanism. Moreover, the knowledge of pK_a values is important for prediction of the effective charge, effective electrophoretic mobility, and migration order of the analytes in CZE, which has become a method of choice for determination of pK_a values [1]. In comparison with the traditional potentiometric and spectrophotometric titration methods, the particular advantage of CZE is that it allows

Correspondence: Dr. Václav Kašička, Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Flemingovo 2, CZ-166 10 Prague 6, Czech Republic

E-mail: kasicka@uochb.cas.cz

Fax: +420-220-183-592

Abbreviations: PMEDAP, 2,6-diamino-9-(2-(phosphonomethoxy)ethyl)-9*H*-purine; PMEG, 9-(2-(phosphono-methoxyethyl)guanine; PMEMAP, 2-amino-9-(2-(phosphonomethoxyethyl)-9*H*-purine

working with a very small amount of sample (nanolitre applied sample volume per analysis) and knowledge of the analyte precise concentration is not necessary. In addition, the analyte need not to be perfectly pure, since its potential admixtures are separated during the analysis.

Newly synthesized amino and (amino)guanidino 9-alkylpurines [2, 3], e.g., 2-amino-6-guanidino-9-isopropylpurine, 6-amino-2-guanidino-9-isopropylpurine, 2- or 6-guanidino-9-isopropylpurine, 2-amino-9-alkylpurine, their acyclic nucleoside phosphonates and alkyl (isopropyl) esters of these acyclic nucleoside phosphonates, bear characteristic pharmacophores contained in various important biologically active substances, such as cytostatics and antivirals. The derivatives of 9-alkylpurines also show a broad spectrum of biological activity; they influence the metabolic pathway of cytokinins and cyclin-dependent kinases. The 6-amino group not only plays an important role in purine derivatives by its Watson–Crick basepairing capacity, but is also essential for substrate/inhibitor binding to diverse enzymes of purine metabolism as well as in important regulatory pathways. The guanidinyl group is closely related to the amino function but it is more basic than amino group. Among others, the inhibitors of the influenza virus neuraminidase, inhibitors of polyamine synthesis and the minor groove binding agent netropsin belong to the biologically active compounds bearing this functional group.

CZE has proved to be a convenient and precise method for determination of dissociation constants of ionogenic groups of various types of compounds, such as different types of drugs [4–9], alkaloids [10], flavonoids [11], amino acids [12], peptides [13–15], morpholinyl group of Mannich ketones [16], cytokinins [17], imidazole derivatives [18], guanosine monophosphate and adenosine monophosphate [19], xanthones [20], enolizable compounds [21], 2-amino-2-oxazolines [22], saccharides [23], heterocyclic aromatic amines [24], and secondary and tertiary amines [25].

The pK_a values of the above types of compounds were mostly determined in aqueous media, but several papers deal with pK_a estimation in nonaqueous [15, 26] or aqueous–organic solvents [27–29]. The most frequently applied detection for CZE estimation of pK_a is the direct UV-absorption detection [6, 7, 9, 10, 13, 30–32]; however, conductometric [12], amperometric [23], and indirect UV-absorption [33] detection schemes have been also used. The pK_a values of the ionogenic groups in both aqueous and nonaqueous media were determined using the different linear or nonlinear regression analysis of the pH dependence of effective electrophoretic mobility of analyzed compounds [1].

The aim of this work was to employ CZE for determination of the thermodynamic dissociation constants (pK_a) of ionogenic groups of amino- and guanidinopurines and their analogs, particularly pK_a of the protonated guanidinyl group in (amino)guanidino 9-alkylpurines, their acyclic nucleoside phosphonates and acyclic nucleoside phosphonate diisopropyl esters, pK_a of phosphonic acid to the second dissociation degree in acyclic nucleoside phosphonates of (amino)guanidino 9-alkylpurines, and pK_a of protonated nitrogen in position 1 (N1) of purine moiety in acyclic nucleoside phosphonates of amino 9-alkylpurines. To achieve this goal, it was necessary to measure the pH dependence of the effective electrophoretic mobilities of the above compounds by CZE performed in the BGEs within a broad pH range, 3.50–11.25, at constant ionic strength and constant temperature.

2 Materials and methods

2.1 Chemicals

All chemicals used were of analytical reagent grade. MES (2-morpholinoethanesulfonic acid), CHES (2-(cyclohexylamino)ethanesulfonic acid), CAPS, and Tris were obtained from Serva (Heidelberg, Germany); phosphoric acid, acetic acid, formic acid, sodium hydroxide, and DMSO were supplied by Lachema (Brno, Czech Republic); SDS and isophorone (3,5,5-trimethyl-2-cyclohexen-1-one) were from Fluka (Buchs, Switzerland); MOPS (4-morpholinopropanesulfonic acid) was obtained from Calbiochem (San Diego, CA, USA); and Tricine ((tris(hydroxymethyl)-methyl)-glycine) was from Merck (Darmstadt, Germany).

2.2 Analyzed compounds

The list of analyzed compounds (analytes), amino- and (amino)guanidinopurine nucleotide analogs, such as acyclic nucleoside phosphonates and acyclic nucleoside phosphonate diesters, their identification numbers and M_r , are presented in Table 1. 6-Guanidinopurines and 2-amino-6-guanidinopurine derivatives were synthesized by reaction of the 6-chloropurine derivatives with guanine solution in DMF (dimethylformamide) in the presence of 1,4-diazo-bicyclo(2,2,2)octane (DABCO) [2]. Acyclic guanidinopurine nucleosides and acyclic guanidinopurine nucleoside phosphonates were obtained by alkylation of the 6-guanidinopurine bases [2]. 9-Alkyl and 9-heteroalkyl substituted derivatives of the 2-amino-6-guanidinopurine were synthesized by alkylation of 2-amino-6-chloropurine and subsequent guanidinolysis [3].

Table 1. List of analyzed compounds, their identification numbers and M_r

Compound	Identification number	M_r
6-Guanidino-9-methyl-9 <i>H</i> -purine	1	191.2
2-Amino-6-guanidino-9-isopropyl-9 <i>H</i> -purine	2	234.3
2-Guanidino-9-isopropyl-9 <i>H</i> -purine	3	219.2
6-Amino-2-guanidino-9-isopropyl-9 <i>H</i> -purine	4	234.3
9-{2-((Diisopropoxy)phosphorylmethoxy)ethyl}-2-guanidino-9 <i>H</i> -purine	5	399.4
6-Amino-9-(2-((diisopropoxy)phosphorylmethoxy)ethyl)-2-guanidino-9 <i>H</i> -purine	6	414.4
2-Amino-9-(2-(phosphonomethoxy)ethyl)-9 <i>H</i> -purine	7 (PMEMAP)	273.2
2,6-Diamino-9-(2-(phosphonomethoxy)ethyl)-9 <i>H</i> -purine	8 (PMEDAP)	288.2
9-(2-Phosphonomethoxyethyl)guanine	9 (PMEG)	289.2
6-Guanidino-9-(2-(phosphonomethoxy)ethyl)-9 <i>H</i> -purine	10	315.3
2-Amino-6-guanidino-9-(2-(phosphonomethoxy)ethyl)-9 <i>H</i> -purine	11	330.2
2-Guanidino-9-(2-(phosphonomethoxy)ethyl)-9 <i>H</i> -purine	12	315.2
6-Amino-2-guanidino-9-(2-(phosphonomethoxy)ethyl)-9 <i>H</i> -purine	13	330.2

2.3 Instrumentation

CZE analyses were carried out in the capillary electrophoretic analyzer P/ACE MDQ (Beckman Coulter, Fullerton, CA, USA) using the software 32 Karat System, version 7.0 (Beckman) for data acquisition and evaluation. Origin 6.1 (OriginLab Corp., Northampton, MA, USA) was used for data plotting and regression analysis. The analyzer was equipped with the internally uncoated fused-silica capillary with outer polyimide coating, total/effective length 394/292 mm, ID/OD 75/360 μm (Polymicro Technologies, Phoenix, AR, USA). The analytes were detected by UV-Vis spectrophotometric photodiode array detector (190–600 nm) set to operate in the range 190–300 nm. Absorbance of analyzed compounds and of EOF markers (DMSO or isophorone) was monitored at 225 and 250 nm, respectively. The analyses were performed at constant temperature of the BGE inside the capillary, 25°C.

The new capillary was gradually flushed with water, 0.1 M NaOH, water, and BGE, each wash for 5 min. Finally, the capillary was conditioned by a 20 min application of the

high voltage to equilibrate the inner surface and to stabilize EOF. Between runs under the same conditions, the capillary was rinsed with the BGE for 1 min. Prior to any change of the BGE the capillary was rinsed with 0.1 M NaOH for 5 min and then repeatedly stabilized. The samples were injected hydrodynamically, with pneumatically induced pressure 6.9–13.8 mbar for 5–10 s. The samples were dissolved in concentrations of 0.1 mmol/L in deionized water or in deionized water slightly alkalized by a small addition of NaOH to ensure solubility of some compounds.

BGE solutions with pH values covering a broad pH range (3.50–11.25) with 0.25 pH unit increment were prepared by mixing the appropriate amounts of stock solutions listed in Table 2 and then diluted to the constant ionic strength 25 mmol/L. The pH was measured at 25°C by pH meter CyberScan pH 2100 (Oakton Instruments, Vernon Hills, IL, USA). The BGEs were filtered through a 0.45 μm pore NC syringe filter (Millipore, Bedford, MA, USA) before use.

Table 2. Composition of the stock solutions and pH range of the BGEs, applied separation voltage, U , and electric current, I , of the CZE analyses

BGE number	Composition of stock solutions	pH range	U range, kV	I range, μA
BGE I–IV	0.95 M HCOOH, 0.7 M Tris	3.50– 4.25	9.8–10.7	26.4–24.1
BGE V–X	1 M CH ₃ COOH, 1 M NaOH	4.25– 5.50	8.6–10.6	30.0–24.4
BGE XI–XV	0.4 M MES, 1 M NaOH	5.50– 6.50	11.0–11.5	24.8–23.4
BGE XVI–XX	0.4 M MOPS, 1 M NaOH	6.50– 7.50	11.3–11.5	23.4–21.7
BGE XXI–XXVI	0.4 M Tricine, 1 M NaOH	7.50– 8.75	11.1–12.3	23.1–19.7
BGE XXVII–XXXI	0.5 M CHES, 1 M NaOH	8.75– 9.75	11.0–11.5	23.5–22.5
BGE XXXII–XXXVIII	0.4 M CAPS, 1 M NaOH	9.75–11.25	10.0–13.0	27.1–19.8

2.4 Determination of the effective electrophoretic mobility

The effective electrophoretic mobility, m_{eff} , of the analyte at pH of the given BGE was calculated from the experimental data of its CZE analysis, using the following equation:

$$m_{\text{eff}} = \frac{l_l l_{\text{eff}}}{U} \left(\frac{1}{t_m} - \frac{1}{t_{\text{eo}}} \right) \quad (1)$$

where l_l is the total capillary length, l_{eff} is the effective capillary length, U is the applied separation voltage, t_m is the migration time of the analyte, and t_{eo} is the migration time of the electroneutral EOF marker.

Electrophoretic mobility is relatively strongly dependent on the temperature: it varies by about 2% per °C in the aqueous solutions. Therefore, it was necessary to ensure that all mobilities were measured at the same temperature, best at the standard temperature of 25°C. It was achieved by thermostating the outer polyimide capillary surface to constant temperature of 24°C and by setting the input power to the value of 0.66 W per meter of capillary length, which causes 1°C temperature increase inside the capillary, resulting in the constant temperature 25°C of the BGE inside the capillary. The value of 0.66 W/m for 1°C temperature increase was determined from the electric conductivity measurements of an aqueous standard solution of potassium chloride in the same capillary and from the known temperature dependence of its conductivity, as described in our previous paper [32].

3 Theory of the acid–base equilibria of the analyzed compounds

From the standpoint of the acid–base behavior in the applied pH range (3.50–11.25) of BGEs four different types of the analyzed compounds can be distinguished.

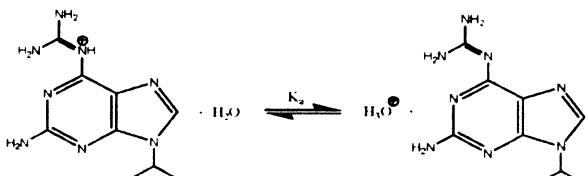
(i) (Amino)guanidino 9-alkylpurines (compounds **1–4**, see Table 1 and Fig. 1A) and their acyclic nucleoside phosphonate diisopropyl esters (compounds **5** and **6**, see Table 1 and Fig. 1B) behave as monovalent bases due to the presence of a single strongly basic (cationogenic) guanidinyl group. (ii) Acyclic nucleoside phosphonates of amino 9-alkylpurines (compounds **7** and **8**, see Table 1 and Fig. 1C) behave as zwitterions with single weakly basic (cationogenic) nitrogen group in position 1 (N1) of purine moiety and two acidic (anionogenic) phosphonic acid groups. (iii) Acyclic nucleoside phosphonates of (amino)guanidino 9-alkylpurines (compounds **10–13**, see Table 1 and Fig. 1D) behave as zwitterions due to the presence of single strongly basic (cationogenic) guanidinyl group and two acidic (anionogenic) phosphonic acid groups. (iv) 9-(2-(Phosphonomethoxy)ethyl)guanine

(PMEG, **9**) behaves as trivalent acid due to the presence of two acidic anionogenic phosphonic acid groups and single enol –OH group in position 6 of purine moiety.

The type (ii) differs from the type (iii) by different strength of the basic group, resulting in different order of the dissociation of the protonized groups. In the type (ii) the proton from nitrogen 1 (N1) dissociates at pH lower than that from the phosphonic acid to the second dissociation degree, whereas in the type (iii) the proton from guanidinyl group dissociates at pH higher than that from the hydrogen-phosphonate (–1) to phosphonate (–2). In both these types of analytes and in type (iv) (PMEG (**9**)) phosphonic acid is dissociated into the first and/or into the second dissociation degree within the whole applied pH range of BGEs.

3.1 (Amino)guanidino 9-alkylpurines and diisopropyl esters of their acyclic nucleoside phosphonates

The (amino)guanidino 9-alkylpurines (compounds **1–4**) and their acyclic nucleoside phosphonate diisopropyl esters (compounds **5** and **6**) are present in the form of single charged cations in acidic, neutral and weakly to medium alkaline solutions due to protonation of the strongly basic guanidinyl group and they are losing their charge in strongly alkaline solutions. The hydroxyl groups of phosphonic acid are blocked in (amino)guanidino 9-alkylpurine phosphonate diisopropyl esters (**5** and **6**) and they cannot participate in the acid–base equilibria. The dissociation equilibrium of (amino)guanidino 9-alkylpurines and their acyclic nucleoside phosphonate diisopropyl esters can be expressed as shown in Scheme 1 using 2-amino-6-guanidino-9-isopropyl-9H-purine (**2**) as an example.



Scheme 1

This acid–base equilibrium is characterized by the thermodynamic dissociation constant, K_a , of the guanidinyl group:

$$K_a = \frac{a_{\text{H}_3\text{O}^+} a_B}{a_{\text{BH}^+}} = \frac{a_{\text{H}_3\text{O}^+} c_B \gamma_B}{c_{\text{BH}^+} \gamma_{\text{BH}^+}} \quad (2)$$

where $a_{\text{H}_3\text{O}^+}$ is the activity of the hydronium ions, and a_B and a_{BH^+} are the activities of the neutral and protonated forms of compound **2** (indicated as a general base B), respectively. In the second part of Eq. (2) the activities of the

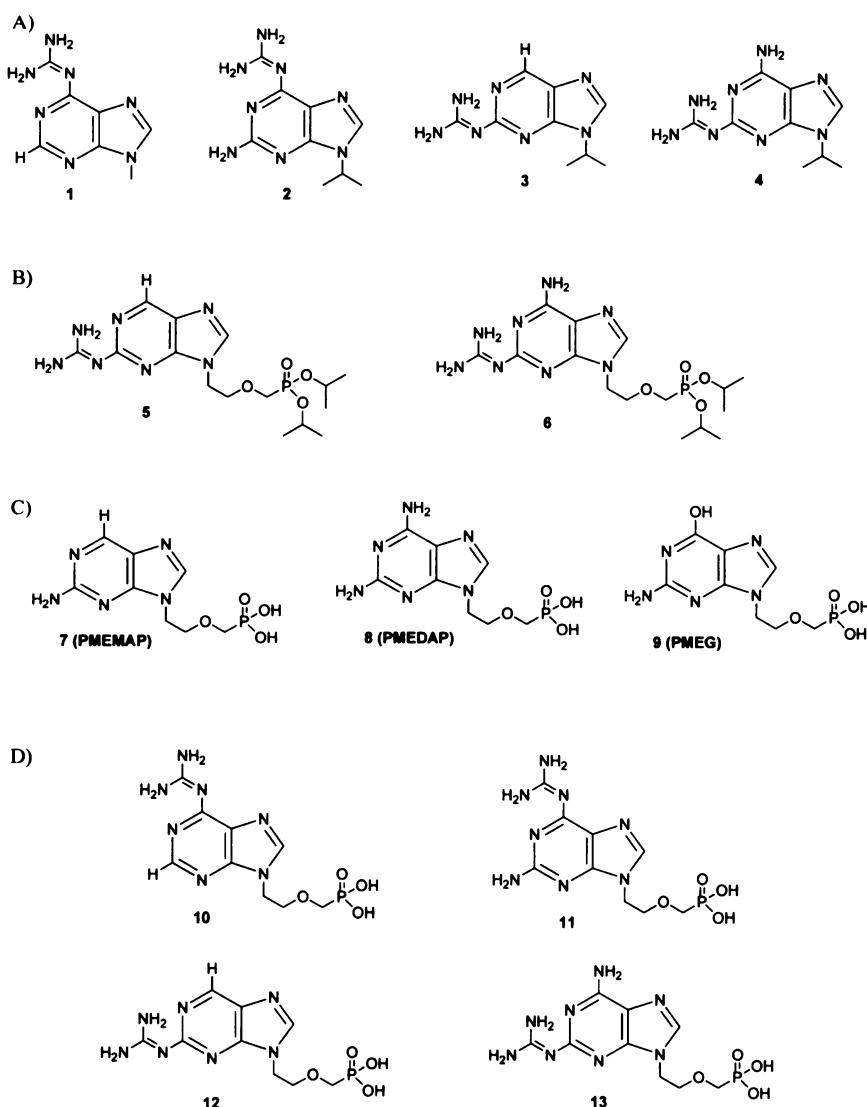


Figure 1. Molecular structures of analyzed compounds (A) guanidino 9-alkylpurines and amino-guanidino 9-alkylpurines; (B) acyclic nucleoside phosphonate diisopropyl esters of 2-guanidino-9-alkylpurines; (C) acyclic nucleoside phosphonates of amino 9-alkylpurines and PMEG; (D) acyclic nucleoside phosphonates of guanidino- and amino-guanidino 9-alkylpurines.

species B and BH^+ , a_B and a_{BH}^+ , are expressed as products of their molar concentrations, c_B and c_{BH}^+ , and corresponding activity coefficients, γ_B and γ_{BH}^+ , respectively. The activity coefficient γ_i of the charged species (ion i) in aqueous solution at 25°C can be calculated from the Debye–Hückel theory according to Eq. (3):

$$-\log \gamma_i = \frac{0.5085 z_i^2 \sqrt{l}}{1 + 3.281 a \sqrt{l}} \quad (3)$$

where z_i is the charge number and a is the effective diameter of the hydrated i th ion. The approximative average value of $a = 0.5 \text{ nm}$ was used in the calculations of the activity coefficients, since the exact diameters of hydrated ions are mostly unknown. The ionic strength of the solution (mol/dm^3), l , is defined by Eq. (4):

$$l = \frac{1}{2} \sum_i c_i z_i^2 \quad (4)$$

where c_i is molar concentration and z_i is the charge number of the i th ion in the solution.

The effective electrophoretic mobility m_{ef} of an analyte consisting of n ionic forms is defined by Eq. (5):

$$m_{\text{ef}} = \frac{\sum_{i=1}^n c_i m_i \text{sign}(z_i)}{c} \quad (5)$$

where c_i , m_i , and z_i , are the molar concentration, actual ionic mobility and charge number of the i th ionic form, respectively, $\text{sign}(z_i)$ is the sign of the charge number z_i and c is the total (analytical) concentration of the analyte. As follows from Eq. (5) effective mobility of the analyte as

a whole is equal to the sum of the products of molar fraction and ionic mobility of all ionic species of the analyte and it is related to molar concentrations of all ionic forms of the analyte and to the total (analytical) molar concentration of the analyte. On the other hand, the other experimentally measured parameter of CZE analyses, pH of the BGE, is related to the activity of the hydroxonium cations H_3O^+ ($\text{pH} = -\log(a_{\text{H}_3\text{O}^+})$). For that reason it is useful to introduce so-called "mixed" dissociation constant, K_a^{mix} , which is expressed by the activity of H_3O^+ cations and by molar concentrations of the ionic forms of the analyte; for the dissociation of the above protonated guanidinyl group of analyte 2 we can then obtain K_a^{mix} as

$$K_a^{\text{mix}} = \frac{a_{\text{H}_3\text{O}^+} \cdot c_B}{c_{\text{BH}^+}} \quad (6)$$

Since the activity coefficient of the electroneutral form of the base is supposed to be unity, the relationship between thermodynamic and mixed dissociation constants is

$$K_a = \frac{K_a^{\text{mix}}}{\gamma_{\text{BH}^+}} \quad (7)$$

or, after logarithmization and considering that $pK_a = -\log K_a$:

$$pK_a = pK_a^{\text{mix}} + \log \gamma_{\text{BH}^+} \quad (8)$$

As follows from Eq. (5) the effective mobility of the univalent base B, $m_{\text{eff},B}$, is equal to

$$m_{\text{eff},B} = m_{\text{BH}^+} \frac{c_{\text{BH}^+}}{c_{\text{BH}^+} + c_B} \quad (9)$$

where m_{BH^+} is actual ionic mobility of the protonated base, BH^+ . The combination of Eqs. (6) and (9) provides the dependence of the effective mobility of base B, $m_{\text{eff},B}$, on pH:

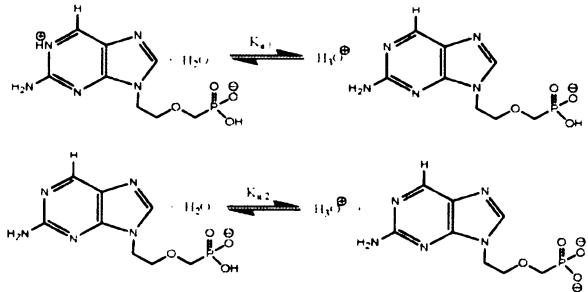
$$m_{\text{eff},B} = \frac{m_{\text{BH}^+}}{1 + 10^{pH - pK_a^{\text{mix}}}} \quad (10)$$

Generally, the actual ionic mobility m_{BH^+} and mixed dissociation constant pK_a^{mix} are dependent on the ionic strength and temperature of the BGE, but if the CZE analyses are performed at constant ionic strength and temperature, then they can be considered as constant parameters of Eq. (10), and the effective mobility, $m_{\text{eff},B}$, expressed by Eq. (10), can be considered as a function of pH only. This function can be then used as a regression function for fitting the pH dependence of the effective mobility obtained from CZE analyses of the investigated compounds in BGEs of different pH. The fitted parameters are the actual ionic mobility, m_{BH^+} , and the mixed dissociation constant, pK_a^{mix} , which can be subsequently corrected for the zero ionic strength, i.e., to the thermodynamic dissociation constant

pK_a (using Eq. 8) or to limiting (absolute) ionic mobilities (using one of the equations describing the concentration dependence of electrophoretic mobilities).

3.2 Acyclic nucleoside phosphonates of amino 9-alkylpurines

The acyclic nucleoside phosphonates of amino 9-alkylpurines (compounds 7 and 8) behave as zwitterions with effective charge close to zero at strongly acidic BGEs due to the positive charge of protonated nitrogen in position 1 (N1) of purine moiety and the negative charge of hydrogenphosphonate (-1) group. With the increasing pH of the BGE these compounds are becoming negatively charged due to the deprotonation of N1 of purine and due to the dissociation of hydrogenphosphonate (-1) to phosphonate (-2). The dissociation equilibria of these type of compounds, using 2-amino-9-(2-(phosphonomethoxy)ethyl)-9H-purine (7, PMEMAP) as an example, are described as follows:



Scheme 2

Thermodynamic and mixed dissociation constants of the dissociation of protonated N1 in purine moiety, $K_{a,1}$ and $K_{a,1}^{\text{mix}}$, respectively, are given by

$$K_{a,1} = \frac{a_{\text{H}_3\text{O}^+} \cdot a_{\text{HA}^- \text{B}}}{a_{\text{HA}^- \text{BH}^+}} = \frac{a_{\text{H}_3\text{O}^+} \cdot c_{\text{HA}^- \text{B}} \gamma_{\text{HA}^- \text{B}}}{c_{\text{HA}^- \text{BH}^+} \gamma_{\text{HA}^- \text{BH}^+}} \quad (11)$$

$$K_{a,1}^{\text{mix}} = \frac{a_{\text{H}_3\text{O}^+} \cdot c_{\text{HA}^- \text{B}}}{c_{\text{HA}^- \text{BH}^+}} \quad (12)$$

where $a_{\text{H}_3\text{O}^+}$ is the activity of the hydroxonium ions, $a_{\text{HA}^- \text{BH}^+}$, ($c_{\text{HA}^- \text{BH}^+}$), and $a_{\text{HA}^- \text{B}}$, ($c_{\text{HA}^- \text{B}}$), are the activities (concentrations) of the zwitterionic and N1-deprotonated forms of acyclic nucleoside phosphonates of aminopurines, respectively.

The substitution of Eq. (12) in Eq. (11) and taking into account the unity value of activity coefficient of the zwitterionic form of the analyte result in the following relation between thermodynamic and mixed dissociation constants:

$$K_{a,1} = K_{a,1}^{\text{mix}} \gamma_{\text{HA}^- \text{B}} \quad (13)$$

or in the logarithmic form:

$$pK_{a,1} = pK_{a,1}^{\text{mix}} - \log \gamma_{HA^-B} \quad (14)$$

Thermodynamic and mixed dissociation constants of dissociation of phosphonic acid group to the second degree (–2), $K_{a,2}$ and $K_{a,2}^{\text{mix}}$, respectively, are given by the following equations:

$$K_{a,2} = \frac{a_{H_3O^+} a_{A^2-B}}{a_{HA^-B}} = \frac{a_{H_3O^+} c_{A^2-B} \gamma_{A^2-B}}{c_{HA^-B} \gamma_{HA^-B}} \quad (15)$$

$$K_{a,2}^{\text{mix}} = \frac{a_{H_3O^+} c_{A^2-B}}{c_{HA^-B}} \quad (16)$$

where a_{HA^-B} , (c_{HA^-B}), and a_{A^2-B} , (c_{A^2-B}), are the activities (concentrations) of the single and double negatively charged forms of acyclic nucleoside phosphonates of aminopurines, respectively.

Combination of Eqs. (15) and (16) provides the following relations between thermodynamic and mixed dissociation constants:

$$K_{a,2} = \frac{K_{a,1}^{\text{mix}} \gamma_{A^2-B}}{\gamma_{HA^-B}} \quad (17)$$

It is worth to mention that in this case the activity coefficients of two ionic species, HA^-B and A^2-B , have to be taken into account. After logarithmization of Eq. (17) and with respect to the relation $\log \gamma_{A^2-B} = 4 \log \gamma_{HA^-B}$ (resulting from Eq. 3) the following relation is obtained for the thermodynamic and mixed dissociation constants:

$$pK_{a,2} = pK_{a,2}^{\text{mix}} - 3 \log \gamma_{HA^-B} \quad (18)$$

For the dependence of the effective mobility of these types of analytes on pH the following equation can be derived:

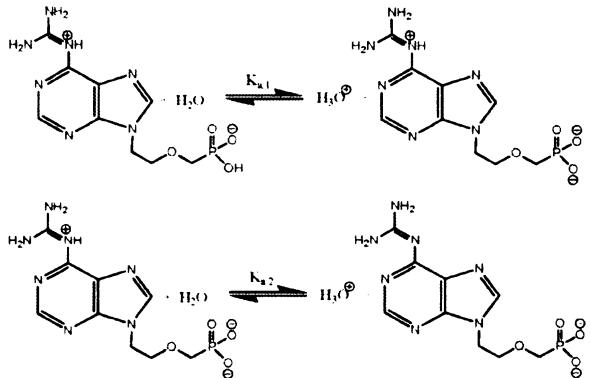
$$m_{\text{eff}} = \frac{m_{HA^-B} 10^{pK_{a,1}^{\text{mix}} - \text{pH}} - m_{A^2-B} 10^{\text{pH} - pK_{a,2}^{\text{mix}}}}{1 + 10^{pK_{a,1}^{\text{mix}} - \text{pH}} + 10^{\text{pH} - pK_{a,2}^{\text{mix}}}} \quad (19)$$

where m_{HA^-B} and m_{A^2-B} are the actual ionic mobilities of the ionic forms HA^-B and A^2-B , respectively.

3.3 Acyclic nucleoside phosphonates of (amino)guanidino 9-alkylpurines

The acyclic nucleoside phosphonates of (amino)guanidino 9-alkylpurines (compounds 10–13) behave as zwitterions with effective charge close to zero in acidic BGEs, where the positive charge of the protonated guanidinyl group is compensated by the negative charge of hydrogenphosphonate. With the increasing pH of the BGE the effective charge of these compounds is becoming negative due to the dissociation of hydrogenphosphonate (–1) to phosphonate

(–2) at around neutral pH and due the deprotonation of the guanidinyl group at alkaline pH. Using 6-guanidino-9-(2-(phosphonomethoxy)ethyl)-9*H*-purine (10) as a representative of these types of compounds, their dissociation equilibria can be described as shown in Scheme 3.



Scheme 3

The corresponding thermodynamic and mixed dissociation constants of phosphonic acid group to the second dissociation degree can be expressed as

$$K_{a,1} = \frac{a_{H_3O^+} a_{A^2-BH^+}}{a_{HA^-BH^+}} = \frac{a_{H_3O^+} c_{A^2-BH^+} \gamma_{A^2-BH^+}}{c_{HA^-BH^+} \gamma_{HA^-BH^+}} = K_{a,1}^{\text{mix}} \gamma_{A^2-BH^+} \quad (20)$$

where $a_{HA^-BH^+}$, ($c_{HA^-BH^+}$), and $a_{A^2-BH^+}$, ($c_{A^2-BH^+}$) are the activities (concentrations) of the zwitterionic and single charged anionic forms of this type of analytes, respectively, and the activity coefficient of the zwitterion, $\gamma_{HA^-BH^+}$, is supposed to be unity.

Similarly as in Section 3.2, the relation between thermodynamic and mixed dissociation constants can be expressed in the logarithmic form as

$$pK_{a,1} = pK_{a,1}^{\text{mix}} - \log \gamma_{A^2-BH^+} \quad (21)$$

Thermodynamic and mixed dissociation constants of the dissociation of the protonated guanidinyl group, $K_{a,2}$ and $K_{a,2}^{\text{mix}}$, respectively, are defined as

$$K_{a,2} = \frac{a_{H_3O^+} a_{A^2-B}}{a_{A^2-BH^+}} = \frac{a_{H_3O^+} c_{A^2-B} \gamma_{A^2-B}}{c_{A^2-BH^+} \gamma_{A^2-BH^+}} = \frac{K_{a,2}^{\text{mix}} \gamma_{A^2-B}}{\gamma_{A^2-BH^+}} \quad (22)$$

where $a_{A^2-BH^+}$, ($c_{A^2-BH^+}$), and a_{A^2-B} , (c_{A^2-B}) are the activities (concentrations) of the single charged and double charged anions of these type of analytes.

In the logarithmic form (and regarding the relationship $\log \gamma_{A^2-B} = 4 \log \gamma_{A^2-BH^+}$) the relation between thermodynamic and mixed dissociation constants can be expressed as

$$pK_{a,2} = pK_{a,2}^{\text{mix}} - 3 \log \gamma_{A^2-BH^+} \quad (23)$$

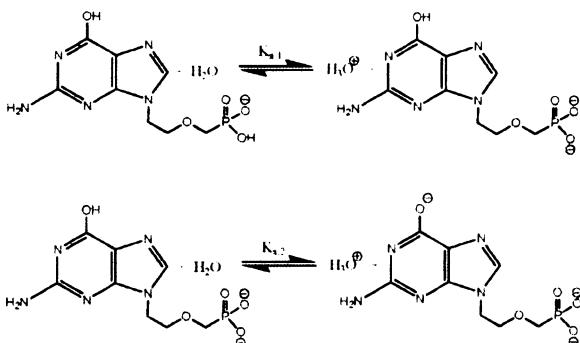
The dependence of the effective mobility of these types of analytes on pH is given by Eq. (24):

$$m_{\text{eff}} = \frac{m_{A^2-B} 10^{pK_{a,2}^{\text{mix}} - \text{pH}} - m_{A^2-BH^+} 10^{\text{pH} - pK_{a,1}^{\text{mix}}}}{1 + 10^{\text{pH} - pK_{a,1}^{\text{mix}}} + 10^{pK_{a,2}^{\text{mix}} - \text{pH}}} \quad (24)$$

where $m_{A^2-BH^+}$ and m_{A^2-B} are the actual ionic mobilities of the ionic forms A^2-BH^+ and A^2-B , respectively.

3.4 9-(2-(Phosphonomethoxy)ethyl)guanine

PMEG (**9**) behaves as a trivalent acid owing to the presence of two acidic anionogenic hydroxyl groups of phosphonic acid and single enol –OH group in position 6 of purine moiety. The phosphonic acid is fully dissociated into the first degree below pH 3.5; thus, in the applied pH range (3.50–11.25), only the dissociation of hydrogen phosphonate (–1) to phosphonate (–2) and the dissociation of the enol –OH group can be monitored by CZE. These two acid-base equilibria of PMEG can be described as shown in Scheme 4.



Scheme 4

The corresponding thermodynamic and mixed dissociation constants of phosphonic acid group to the second dissociation degree can be expressed as

$$K_{a,1} = \frac{a_{H_3O^+} a_{HA^{2-}}}{a_{H_2A^-}} = \frac{a_{H_3O^+} c_{HA^{2-}} \gamma_{HA^{2-}}}{c_{H_2A^-} \gamma_{H_2A^-}} = K_{a,1}^{\text{mix}} \frac{\gamma_{HA^{2-}}}{\gamma_{H_2A^-}} \quad (25)$$

where $a_{HA^{2-}}$, ($c_{HA^{2-}}$) and $a_{H_2A^-}$, ($c_{H_2A^-}$) are activities (concentrations) of double and single charged anions of **9** (PMEG), respectively.

Similarly as in the previous Sections 3.2 and 3.3 (regarding the relation $\log \gamma_{HA^{2-}} = 4 \log \gamma_{H_2A^-}$) the relation between thermodynamic and mixed dissociation constants can be expressed as:

$$pK_{a,1} = pK_{a,1}^{\text{mix}} - 3 \log \gamma_{H_2A^-} \quad (26)$$

Thermodynamic and mixed dissociation constants of the dissociation of the enol hydroxyl group at position 6 of purine, $K_{a,2}$ and $K_{a,2}^{\text{mix}}$, respectively, can be expressed as

$$K_{a,2} = \frac{a_{H_3O^+} a_{A^{3-}}}{a_{HA^{2-}}} = \frac{a_{H_3O^+} c_{A^{3-}} \gamma_{A^{3-}}}{c_{HA^{2-}} \gamma_{HA^{2-}}} = K_{a,2}^{\text{mix}} \frac{\gamma_{A^{3-}}}{\gamma_{HA^{2-}}} \quad (27)$$

where $a_{A^{3-}}$, ($c_{A^{3-}}$), and $a_{HA^{2-}}$, ($c_{HA^{2-}}$) are the activities (concentrations) of the triple charged and double charged anions of **9** (PMEG), respectively.

Following the above procedure and regarding the relationships $\log \gamma_{HA^{2-}} = 4 \log \gamma_{H_2A^-}$ and $\log \gamma_{A^{3-}} = 9 \log \gamma_{H_2A^-}$, the relation between thermodynamic and mixed dissociation constants can be expressed as

$$pK_{a,2} = pK_{a,2}^{\text{mix}} - 5 \log \gamma_{H_2A^-} \quad (28)$$

The dependence of the effective mobility of this type of analyte on pH is given by Eq. (29):

$$m_{\text{eff}} = \frac{m_{HA^{2-}} 10^{pK_{a,1}^{\text{mix}} - \text{pH}} + m_{A^{3-}} 10^{pK_{a,2}^{\text{mix}} - \text{pH}}}{1 + 10^{pK_{a,1}^{\text{mix}} - \text{pH}} + 10^{pK_{a,2}^{\text{mix}} - \text{pH}}} \quad (29)$$

where $m_{A^{3-}}$ and $m_{HA^{2-}}$ are the actual ionic mobilities of the ionic forms A^{3-} and HA^{2-} , respectively.

4 Results and discussion

4.1 Selection of experimental conditions

For pK_a determination by CZE it is important to select experimental conditions in such a way that the measured effective mobilities are obtained as precisely and accurately as possible. As stated above, the actual ionic and effective mobilities depend on ionic strength and temperature. Consequently, the mobilities should be measured in the BGEs of constant ionic strength and at constant temperature. Measurements of effective mobilities in the pH range 3.50–11.25 allowed us to use the BGEs of constant ionic strength, 25 mM. The appropriate mixtures of the buffering anionic constituents with pK_a values close to pH of the BGE ($pH_{\text{BGE}} = pK_a \pm 0.7$) and strong cationic counterion (Tris for formic acid-based BGEs, pH 3.50–4.25, sodium for the other BGEs) were used for preparation of BGEs with this constant ionic strength and also with sufficient buffering capacity. The mobility measurement at constant temperature of 25°C was ensured by thermostating the outer polyimide capillary surface to constant temperature of 24°C and by setting the input power to the value of 0.66 W per meter of the capillary length, which causes 1°C temperature increase inside the capillary, resulting in the constant temperature of 25°C inside the capillary (see Section 2.4).

Another important factor is selection of the detection wavelength of the UV-absorption detector. With the photodiode array detector it was possible to detect the analytes in the broad wavelength range 190–300 nm. The detection wavelength of the analyzed compounds was set at their absorption maximum, at 225 nm, allowing their detection at low-concentration level below 0.1 mM, whereas the optimal detection wavelength for determination of migration time of the EOF marker (DMSO or isophorone) was 250 nm. The examples of CZE analyses of one of the investigated analytes, 2,6-diamino-9-(2-(phosphonomethoxy)ethyl)-9*H*-purine (compound 8, PMEDAP) in three different BGEs are shown in Fig. 2. Figure 2A shows the analysis of this compound in the strongly acidic Tris-formate BGE, pH 3.75, in which PMEDAP is present mostly in its zwitterionic form with slightly negative effective charge resulting in counter EOF anionic migration with migration time close behind that of the EOF marker. In the weakly acidic sodium/MOPS BGE, pH 6.5, the effective charge of PMEDAP is close to -1 elementary charge due to the deprotonation of the N1 of purine resulting in the higher effective mobility and more distant position of the PMEDAP peak behind the EOF marker peak in Fig. 2B. CZE analysis of PMEDAP in alkaline sodium/CAPS BGE, pH 10.5 (see Fig. 2C) shows further increase of the relative migration time difference between PMEDAP and EOF marker peaks as a result of the increased PMEDAP effective charge and mobility due to the dissociation of the phosphonic acid group to the second degree. All these figures show the presence of small amounts of nonidentified admixtures (peaks X) in the synthetic PMEDAP preparation, which were not detected by HPLC analysis of this compound. This confirms complementarity of CZE and HPLC separation methods.

4.2 Estimation of effective charge and effective electrophoretic mobility

In the applied pH range the effective charge of (amino)-guanidino 9-alkylpurines (**1–4**) and acyclic nucleoside phosphonate isopropyl diesters of (amino)-guanidinopurines (**5** and **6**) was changed from +1 elementary charge (e) at pH 3.50 to charge zero at pH 11.25 due to the deprotonation of the guanidinyl group. Effective mobility of the fully protonated cationic form of these compounds at pH 3.5 was in the range $(20.7\text{--}24.5)\times 10^{-9}\text{ m}^2\text{V}^{-1}\text{s}^{-1}$ for (amino)guanidino 9-isopropylpurines (**1–4**) and around the $16.3\times 10^{-9}\text{ m}^2\text{V}^{-1}\text{s}^{-1}$ value for acyclic nucleoside phosphonate diisopropyl esters (**5** and **6**), respectively. The lower mobility of **5** and **6** compared to the mobility of **1–4** reflects the lower charge to mass ratio of the former compounds due to their higher M_r at approximately the same charge as that of the latter compounds. The effec-

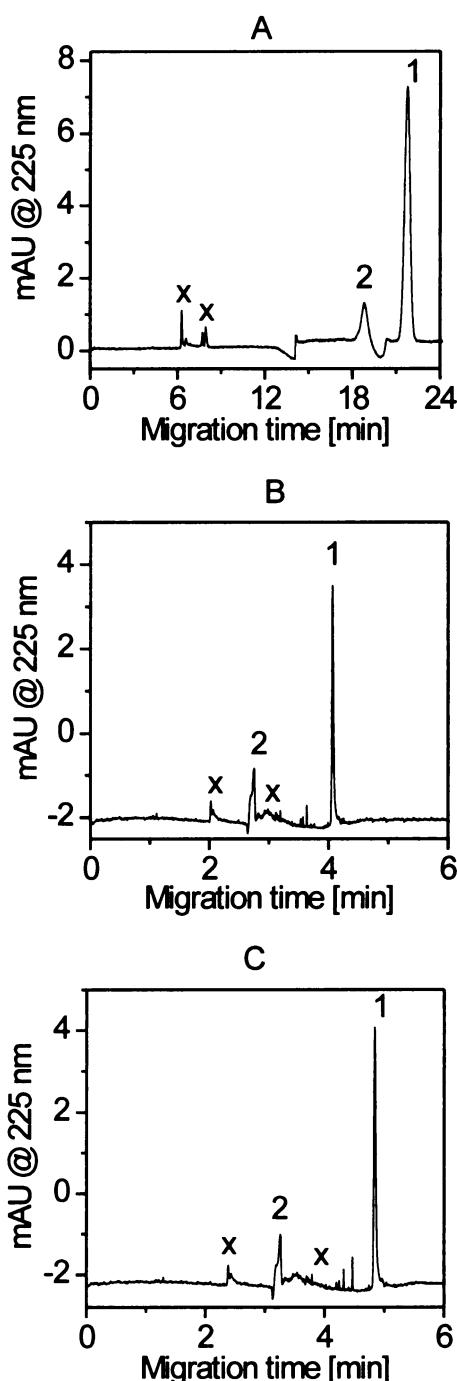


Figure 2. Selected records of CZE analysis of compound 8 (PMEDAP) in three different BGEs: (A) 47 mM HCOOH + 25 mM Tris, pH 3.75; (B) 132 mM MOPS + 25 mM NaOH, pH 6.50; (C) 41 mM CAPS + 25 mM NaOH, pH 10.50. Capillary total/effective length 394/292 mm, ID/OD 75/360 μm ; hydrodynamic sample injection 6.9–13.8 mbar for 5–10 s; UV-absorption detection at 225 nm; sample dissolved in deionized water in concentration 0.1 mM. 1, PMEDAP; 2, EOF marker; X, nonidentified admixtures.

tive charge and the effective mobility of the acyclic nucleoside phosphonates of aminopurines (**7** and **8**) were close to zero at pH 3.5, resulting from their zwitterionic forms, in which the positive charge of protonated nitrogen in position 1 (N1) of purine moiety is compensated by the negative charge of the hydrogenphosphonate (−1) group. Effective charge of these compounds at alkaline pH 11.25 has changed to −2 e, and effective mobility was in the range $(31.5\text{--}32.1)\times 10^{-9}\text{ m}^2\text{V}^{-1}\text{s}^{-1}$.

Effective charge of **9** (PMEG) was negative in the whole applied pH range of BGEs being close to −1 e at pH 3.5 due to the dissociation of phosphonic acid group to the first degree and possessing increasing negative charge with increasing pH; at weakly alkaline BGEs the charge achieved a value of −2 e due to the dissociation of hydrogenphosphonate (−1) to phosphonate (−2), and at strongly alkaline pH 11.25 the charge was close to −3 e, since at this pH also the weakly acidic enol −OH group at position 6 of purine moiety has been almost fully dissociated. Consequently, the effective mobility of this analyte was also negative within the whole applied pH range achieving values of $-17.9\times 10^{-9}\text{ m}^2\text{V}^{-1}\text{s}^{-1}$ for monovalent anion, $-29.5\times 10^{-9}\text{ m}^2\text{V}^{-1}\text{s}^{-1}$ for divalent anion and $-39.7\times 10^{-9}\text{ m}^2\text{V}^{-1}\text{s}^{-1}$ for trivalent anion.

The effective charge of acyclic nucleoside phosphonates of (amino)-guanidino purines with guanidinyl group in position 2 or 6 of purine moiety (compounds **10**–**13**) was close to zero at pH 3.5, resulting from their zwitterionic forms, in which the positive charge of protonated guanidinyl group is compensated by the negative charge of hydrogenphosphonate (−1) group. Effective charge of these compounds at weakly alkaline BGEs was close to −1 e with effective mobility in the range $(-12.6\text{ to }-15.4)\times 10^{-9}\text{ m}^2\text{V}^{-1}\text{s}^{-1}$ and at alkaline pH 11.25 the charge was close to −2 e, and effective mobility was in the range $(-22.5\text{ to }-27.4)\times 10^{-9}\text{ m}^2\text{V}^{-1}\text{s}^{-1}$. The values of effective charges and effective mobilities at 25°C in BGEs of 25 mM ionic strength and at pH 3.5 and 11.25 are summarized in Table 3.

4.3 Determination of thermodynamic dissociation constants

The calculation of mixed dissociation constants was accomplished by nonlinear fitting of the sets of experimentally determined effective electrophoretic mobilities within a broad pH range using the computer program Origin with appropriate regression functions, Eqs. (9), (19), (24), and (29). The courses of these functions for monovalent bases of (amino)guanidino 9-alkylpurines (**1**–**4**) and the acyclic nucleoside phosphonate diisopropyl esters (**5** and **6**) are depicted in Fig. 3, for the zwitterionic

Table 3. Effective electrophoretic mobilities, m_{eff} , and effective charges, q , at pH 3.5 and 11.25

Compound number	$m_{\text{eff}}, 10^{-9}\text{ m}^2\text{V}^{-1}\text{s}^{-1}$		$q, (\text{e})$	
	pH 3.5	pH 11.25	pH 3.5	pH 11.25
1	24.5	0	+1.0	0
2	21.3	0	+1.0	0
3	21.7	0	+1.0	0
4	20.7	0	+1.0	0
5	16.9	0	+1.0	0
6	16.4	0	+1.0	0
7 (PMEMAP)	0	-31.4	0	-2
8 (PMEDAP)	0	-30.4	0	-2
9 (PMEG)	-17.9	-39.7	-1.0	-3
10	7	-26.9	0	-2
11	0	-26.7	0	-2
12	0	-28.0	0	-2
13	0	-23.1	0	-1.8

a) Related to standard temperature 25°C and ionic strength 25 mM.

to divalent anionic acyclic nucleoside phosphonate of aminopurines (**7** and **8**) and for the trivalent anionic PMEG (**9**) in Fig. 4, and for the zwitterionic to divalent anionic acyclic nucleoside phosphonates of (amino)guanidino 9-alkylpurines (**10**–**13**) in Fig. 5. The values of correlation coefficient, mixed dissociation constants and actual cationic or anionic mobilities of the individual ionic species of the analyzed compounds are presented in the text parts of the figures. Thermodynamic dissociation constants, pK_a , were recalculated from mixed dissociation constants using Eq. (8) for dissociation of protonated guanidinyl group in amino-guanidino-9-alkylpurines (**1**–**4**) and the acyclic nucleoside phosphonate diisopropyl esters (**5** and **6**), Eqs. (14) and (18) for dissociation of protonated N1 of purine moiety and dissociation of phosphonic acid group to the second degree, respectively, in acyclic nucleoside phosphonates of aminopurines (**7** and **8**), Eqs. (21) and (23) for dissociation of phosphonic acid group to the second degree and dissociation of protonated guanidinyl group, respectively, in the acyclic nucleoside phosphonates of (amino)-guanidino 9-alkylpurines (**10**–**13**), and Eqs. (26) and (28) for dissociation of phosphonic acid group to the second degree and for dissociation of enol −OH group, respectively, in PMEG (**9**).

Thermodynamic pK_a of the protonated guanidinyl group was determined to be in the range 7.77–9.64 for (amino)-guanidino 9-alkylpurines (**1**–**4**), 9.85 and 9.26 for their acyclic nucleoside phosphonate diisopropyl esters (**5** and **6**) and 8.29–10.32 for their acyclic nucleoside phosphonates, pK_a of a phosphonic acid group to the second dissociation degree (−2) was found to be in the range

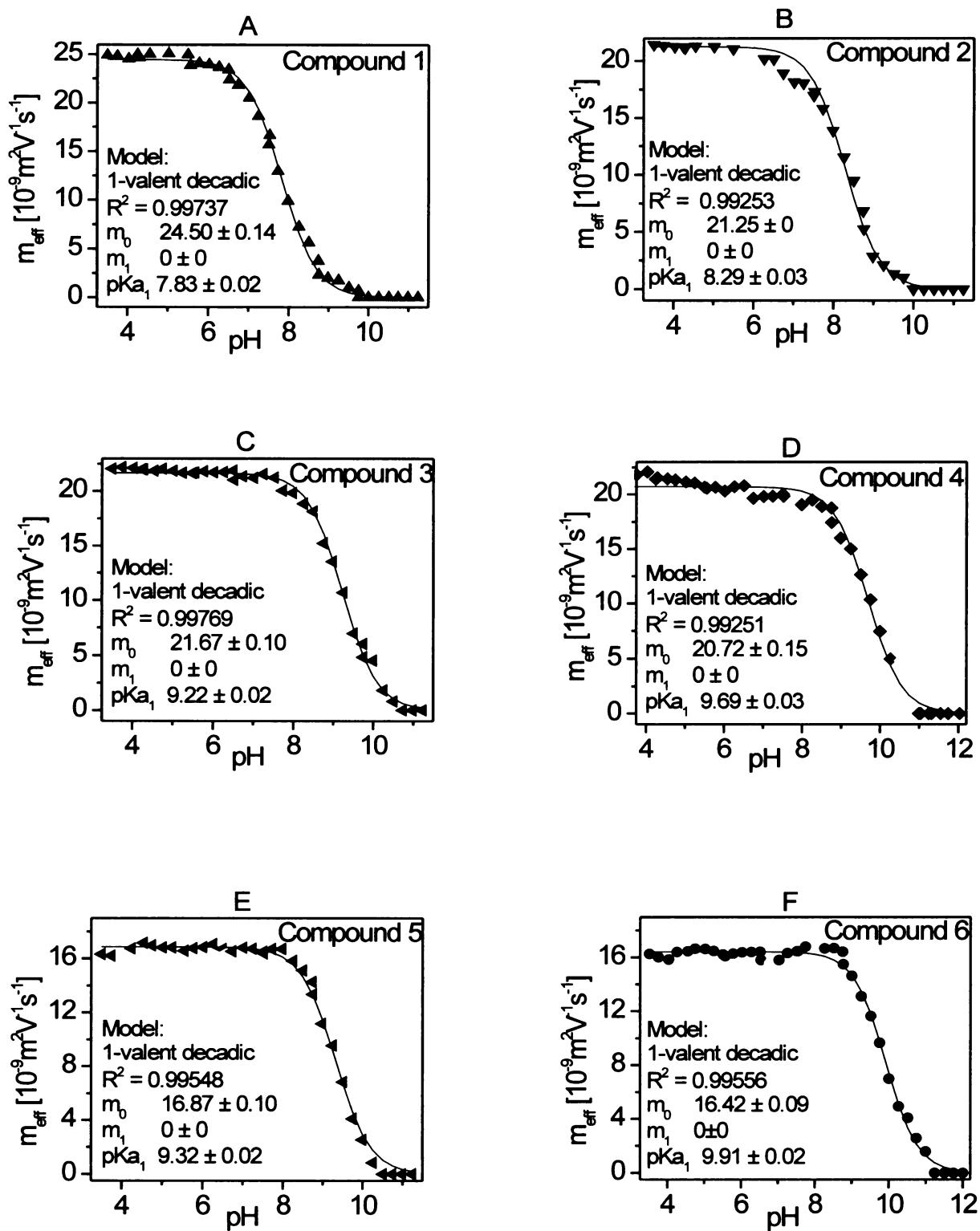


Figure 3. Dependencies of effective electrophoretic mobilities, m_{eff} , of (amino)guanidino 9-alkyl purines (**1–4**) and the acyclic nucleoside phosphonate diisopropyl esters (**5** and **6**) on pH. R , correlation coefficient; m , actual ionic mobility; pK_a , mixed dissociation constant.

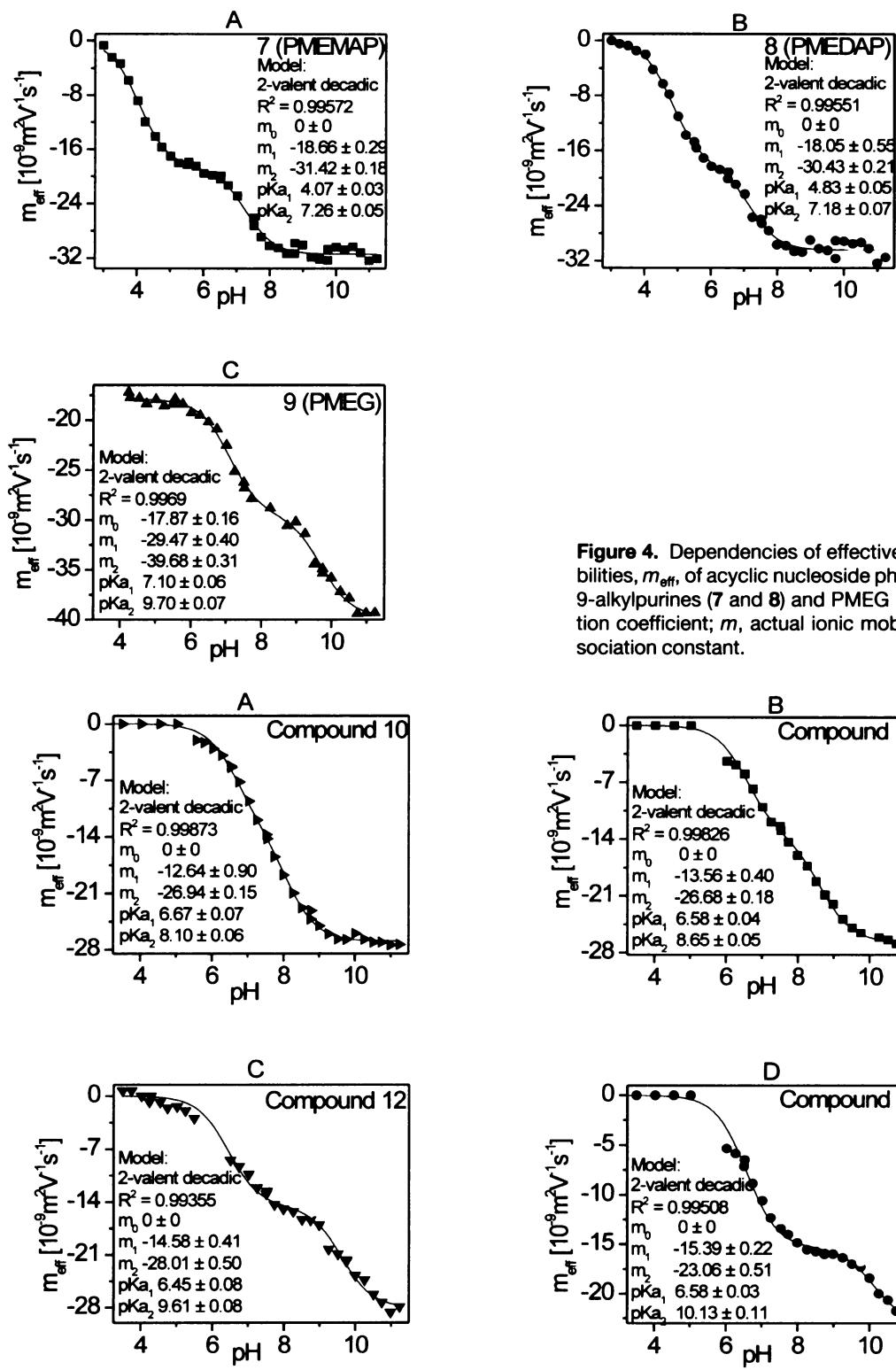


Figure 4. Dependencies of effective electrophoretic mobilities, m_{eff} , of acyclic nucleoside phosphonates of amino 9-alkylpurines (7 and 8) and PMEG (9) on pH. R , correlation coefficient; m , actual ionic mobility; pK_a , mixed dissociation constant.

Figure 5. Dependencies of effective electrophoretic mobilities, m_{eff} , of acyclic nucleoside phosphonates of (amino)guanidino 9-alkylpurines (10–13) on pH. R , correlation coefficient; m , actual ionic mobility; pK_a , mixed dissociation constant.

Table 4. Thermodynamic pK_a values of ionogenic groups of analyzed compounds (at 25°C)

Compound number	pK_a		
	(N1)H ⁺ /(N1)	P(OH)O ⁻ /P(O) ₂ ²⁻	(G)H ⁺ /(G)
1	n.d.	n.p.	7.77 ± 0.02
2	n.d.	n.p.	8.23 ± 0.03
3	n.d.	n.p.	9.16 ± 0.02
4	n.d.	n.p.	9.64 ± 0.03
5	n.d.	n.p.	9.26 ± 0.02
6	n.d.	n.p.	9.85 ± 0.03
7 (PMEMAP)	4.13 ± 0.04	7.46 ± 0.06	n.p.
8 (PMEDAP)	4.89 ± 0.05	7.37 ± 0.07	n.p.
9 (PMEG)	n.d.	7.29 ± 0.06	10.02 ± 0.07 (enol) ^a
10	n.d.	6.86 ± 0.06	8.29 ± 0.06
11	n.d.	6.77 ± 0.04	8.84 ± 0.05
12	n.d.	6.64 ± 0.08	9.80 ± 0.08
13	n.d.	6.77 ± 0.03	10.32 ± 0.11

a) The value belongs to the dissociation of the enol hydroxyl group of PMEG, guanidinyl group is not present in PMEG.

(N1)H⁺/(N1), dissociation of protonated N1 in purine moiety; P(OH)O⁻/P(O)₂²⁻, dissociation of phosphonic acid group to the second degree (−2); (G)H⁺/(G), dissociation of protonated guanidinyl group; n.d., not determined; n.p., not present.

6.64–6.86 for acyclic nucleoside phosphonates of (amino)-guanidinopurines (**10–13**) and in the range 7.29–7.46 for acyclic nucleoside phosphonates of aminopurines (**7** and **8**). pK_a of protonated nitrogen in position 1 (N1) of purine moiety was estimated to be 4.13 and 4.89 for acyclic nucleoside phosphonates of aminopurines (**7** and **8**). The values of thermodynamic pK_a with their SD are presented in Table 4.

From the comparison of these pK_a values the following conclusions can be drawn: Basicity of the guanidino derivatives (compounds **1–6** and **10–13**) with pK_a values in the range 7.77–10.32 is much higher (by three to six orders of magnitude) than that of the amino derivatives (**7** and **8**) with pK_a equal to 4.13 and 4.89, respectively. The stronger basicity of the guanidino derivatives compared to amino derivatives is reflected by the increased acidity of phosphonic acid in the former derivatives, see the pairs **12** (pK_a 6.64) versus **7** (pK_a 7.46), and **13** (pK_a 6.77) versus **8** (pK_a 7.37). Basicity of the derivatives with guanidinyl group in position 2 of purine is significantly larger (ca. by 1.5 unit difference in pK_a) than that of the compounds having guanidinyl group in position 6 of purine, see the pairs **3** (pK_a 9.16) versus **1** (pK_a 7.77), **4** (pK_a 9.64) versus **2** (pK_a 8.23), **12** (pK_a 9.80) versus **10** (8.29), and **13** (pK_a 10.32) versus **11** (pK_a 8.84). Basicity of the amino-guanidino derivatives is higher than that of the guanidino derivatives (ca. by the 0.5 unit difference in pK_a), see the pairs **2** (pK_a 8.23) versus **1** (pK_a 7.77), **4** (pK_a 9.64) versus **3** (pK_a 9.16), **6** (pK_a 9.85) versus **5** (pK_a 9.26), **11** (pK_a 8.84) versus **10** (pK_a 8.29), **13** (pK_a 10.32) versus **12** (pK_a 9.80).

Similarly, the basicity of PMEDAP (**8**) (pK_a 4.89) with two amino groups in positions 2 and 6 of purine is higher than that of PMEMAP (**7**) (pK_a 4.13) with single amino group at position 2 of purine. As it was expected the basicity of the guanidinyl derivatives with free phosphonic acid group was found higher, ca. by 0.5–0.6 unit of pK_a , than that of 9-alkyl derivatives, see the pairs **10** (pK_a 8.29) versus **1** (pK_a 7.77), **11** (pK_a 8.84) versus **2** (pK_a 8.23), **12** (pK_a 9.80) versus **3** (pK_a 9.16), **13** (pK_a 10.32) versus **4** (pK_a 9.64). Approximately the same difference of basicity (0.5 unit of pK_a) was found in favor of the guanidinyl derivatives with free phosphonic acid when compared with their diisopropyl esters, see the pairs **12** (pK_a 9.80) versus **5** (pK_a 9.26), and **13** (pK_a 10.32) versus **6** (pK_a 9.85). In these last two cases apparently the basicity of the guanidinyl group is enlarged by the presence of anionogenic phosphonic acid group and by the potential of these compounds to form zwitterions. Basicity of the non-anionogenic diisopropyl esters of guanidino derivatives was only slightly higher (ca. 0.1–0.2 unit of pK_a) than that of the also nonanionogenic alkyl derivatives, see the pairs **5** (pK_a 9.26) versus **3** (pK_a 9.16), and **6** (pK_a 9.85) versus **4** (pK_a 9.64).

The determined pK_a values of the protonated nitrogen in position 1 of amino 9-alkylpurines, pK_a values of phosphonic acid to the second dissociation degree and pK_a values of enol hydroxyl group of PMEG were in a good agreement with pK_a values of these ionogenic groups in the related compounds determined by potentiometric pH titration [34].

In conclusion, CZE has proved to be a suitable and useful method for determination of dissociation constants (pK_a) of amino and (amino)guanidinopurine nucleotide analogs, such as acyclic nucleoside phosphonate, acyclic nucleoside phosphonate diesters and other related compounds, in a microscale, applying only few nanoliter sample volumes of 0.1 mM analyte solution per analysis. The determination of pK_a values of a series of the above related compounds allowed to quantify their basicity and to detect certain correlations between the basicity and the structure of these analytes.

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Veronika Šolínová
Václav Kašička

Institute of Organic Chemistry
and Biochemistry, Academy of
Sciences of the Czech Republic,
Prague, Czech Republic

Review

Recent applications of conductivity detection in capillary and chip electrophoresis

The review provides a comprehensive survey of the recent applications of contact and contactless conductivity detection in capillary electrophoretic and chip electrophoretic analyses of a broad scale of compounds, from low-molecular-mass highly mobile small inorganic and organic ions, via medium-molecular-mass peptides and oligo- and polynucleotides up to high-molecular-mass biopolymers, proteins and nucleic acids fragments. The review presents also the recent developments in the construction of different types of conductivity detectors (detectors with galvanic contact of the sensing electrodes with the BGE and sample components, contactless conductivity detectors with capacitively coupled tubular and semitubular electrodes and combined conductivity/optical detectors) applied in the capillary electromigration methods performed in classical fused silica, polytetrafluoroethylene, and polyetheretherketone capillaries or on glass and polymethylmethacrylate microchips. In addition, the principle and theoretical bases of conductivity detection in capillary electromigration techniques, zone electrophoresis, ITP, micellar EKC, and electrochromatography are briefly described.

Keywords: Capillary electrophoresis / Chip electrophoresis / Conductivity detection / Contactless conductivity detection

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1 Introduction

Contact conductivity detection and especially the contactless conductivity detection (CLCD) have become within the last years frequently used detection techniques in capillary electromigration methods [1–4]. The recent improvements in the construction of the capaci-

Correspondence: Dr. Václav Kašička, Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Flemingovo 2, 166 10 Prague 6, Czech Republic.
E-mail: kasicka@uochb.cas.cz.
Fax: +420-220-183-592.

Abbreviations: AA, amino acid; AC, alternating current; AcOH, acetic acid; AMP, 2-amino-2-methyl-1-propanol; AMPD, 2-amino-2-methyl-1,3-propanediol; BTP, Bis-Tris propane; 18C6E, 18-crown-6-ether; 18C6EH₄, 18-crown-6-ether-tetracarboxylic acid; CLCD, contactless conductivity detection; CTAH, cetyltrimethylammonium hydroxide; DC, direct current; DMAA, N,N-dimethylacetamide; FI-CE, flow injection-CE system; FS, fused silica; HDB, hexadimethrine bromide; HEC, hydroxyethylcellulose; HF, high frequency; HIBA, α -hydroxyisobutyric acid; HP- β -CD, hydroxypropyl- β -CD; IE-CEC, ion-exchange CEC; MeOH, methanol; NACE, nonaqueous CE; PC, propylene carbonate; PMMA, poly(methylmethacrylate); p-p, peak to peak; TAPS, N-tris(hydroxymethyl)-methyl-3-aminopropanesulfonic acid; TE, terminating electrolyte; TRH, thyrotropin releasing hormone; TTAB, tetradecyltrimethylammonium bromide; Tween 20, polyoxyethylene sorbitan monolaurate

tively coupled contactless conductivity detectors and their electronic circuitry have led to the higher sensitivity and robustness of these detectors, and also to their commercial utilization. In addition, due to their miniature dimensions and relatively simple construction, the conductivity detectors are also successfully integrated in the microfluidic chip electrophoresis devices. The increasing number of applications shows that conductivity detection is suitable not only for detection of chromophore- and fluorophore-free small inorganic and organic ions but also for other types of ionogenic compounds, including medium- and high-molecular-mass biomolecules, such as peptides, proteins, oligo- and polynucleotides, and nucleic acids fragments.

Conductivity detection in CE is performed in two modes either with or without direct contact of the sensing electrodes with the BGE and the sample components. The first conductivity detectors used in the CE methods had the electrodes in contact with the BGE. They were developed already in the 1970s for capillary ITP (CITP) [5, 6]. They have been much more sensitive than the previously used temperature detectors but their great disadvantage was that during the electromigration passage through the detector the BGE constituents and sample components interacted electrochemically or by a simple adsorp-

tion with the surface of the electrodes, which caused defects and low reproducibility of the detection signal. These unfavorable phenomena are avoided in contactless conductivity detectors that have been first used also for CITP [7, 8] performed in PTFE (Teflon) capillaries. Later on, in 1998, using a new concept of capacitively coupled in-series placed two open tubular electrodes with detection cell between them, Zemann *et al.* [9] and da Silva and do Lago [10] have introduced the application of CLCD also for CZE performed in the fused-silica (FS) capillaries. In 2001, the contactless conductivity detector was first integrated on a chip electrophoresis device [11].

This review presents a short introduction to the principle and theoretical bases of conductivity detection in CE, describes the recent developments of the conductivity detectors and provides a comprehensive survey of applications of conductivity detection to monitor separations and analyses of different types of analytes by capillary electromigration methods performed in classical capillary format and in microchips.

2 Basic principles of conductivity detection in CE

In general, conductivity detection is based on the application of alternating current (AC) voltage of few kHz to few MHz frequency between two electrodes. The generated electric current (indirectly proportional to the resistance or directly proportional to conductance of the solution between the electrodes according to Ohm's law) is measured and yields the resistance or conductivity, respectively. Conductance of the BGE, G_{BGE} , containing all ionic species E^+ , E^- , depends on their equivalent conductivities, λ_{E^+} , λ_{E^-} , their concentrations in the BGE, $c_{\text{E}^+}^C$, $c_{\text{E}^-}^C$, and on their degrees of dissociation, α_{E^+} , α_{E^-}

$$G_{\text{BGE}} = \frac{\lambda_{\text{E}^+} c_{\text{E}^+}^C \alpha_{\text{E}^+} + \lambda_{\text{E}^-} c_{\text{E}^-}^C \alpha_{\text{E}^-}}{10^{-3} K} \quad (1)$$

K is the constant of the detection cell, which depends on area and distance of the electrodes. Further, in order to simplify the presented equations, only the fully dissociated electrolytes are considered. The conductance of analyte zone, G_s , containing an anionic analyte A^- in the medium of the BGE solution, is determined by the concentration of the BGE ionic species E^+ , c_{E^+} , and E^- , c_{E^-} , and concentration of the anionic analyte A^- , c_{A}^S , and their equivalent conductivities, λ_s

$$G_s = \frac{\lambda_{\text{E}^+} c_{\text{E}^+}^S + \lambda_{\text{E}^-} c_{\text{E}^-}^S + \lambda_s c_{\text{A}}^S}{10^{-3} K} \quad (2)$$

The counter cation A^+ of the analyte is not considered since in the applied electric field it migrates in a direction opposite to that of the anion and does not reach the detection cell. The concentrations of E^+ and E^- in the BGE

and in the sample zone are related according to the following equations:

$$c_{\text{E}^+}^S = c_{\text{E}^+}^C + (1 - k_A) c_A^S \quad (3)$$

$$c_{\text{E}^-}^S = c_{\text{E}^-}^C - k_A c_A^S \quad (4)$$

where k_A is the transfer ratio of the analyte anion A^- , which is equal to the number of equivalents of the BGE co-ions displaced by an equivalent of the analyte ions. Consequently, the conductance of the sample zone can be expressed as

$$G_s = \frac{\lambda_{\text{E}^+} [c_{\text{E}^+}^C + (1 - k_A) c_A^S] + \lambda_{\text{E}^-} [c_{\text{E}^-}^C - k_A c_A^S] + \lambda_s c_A^S}{10^{-3} K} \quad (5)$$

The detector signal of the sample zone originates from the difference in conductance ΔG of sample zone and of the BGE

$$\Delta G = G_s - G_{\text{BGE}} = \frac{c_A^S [\lambda_{\text{E}^+} (1 - k_A) - \lambda_{\text{E}^-} k_A + \lambda_s]}{10^{-3} K} \quad (6)$$

From Eq. (6) it follows that the detector signal, which is directly proportional to ΔG , depends on the equivalent conductivities of the analyte anion A^- , of the BGE co-ion E^+ and of the BGE counterion E^- . ΔG is also a function of the transfer ratio k_A , which is determined by the Kohlrausch regulating function, ω

$$\omega = \sum_i \frac{c_i z_i}{|m_i|} \quad (7)$$

where c_i , z_i , and m_i are molar concentrations, charge number and electrophoretic mobility of the i -th ion.

For the monovalent strong anions, the transfer ratio can be derived from the Kohlrausch's regulating function as

$$k_A = \frac{m_E (m_A + m_{E^-})}{m_A (m_E + m_{E^+})} \quad (8)$$

where m indicates electrophoretic mobilities of the respective ions. Taking into account the relation between conductivities and mobilities of species X , $\lambda_X = Fm_X$, where F is the Faraday constant, Eq. (6) can be rewritten as

$$\Delta G = G_s - G_{\text{BGE}} =$$

$$\frac{c_A^S (m_A - m_E)(m_A + m_{E^-})}{m_A} \cdot \frac{F}{10^{-3} K} \quad (9)$$

Equation (9) clearly shows that the sensitivity of the conductivity detection is directly proportional to the difference in mobilities of the analyte and the BGE co-ion as well as the mobilities of the analyte and counterion. Hence, for maximal sensitivity of the conductivity detection, *i.e.*, for the highest S/N, theoretically it would be most suitable to use the BGE composed of co-ion and counterion the mobilities of which differ as much as pos-

sible from the mobilities of the sample ions. However, this counteracts the other conditions for high-efficient CE separations. The separation efficiency is maximized (electromigration dispersion is minimized) if the difference between analyte ion and BGE co-ion mobilities is minimized. With the increasing difference between sample ions and BGE co-ions, the peak symmetry as well as separation efficiency is falling down. If the BGE co-ion mobility is lower or higher than that of sample ion, the separation efficiency is reduced by peak fronting or peak tailing, respectively. The use of highly mobile BGE counterion is also problematic, since from one point of view it increases the detector signal but on the other hand it increases the conductivity of the BGE resulting in a limitation of using a high intensity of the electric field to prevent Joule heating effects (temperature increase and gradients) inside the capillary. Thus, in practical CE separations with conductivity detection, a compromise between detection sensitivity and separation efficiency has to be chosen for the BGE composition.

3 Conductivity detectors

3.1 Contact conductivity detectors

The first conductivity detectors used in the capillary electromigration methods had the electrodes in galvanic contact with the BGE. They were developed already in 1970s for CITP [5, 6], which was the first electromigration technique performed in the capillary format. The platinum microwire electrodes (od 0.05 mm) were sealed in the thin microholes, which were cut out in the walls of PTFE capillaries (id/od 0.45/0.7 mm). AC voltage of a few kHz frequency was applied on the electrodes and from the produced electric current the resistance or the conductivity of the migrating sample zones was measured. Alternatively, the direct current (DC) voltage (potential gradient) between the two microelectrodes placed in series (od 0.05 mm) with 0.1 mm distance was measured utilizing the DC driving current of ITP analysis to monitor the conductivity of ITP zones.

Contact conductivity detectors for modern high-performance capillary electromigration methods (HPCE) performed in the FS capillaries with internal diameter below 100 µm are implemented either in on-column or in the end-column mode. In the on-column mode, the two microelectrodes are fixed in the microholes drilled through the capillary wall whereas in the end-column mode one microelectrode is placed at the capillary outlet and the second electrode is placed close to the first electrode in the electrode vessel. The first on-column conductivity detector for CZE was designed by Zare and coworkers [12]. The platinum microwires were aligned and sealed in two diametrically positioned 40 µm holes made by drilling the FS capillary wall by a CO₂ laser. The con-

ductivity changes of the BGE and sample zones were monitored by applying an AC voltage with oscillation frequency of 3.5 kHz between the electrodes. A transformer was used as a galvanic insulator between the sensing electrodes, which have a high DC potential to ground. Few years later in the same group the end-column conductometric detector was developed [13]. The sensing Pt microelectrode was centered at the capillary outlet and held by epoxy in the protective plastic jacket (1 cm long FS capillary), which was placed inside the outlet reservoir containing the grounding electrode. The conductivity measurement is made between the sensing microelectrode and the grounding electrode using an AC circuit, which was the same as that for the on-column detector. The end-column conductivity detection is technologically a simpler approach, since only a single metal microelectrode is placed, as close as possible to the capillary outlet. Conductivity changes are then measured between this sensing electrode and a second electrode, which is also positioned close to the capillary outlet but in fact already in the electrode vessel containing the BGE.

Another construction of the end-column conductivity detector was designed by Opekar and coworkers [14]. A Pt ring electrode was chemically deposited at the capillary end and at the other electrode, a Pt wire was immersed together with the capillary in the electrode vessel. This detector was further modified by the usage of a hydrophilic strip to ensure conductive contact between the BGE solution at the capillary outlet and the BGE in the electrode vessel as well as between the BGE at the capillary outlet and the sensing electrode [15, 16]. The cellophane strip was cemented to the end of a thin, tin-covered wire using a small drop of an electrically conductive silver epoxy resin. This drop served as one of the two conductometric electrodes. The wire with the cemented cellophane strip was fixed at the end of the separation capillary by thin PTFE tubing or by a PTFE ribbon. The free end of the cellophane strip was immersed in the BGE solution in the electrode vessel, where also the second Pt wire was placed. The conductivity was measured by a simple conductometer using an alternating voltage with an effective value of 1.0 V and a frequency of 1.0 kHz.

In the mid 1990s the contact conductivity detector has become commercially available for the postcolumn conductivity detection in CE devices. The applications of contact conductivity detectors in CE methods are summarized in Table 1.

3.2 Contactless conductivity detectors in capillaries

CLCD or more exactly capacitively coupled contactless conductivity detection (C²D) is a particular type of electric conductivity measurement based detection where the electrodes are not in direct contact with the meas-

Table 1. Application of contact conductivity detectors in CE

Analyte (LOD or concentration range)/matrix ^{a)}	CE mode BGE composition	Capillary material and diameters (id/od)	Electrodes arrangement Frequency	References
K ⁺ , Rb ⁺ , Na ⁺ , Li ⁺ (0.1 μM), Arg, His, tetramethylammonium, triethylamine/human serum	CZE 20 mM MES, 20 mM His, pH 6.1 5 mM potassium acetate, pH 5.4	FS: 50, 75/320 μm	On-column, Pt wires covered by epoxy resin $f = 3.5$ kHz	[12, 106, 107]
Ions of carboxylic acids: formate, acetate, propanoate, butanoate, pentanoate, hexanoate, heptanoate	10 mM MES, 10 mM His, pH 6.0. 0.5 mM TTAB 5 mM Cl ⁻ adj. to pH 7.1 by Tris, 0.5 mM TTAB			
Fumarate, methylene succinate, malate, methyl malate	10 mM MES, 10 mM His, pH 6.0. 0.5 mM TTAB			
Tartarate, malate, succinate, citrate, acetate, lactate/wine α-ketoglutarate, malate	7 mM MES, 7 mM His, pH 6.0, 0.5 mM TTAB, 30% MeOH 5 mM Cl ⁻ adj. to pH 7.1 by Tris, 0.4 mM TTAB 5 mM Cl ⁻ adj. to pH 3.1 by Gly, 0.6 mM TTAB	FS: 80/355 μm	End-column, Pt wires covered by epoxy resin, $f = 3.5$ kHz	[13]
Carboxylate ions: formate, acetate, propanoate, butanoate, pentanoate, hexanoate	CZE 20 mM MES, 20 mM His, pH 6.0, 1 mM TTAB	FS: 75/320 μm	Deposited Pt rings, AC current 5 μA $f = 1$ kHz	[14]
K ⁺ (3 nM)	CZE 20 mM sodium tetraborate	FS: 75/375 μm	End-column: 1. Cellophane strip, on Sn-covered Cu wire covered by Ag epoxy resin, 2. Pt wire $f = 1$ kHz	[15, 16]
K ⁺ (26 μM), Na ⁺ (52 μM)/mineral waters	CZE 30 mM MES, 15 mM LiOH, pH 6.1			

^{a)} Matrix is specified for real world samples only, in the other cases model analyte mixtures are involved.

ured solution. The fundamentals as well as important factors influencing the characteristics of CLCD in capillary and microchip electrophoresis were recently reviewed by Geraldo *et al.* [17, 18].

The first prototype of CLCD was constructed in the early 1980s for CITP in the research group of Vacík and Gaš [7, 8, 19]. The detector consisted of a capacitive cell formed by four electrodes (copper enameled wires) arranged perpendicularly and equiplanar around the circumference of the PTFE capillary (id/od 0.45/0.70 mm). The input signal was produced by the high frequency (HF) generator (1 MHz) and led to the two emitting electrodes. The output signal was picked up by the other two electrodes and amplified by the receiver. This four-electrode arrangement of the high-frequency CLCD detector was used later with some modifications in the working group of Kaniansky not only for CITP but also for CZE performed both in the PTFE (id/od 300/500 μm) and FS capillaries (id/od 300/430 μm) [20–22]. A little bit higher sensitivity was achieved in FS than in the PTFE capillaries due to their thinner capillary walls and more suitable capacitance values.

In 1998, a new approach for CLCD in CE has been introduced Zemann *et al.* [9]. The detector consisted of two tubular electrodes for capacitive coupling, which were

placed cylindrically around the outer surface of the FS capillary (see Fig. 1). The electrodes were of 30 mm length, cut from the syringe needles and they were separated with a detection gap of 2 mm. This design was further modified by the application the copper foil between the two electrodes, which had a shielding function [23]. The sensing electrodes were connected to an oscillator through a resistor. A high audio or low ultrasonic frequency for coupling of the AC voltage was used.

In the same year, almost simultaneously and independently, the same approach for CLCD detection in CZE was reported by da Silva and do Lago [10]. In their design, the sensing electrodes were painted directly on the outer capillary wall with the silver varnish. And the FS capillary was placed between poly(methylmethacrylate) (PMMA) bases and silicone septas. A ground plane was inserted between the two sensing electrodes. A function generator was connected with an electrode, which generated a HF sinusoidal signal, and the other one was connected to the input of a current-to-voltage converter. Few years later da Silva *et al.* [24] presented the improved detector, which was smaller and easier to operate. The detector could be placed both at the low- or high-voltage end of the capillary. Amplitude and frequency of the input signal should be optimized for each capillary diameter and BGE composition.

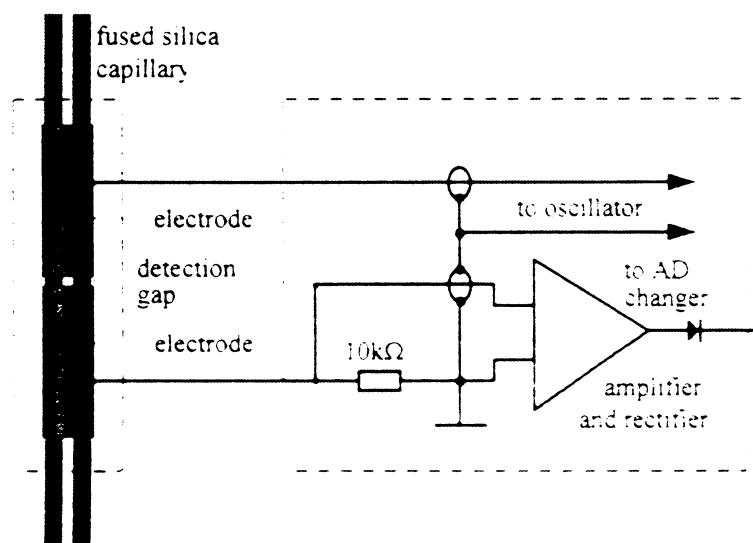


Figure 1. Schematic drawing of the capacitively coupled conductivity detector. Reprinted with permission from [9].

This approach with axial electrode arrangement was in the next years followed by several research groups and some other modifications, and refinements of both mechanic and electronic parts of the CLCD detector were reported.

Túma *et al.* [25] developed a new prototype of CLCD using the strips of aluminum foil as electrode material. The foil strips formed semitubular shape electrodes between two PMMA blocks, where one of these two bases was movable. This construction allowed very easy exchange of the separation FS capillary. The qualitative and quantitative parameters of this detector with semitubular electrodes were found to be comparable with those achieved by the detector with tubular electrodes. After the studies testing the influence of geometry of the detection cell, especially width of electrodes and detection gap, on the sensitivity [26], the arrangement of the detection cell was further modified. The movable PMMA plate was divided into two parts, each containing a single electrode, which makes possible to change easily the gap between the two electrodes, *i.e.*, the length and volume of the detection cell. The effects of electrode geometry, planar, semitubular placed one beside the other, semitubular placed one against the other, tubular shape, on the detector characteristics were studied prior to finding the optimal arrangement [27].

Gaš and coworkers [28] have used two metal cylinders as tubular electrodes that surrounded the capillary in series for his new construction of CLCD. The input signal was provided by a HF voltage supply with a frequency of 625 kHz. A metal disk placed between the electrodes was connected to the ground. The detector has been further optimized and its construction has been improved in

such a way that the two cylindrical brass electrodes were set to be 2.5 mm long, the detection gap between them was 1.5 mm and a copper disk with a hole for capillary placed between electrodes was used as a shielding. The output voltage of a HF generator was 50 V_{eff} [29]. The same parameters of the cell and electric circuit have been applied also for a double cell arrangement of the CLCD [30], which is suitable for investigation of dynamics of the electroseparation processes and for precise measurements of electrophoretic mobilities.

Zemann and coworkers [31] continued in the development and application of CLCD. A new prototype of CLCD with some small construction changes was used to detect fast separations of inorganic cations and anions, which were injected into the capillary from its both ends. The CLCD could be placed at various positions along the capillary length. The electrodes of this detector were 1 cm long, formed by stainless steel tubing, and placed at a distance of 1 mm from each other. Movable CLCD was miniaturized and built in commercial CE instrument [32]. The tubular electrodes were made of stainless steel with dimensions 0.4 mm wide, 8 mm long and were partly covered with heat-shrink tubing. The grounded copper plate was between electrodes. The rounded body of the cell was formed by epoxy resin covered by conductive nail assembled onto the capillary with dimensions 42 mm × 20 mm. The performance characteristics (noise, sensitivity, and peak width) were compared with a large cell and no significant differences were observed. In addition, these two miniaturized detectors placed on a single capillary were successfully tested for dual channel detection in CZE.

The original, full-size two-electrode CLCD was slightly modified (the detection gap was set 1 mm and the electrodes were cut down to 5 mm) and it was found suitable for detection of conductivity changes of the eluate in ion-exchange capillary chromatography [33].

Kubáň *et al.* [34, 35] have developed CLCD for simultaneous determination of inorganic or organic cations and anions, where samples were injected from both capillary ends. In the construction the authors followed the model of da Silva. The flexible detection cell comprised two 2 cm × 2 cm × 2.5 cm PMMA blocks. CLCD had two tubular silver electrodes, a thick leaf of copper foil was sandwiched between the electrodes, and the detection window was experimentally chosen as 2 mm long. The operating frequency of the detector was 290 kHz. LODs of inorganic cations and anions were simultaneously determined and found comparable with CZE, where samples were injected from one end of the capillary.

Hauser and coworkers [36] have constructed a modified CLCD, analogous to that developed by Zemann [9, 23]. Two 4 mm long electrodes were made of steel rings and placed 1 mm from each other around the easily exchangeable FS capillary. The detector operated up to a maximal frequency of 450 kHz and voltages of 25, 100, and 250 V_{p-p}. The usual working frequency was 190 kHz and voltage was 250 V_{p-p}. When the detector was placed in a Faraday cage the optimal frequency was changed to 100 kHz and the voltage was increased up to 300 V_{p-p}. A slightly modified detector [37] has been tested for CZE separation of inorganic ions in polyetheretherketone (PEEK) capillary. The detection window was enlarged to 2 mm and the detector operated at 100 kHz and 450 V_{p-p}.

Vuorinen *et al.* [38] developed CLCD following the model of da Silva *et al.* [24] and installed it in the commercial CE device. CLCD consisted of two silver covered copper coil electrodes, 7 mm long and with detection gap 0.4 mm. Detector operated at optimized frequency 291 kHz and voltage 20–25 V_{p-p}. Tan *et al.* [39] designed modified CLCD consisting of two 5 mm long stainless-steel tubes placed 2 mm apart from each other on the capillary. A thin grounded copper plane was placed between the electrodes to minimize the coupling capacitance between the electrodes in air. The electric circuitry was analogous to that described in [10], the detector was operated at the frequency of 200 kHz and with amplitude 220 V_{p-p}.

Baltussen *et al.* [40] presented two conductivity contactless cells. Both geometries consisted of electrodes placed cylindrically around the FS capillary. A simpler geometry configuration was based on two 2 cm long electrodes with detection gap of 2 mm. A slightly more complicated arrangement included a 0.25 mm thick shielding electrode, situated between two sensing electrodes. The electric circuitry was similar to that described in [9] with

addition of lock-in amplifier. The detector operated at 100 kHz.

Wang and Fu [41] employed their CLCD in a miniaturized CE system with flow injection sample introduction (FI-CE device) [41]. The detection cell consisted of two 2 mm long tubular electrodes made from 100 µm thick Pt foil and positioned near the outlet of the separation capillary with 1 mm distance from each other. A 100 µm thick-grounded copper foil was sandwiched between the electrodes and the capillary passed through a 400 µm hole drilled through the foil. The detector operated at sine wave frequency 200 kHz and amplitude 10 V_{p-p}.

The applications of CLCD in CE methods are summarized in Table 2.

3.3 Combined contactless conductivity detectors in capillaries

Conductivity detection is in its principle a universal detection mode in CE methods. However, in spite of that, its combination with other detectors, is highly required, especially for analysis of complex samples containing both small fast inorganic and organic ions and large biopolymers, such as peptides, proteins and nucleic acids. Various combinations of CE detection techniques have been described, but almost always the detectors were placed in series. The special feature of the CLCD, particularly a gap between the two sensing electrodes, allows the measurement of the absorbance or fluorescence of the solution in the place of this gap, *i.e.*, in the same place where the conductivity of the solution is measured. This possibility has been utilized in some detector designs where CLCD was combined with photometric UV-absorption or fluorescent detection.

Chvojka *et al.* [42] designed a combined dual photometric-CLCD, in which both detection modes were independent and both static and dynamic properties were fully comparable with the characteristics of the individual detectors. Optical fibers were used to carry the photometric signal, whereas the conductivity signal is obtained from electrodes placed on the left- and right-hand side of the fiber using AC voltage in the range 100–500 kHz. Later an improved dual photometric-CLCD has been developed [43, 44] where the CLCD was based on the previously developed semitubular electrodes [25, 26]. The optical part of the detector consisted of a single optical fiber fixed between the conductivity electrodes brought the light to the cell and a photodiode fixed close to the capillary, which recorded the light passing through the cell.

Tan *et al.* [45] presented another type of dual detector, which combined CLCD and fluorescent detection. The CLCD part of this detector was identical with that described in [39] with the exception that the window gap

Table 2. Application of contactless conductivity detectors in CE

Analyte (IOD or concentration range)/matrix ^{a)}	CE mode BGE composition	Capillary material and diameters (id/od)	Electrodes arrangement Frequency/excit. voltage	References
Cl ⁻ , ClO ₃ ⁻ , F ⁻ , IO ₃ ⁻ , glutamate	IIP Leading electrolyte (LE): 1 mM His, 1 mM His.HCl Terminating electrolyte (TE): 1 mM Glu	PTFE (teflon): 0.45/0.8 mm	Planar arrangement of four electrodes of capacitive pile, Cu enameling wire 0.2 mm, $f = 1$ MHz	[6–8, 19]
Rb ⁺ , Ca ²⁺ , Na ⁺ , Mg ²⁺ , Li ⁺ (4 ppb), Mn ²⁺ , Br ⁻ , Cl ⁻ , NO ₂ ⁻ , NO ₃ ⁻ , SO ₄ ²⁻ , F ⁻ (13 ppb), C ₂ O ₄ ²⁻ (all 200 ppb), acet- ate, lactate, butyrate (all 50 ppb)	CZE 20 mM MES, 20 mM His, pH 6.0 (0.0001% hexadimethrine bromide (HDB)) 7.5 mM sorbic acid, 15 mM Arg, 0.0007% HDOH, pH 8.9 20 mM MES, 20 mM His, 1 mM 18C6E, pH 6.1	FS: 50/375 μm, tested id 5–100 μm	Cylindrical Ag rings or synergic needles, 30 (15– 50) mm long, detection gap 2–5 mm, $f = 40$ –100 kHz, amplitude 7–10 V _{p-p}	[9, 23]
K ⁺ (5 μM)				
Ba ²⁺ , K ⁺ (17 ppb), Ca ²⁺ (15 ppb), Na ⁺ (9.4 ppb), Mg ²⁺ (24 ppb), Li ⁺ (1.5 μM)	CZE 10 mM MES/His, pH 6.0 (0.2 mM CTAB) 20 mM His/lactate, pH 4.9	FS: 75/360 μm	Tubular 2 mm long Ag rings painted around capillary, detection gap 1 (0.7) mm, grounding between electrodes, $f = 0.6$ or 1.1 MHz, amplitude 20 V _{p-p}	[10, 24, 68, 80, 81, 85, 87, 88, 105]
Tetramethyl(ethyl, butyl)-ammo- nium, (4.2–10.4 μM)	5 mM potassium acetate, pH 5.2			
HCOO ⁻ , Cl ⁻ (48 ppb), NO ₃ ⁻ (96 ppb), CH ₃ COO ⁻ , SO ₄ ²⁻ (115 ppb), NH ₄ ⁺ (10 ppb), K ⁺ , Ca ²⁺ , Na ⁺ , Mg ²⁺ /rain water	20 mM MES, 20 mM His, pH 6.2 (2.5 mM 18C6E, 0.2 mM CTAB)			
Cl ⁻ (2.5 μM), SO ₄ ²⁻ (1.25 μM), PO ₄ ³⁻ (5.26 μM), malate (0.97 μM), as- corbate (1.2 μM), K ⁺ (0.05 μM), Ca ²⁺ (0.12 μM), Na ⁺ (0.17 μM), Mg ²⁺ (0.08 μM)/coconut water	20 mM MES, 20 mM His, pH 6.0, 0.2 mM CTAB 7.5 mM His, 30 mM lactic acid, 0.3 mM CTAH, pH 3.7 20 mM His, 20 mM lactic acid, 2.5 mM, pH 4.9			
Mono- and di-saccharides: fructose (16 μM), fructose (30 μM), glucose (41 μM), sucrose (26 μM), galactose (18 μM)/coconut water, soft drinks, juices	10 mM NaOH, 4.5 mM Na ₂ HPO ₄ , 0.2 mM CTAB			
Aliphatic alcohols: propanol (1.0 mM), 2-methyl-propanol (0.8 mM), butanol (1.0 mM), pentanol (1.5 mM), hexanol (3.6 mM)/fuel	MEKC 20 mM MES, 20 mM His, pH 6.2, 0.2 mM CTAH 20 mM His/lactate, pH 4.9, 2.5 mM 18C6E			
Cl ⁻ (0.99 μM), F ⁻ (0.89 μM), I ⁻ (0.97 μM), NO ₂ ⁻ (1.11 μM), NO ₃ ⁻ (0.87 μM), SO ₄ ²⁻ (0.46 μM), PO ₄ ³⁻ (1.40 μM)/milk	CZE 10 mM PEG-DC, 4.11 mM BTP, pH 3.5 7 mM succinate, 0.5 mM BTP, 0.2% methylhydroxyethylcellulose (MHEC), pH 3.55, (+75 mM βCD)	PTFE: 300/500 μm FS	Four electrodes, HF genera- tor (4 MHz) according to [7, 8]	[20–22]
K ⁺ (0.5 μM), Ba ²⁺ , Ca ²⁺ , Na ⁺ , Mg ²⁺ , Li ⁺ model mixture	CZE 20 mM MES, 20 mM His, pH 6.0	FS: 75/375 μm	Semitubular from Al foil, 2 strips around capillary,	[25, 27, 78]
Ca ²⁺ (4.1 mg/L), Na ⁺ , Mg ²⁺ (9.4 mg/L), K ⁺ /mineral water	20 mM MES, 20 mM His, 0.1 mM TTAB, pH 6.0		2 mm long, detection gap 1 mm, $f = 200$ kHz, ampli- tude ± 10 V _{p-p}	
K ⁺ (10–100 μM), Cs ⁺ (10–100 μM)	CZE 20 mM citric acid, 10 mM LiOH, 1% HEC, pH 3.1	FS: 75/375 μm	Semitubular from Al foil, 2 strips around capillary, 2–10 mm long, detection gap 1, 2, 3, 5.5 mm $f = 460, 230, 115, 58$ kHz, am- plitude ± 10 V _{p-p}	[26]
Aliphatic amines and ammonium ions: tetrapropylammonium, N-methylcyclohexylammonium, N,N-dimethyldecylammonium, tetramethylammonium, propyl- ammonium, dipropylammo- nium, tetrabutylammonium, dodecylammonium	CZE-NACE 10 mM AcOH, 10 mM Tris acetate, pH 4.75 10 mM 2,6-dihydroxybenzoic acid, 10 mM tetraethylammonium 2,6-dihydroxy-benzoate in DMF, DMAA, PC as solvent CZE-NACE 25 mM perchloric acid in PC 50 mM butylamine, 25 mM perchloric acid, PC	FS: 75/360 μm	Axial arrangement of two brass cylinders with shield- ing (grounding) Cu disk between them, $f = 625$ kHz, amplitude ± 50 V _{p-p}	[28–30, 73, 89, 91–94, 99, 101–104]

Table 2. Continued ...

Analyte (LOD or concentration range)/matrix ^a	CE mode BGE composition	Capillary material and diameters (id/od)	Electrodes arrangement Frequency/excit. voltage	References
Amitriptylin, doxepin, nortryptylin I^- K^+ , Cs^+ , Ca^{2+} , Na^+	25 mM tetraethylammonium acetate, 250 (2.5) mM AcOH in PC EACA/HCl in MeOH and water tetrapropylammonium perchlorate in ACN CZE			
Chlorobromoacetic acids: mono-, di-, tri-chloroacetic, mono-, di-, tri-bromoacetic, mono-, di-bromo-chloroacetic, bromo-dichloroacetic (all 0.1 ppm)/water	10 mM AcOH, 5 mM LiOH, pH 4.7 6.25 mM NaH_2PO_4 , 6.25 mM Na_2HPO_4 , 5 mM diethylenetriamine (DETA), pH 9.4 50 mM citric acid, 10–70 mM LiOH, 5 ppm HDB, pH 2.63–4.61 100 mM boric acid, 80 mM Tris, 3 mM DETA, pH 8.62			
AAs (9.1–29 μ M)/Budvar beer, pan-gamin, yeast, human urine, saliva, extract from herbs	70–14.2 mM chloracetic acid, 0–9.3 mM LiOH, pH 2.0–3.2 2.3 M AcOH, 0.1% HEC, pH 2.1			
AAAs: 18 proteinogenic, Orn, Cit, methyl-histidine (Me-His)(all 4.3–42.9 μ M), creatinine/human plasma	1.7 M AcOH, 0.1% HEC pH 2.2 40 mM chloracetic acid, 0.1% HEC, pH 2.2			
AAAs: Lys, His, Gly, Ala, Ser, Val, Ile, Leu, Asn, Thr, Glu, Phe, Tyr, Pro, OH-Pro/digests of collagen, egg white, milk casein, linseed oil-stand oil, gum arabic, animal glues (binding media)	51.9 mM chloracetic acid adj. with LiOH, pH 2.26			
Long-chain fatty acids: palmitic, stearic, linolic, linolenic, oleic, dicarboxylic acids: pimelic 7di, suberic 8di, azelaic 9di, sebaic 10di/drying oils used as binding media	CZE-NACE 5 mM phosphate buffer, pH 6.86. 4 mM sodium dodecylbenzenesulfonate, 10 mM Brij, 2% (v/v) 1-octanol, 45% (v/v) ACN 10 mM salicylic acid, 20 mM His, pH 5.85, 0.2 mM CTAB			
Fosfomycin (0.6–2 μ g/mL)/plasma, microdialysis samples	25 mM benzoic acid, 0.5 mM CTAB, adj. with 1 M Tris, pH 6.95, 8.05			
Cl^- (11 μ g/L), F^- (6 μ g/L), I^- (64 μ g/L), Br^- (160 μ g/L), NO_2^- (92 μ g/L), NO_3^- (21 μ g/L), SO_4^{2-} (29 μ g/L), PO_4^{3-} (95 μ g/L)	Ion-exchange CEC (IE-CEC) 10 mM <i>p</i> -toluenesulfonic acid, 20 mM His, pH 8.05	FS: 75/360 μ m	Length 5 mm, detection gap [33] 1 mm $f = 2$ –200 kHz amplitude 25 V_{p-p}	
Inorganic ions: NH_4^+ , K^+ , Na^+ , Mg^{2+} , Li^+ (70 ppb, 200–100 ppb)	CZE 50 mM MES, 50 mM His, 1 mM 18C6E, pH 6.1	FS: 50/375 μ m	10 mm long cylinders stain- less steel, detection gap [31] 1 mm $f = 100$ kHz	
Br^- , NO_2^- , NO_3^- , SO_4^{2-} , $C_2O_4^{2-}$, Cl^- (70 ppb, 200–100 ppb)/mineral water	50 mM MES, 50 mM His, 0.001% sodium polyanetholsulfonate (SPAS), pH 6.1			
K^+ , Na^+ , Li^+ , NO_3^- , BrO_3^- , Br^-	CZE: 50 mM MES, 50 mM His, pH 6.0	FS: 50/360 μ m		
Cl^- , NO_2^- , NO_3^- , SO_4^{2-} , PO_4^{3-} , COO^- , Br^- , K^+ , Mg^{2+} , Na^+ , NH_4^+ , Ca^{2+} , Mn^{2+} , Li^+ , Co^{2+} , Zn^{2+} (all 7.5–65 μ g/L), Cd^{2+} , F^- (all 125 μ g/L), Ba^{2+} (90 μ g/L), Cr^{3+} , Cr^{6+} /drainage, tap, rinse, rain, surface, lysimetric water, plant exudates and extracts, ore leachates	20 mM MES, 20 mM His, 1.5 mM 18C6E, 20 μ M CTAB, pH 6.0 8 mM His, 2.8 mM HIBA, 0.32 mM 18C6E, pH 4.25 9 mM His, 4.6 mM lactic acid, 0.38 mM 18C6E, pH 4.25 5 mM His, 5 mM HIBA, adj. by AcOH, pH 3.75 4.5 mM His, adj. with AcOH, pH 3.75		Two tubular stainless steel [32] electrodes, 0.4 mm \times 8 mm, partly covered with heat- shrink tubing, grounded Cu plate	
Se^{4+} (190 μ g/L), Se^{6+} (7.5 μ g/L)/soil sample	FI-CE 8.75 mM His, adj. with AcOH, pH 4.0			
Tetramethylammonium, benzyl ammonium, tetrabutylammonium	CZE 6 mM ammonium acetate, pH 6.8			
Phenolic acids: benzoic, 4-hydroxy-benzoic, ferulic, sinigrin, cinnamic, sinapic, vanillic, 2,4-di-hydroxybenzoic, p-coumaric acids (2.3–3.3 μ M, after sample stacking 0.12–0.17 μ M)	CZE 150 mM AMP, pH 11.6 30 mM MES, 30 mM His, 30 μ M CTAB, 10% MeOH, pH 6.0			[34, 35, 71, 77, 20 (5–30) mm long, shield- ing Cu foil, detection gap 2 (0.6–5.1) mm, $f = 290$ kHz, amplitude 2–20 V_{p-p} , [79, 82–84]

Table 2. Continued ...

Analyte (LOD or concentration range)/matrix ^{a)}	CE mode BGE composition	Capillary material and diameters (id/od)	Electrodes arrangement Frequency/excit. voltage	References
Cr ³⁺ (10 µg/L), Cl ⁶⁺ (39 µg/L), NH ₄ ⁺ (1 µg/L), K ⁺ (4 µg/L), Ca ²⁺ (3 µg/L), Mg ²⁺ (1.5 µg/L), Na ⁺ (4 µg/L), Cl ⁻ (6 µg/L), NO ₃ ⁻ (10 µg/L), SO ₄ ²⁻ (6 µg/L), Se ⁴⁺ (190 µg/L), Se ⁶⁺ (7.5 µg/L), HPO ₄ ²⁻ (200 µg/L), NO ₂ ⁻ (150 µg/L)/mineral, tap, drainage, surface, rain waters	FI-CE 12 mM His, 0.5 mM HIBA, 1 mM 18C6E, adj. with 10% v/v AcOH, pH 4.05 7.5 mM His, 1 mM HIBA, 1 mM 18C6E, adj. with AcOH, pH 2.8 12 mM His, adj. with AcOH, pH 4.00	FS: 50, 75/360 µm	Two tubular steel electrodes, [36, 60, 60–4 mm long, detection gap 1 mm, f = 100 or 190 kHz, (tested to 450 kHz), amplitude 250, 300, 450 V _{p-p} , (tested 25, 100, 200, 300, 400 V _{p-p})	[36, 60–69, 75, 76, 86, 90, 96–98]
K ⁺ , Mg ²⁺ , Na ⁺ , Li ⁺ , NO ₃ ⁻ , SO ₄ ²⁻ , PO ₄ ³⁻ , Br ⁻ , Ba ²⁺ , Ca ²⁺ , Mn ²⁺ , Rb ⁺ , Co ²⁺ , Cd ²⁺ , Zn ²⁺ , Fe ³⁺ , Cs ⁺ , Sr ²⁺ , Pb ²⁺ , NH ₄ ⁺ , Cu ²⁺ , Ni ²⁺ (all 0.089–0.27 µM, 1–200 µM, heavy metal ions 1–5 µM)/river water	CZE 0 (20) mM MES/His, 0.00015% HDB, pH 6.0 20 mM MES/His, 1 mM 18C6E, pH 6.1 10 mM His, adj. with AcOH, pH 2.75 10 mM CAPS/Arg			
Citrate, malate, acetate (0.5 mg/L), lactate (0.5 mg/L), benzoate (0.5 mg/L), sorbate (0.5 mg/L), ascorbate (3 mg/L)/vitamin C tablet, soft drink	CZE 0.135 mM tartaric acid, 10 mM His, pH 6.5, (0.1 mM CTAB and 0.025% HP-β-CD)			
Amines: (0.52 µM), ephedrine, pin-dolol (0.06 µM), atenolol (0.48 µM), metoprolol, lebetanol (0.96 µM), acebutolol, adrenaline, noradrenaline, isoproterenol, doxylamine, octopamine/pharmaceuticals: ternormin, sanalepsi, vick medi-nite	CZE 10 (20) mM MES/His, pH 6.2, (6.1) 10 mM citric acid, pH 3.0 20 mM lactic acid, pH 2.83 100 mM lactic acid, 5 mM His, pH 2.75 100 (250) mM AcOH, pH 2.91, 2.71 100 mM AcOH, 1 mM His			
Human IgM (0.15 ng/ml), L,D-doxyl-amine, L,D-adrenaline (0.28 µM), L,D-isoproterenol (0.42 µM), L,D-noradrenaline (0.44 µM), L,D-praproanol, 1S,2S(+),1R,2R(-)-pseudoephedrine (0.3 µM)	CZE 20 mM TAPS, 20 mM AMPD, pH 8.7, 0.01% Tween 20 100 mM lactic acid, 5 mM His, 40 mM HP-β-CD, pH 2.75 100 mM lactic acid, 5 mM His, 30 mM HP-β-CD, pH 2.75 60 mM lactic acid, 7.5 mM His, 10 mM HP-β-CD, pH 3.06 20 mM lactic acid, 20 mM HP-β-CD, pH 2.45			
NH ₄ ⁺ , K ⁺ , Na ⁺ , Ca ²⁺ , Mg ²⁺ , Cl ⁻ , NO ₃ ⁻ , SO ₄ ²⁻ /human blood serum, urine	CZE 6.5 (13) mM maleic acid, 7.5 (15) mM Arg, 1.5 mM 18C6E, pH 5.5			
(R/S)-1-cyclohexyl ethylamine, (R/S)-1-phenylethylamine (0.5 µM)	10 mM 18C6EH4, 10 mM citrate/Tris, pH 2.2 25 mM citric acid, 5 mM DM-β-CD, 5 mM 18C6E, pH 2.4			
Chlorhexidine digluconate (0.4 µg/ml), polyhexamethylene biguanide (4 µg/ml)/eye drops	CZE 2.3 M AcOH, pH 2.1, 0.05% Tween 20 15 mM Arg, 10 mM AcOH, pH 8.3			
Amines (all 1–2 µM): short-chained aliphatic primary, secondary, tertiary amines, branched aliphatic amines, diamines, hydroxyl-substituted amines	10 mM MES/His, pH 6.0 50 mM AcOH, 10 mM Bis-Tris, pH 4.0 25 mM AcOH, pH 3.0 0.5 M AcOH, pH 2.5 0.5 M AcOH, pH 2.5, 50 mM 18C6E, pH 2.5			
Carbohydrates: D-sucrose, D-galactose, D-glucose, D-fructose	10 mM NaOH, 4.5 mM Na ₂ HPO ₄ , 0.00015% HDB, pH > 12			
AAs: Thr, Met, Leu (all 1 µM)	10 mM NaOH, 4.5 mM Na ₂ HPO ₄ , 1 mM CTAB, pH < 12			
NH ₄ ⁺ , K ⁺ , Mg ²⁺ , Na ⁺ , Li ⁺ , NO ₃ ⁻ , Cl ⁻ , Br ⁻ , SO ₄ ²⁻ , oxalate, tartate, citrate (all around 10 µM)/red wine, tap water	CE with dual opposite injection (CE-DOL) 10 mM MES, 10 mM His, 1 mM 18C6E, pH 6.0	FS, PEEK: 50, 75/360 µm	Two tubular steel, 4 mm long, detection gap 2 mm, f = 100 kHz, amplitude 450 V _{p-p}	[37, 74]
Underivatized AAs: (50–100 mM), Arg (20 µM), Lys (30 µM), His (40 µM)/urine, beer	6 mM lactic acid, pH 2.4 50 mM AMP(AMPD), pH 10.8 50 mM AMP, 5 (10) mM CAPS, pH 11.1 (10.8)			

Table 2. Continued ...

Analyte (LOD or concentration range)/matrix ^a	CE mode BGE composition	Capillary material and diameters (id/od)	Electrodes arrangement Frequency/excit. voltage	References
K ⁺ (0.24 mg/L), Ba ²⁺ (2.14 mg/L), Ca ²⁺ (0.15 mg/L), Na ⁺ (0.12 mg/L), Mg ²⁺ (0.18 mg/L), Mn ²⁺ (0.34 mg/L), Li ⁺ (0.22 mg/L)	CZE 20 mM MES, 20 mM His, pH 6.0	FS: 50/150 µm	Two Cu wire coil electrodes covered by Ag, 7 mm long, detection gap 0.4 mm, f = 291–300 kHz, amplitude 20–25 V _{p-p} .	[38]
Catecholamines: 4-hydroxy-3-methoxybenzylamine hydrochloride, (9.0 mg/L), dopamine (11.2 mg/L), D,L-normetanephrine (11.2 mg/L), D,L-metanephrine (10.1 mg/L)	CZE 10 mM ammonium acetate, pH 4.0			
Zn ²⁺ (5 nM), Co ²⁺ (10 nM), Cu ²⁺ (30 nM), Ni ²⁺ (15 nM), Na ⁺ , K ⁺ , Mg ²⁺ , Ca ²⁺ /tap water	FASI-CE 0–10 mM MES, 0–10 mM His, adj. with AcOH, pH 4.9 10 mM MES, 10 mM His, 15% ACN, pH 4.9	FS: 50/375 µm	Two stainless-steel tubes, 5 mm long, gap 1 mm, thin-grounded Cu plane, f = 200 kHz, amplitude ± 220 V _{p-p} .	[39]
K ⁺ , Li ⁺ (both 0.3 µM)	CZE 20 mM sodium borate, pH 7.5	FS: id 50 µm	Two cylindrical, 2 cm long, 0.25 mm thick, detection gap 2 mm, f = 250 kHz	[40]
Peptides: bradykinin, angiotensin II, CZE α-melanocyte stimulating hormone, luteinizing hormone releasing hormone, TRH, [Leu]enkephalin, bombesin, oxytocin, [Met]enkephalin (2–0.5 µM)	100 mM phosphate buffer, pH 2.5			
NH ₄ ⁺ (2.6 µM), Na ⁺ (2.7 µM), Mg ²⁺ (3.5 µM), K ⁺ (2.5 µM), Ca ²⁺ (2.8 µM)/surface water	Miniaturized FI-CE 20 mM MES, 20 mM His, 1.5 mM 18C6E, pH 6.0	FS: 25/375 µm	2 mm long tubular from Pt foil, detection gap 1 mm, 100 µm thin grounded Cu foil between electrodes with 400 µm hole for capillary, f = 200 kHz, amplitude 10 V _{p-p} .	[41]

^a) Matrix is specified for real world samples only, in the other cases model analyte mixtures are involved.

Table 3. Application of contactless conductivity detectors combined with optical detectors in CE

Analyte (LOD or concentration range)/matrix ^a	CE mode BGE composition	Detection type Capillary material: (id/od) Detection wavelength	Electrodes arrangement Frequency/excit. voltage	References
K ⁺ (10 µM), Na ⁺ , salicylate (0.7 µM), acetylsalicylate, benzoate, picrate, benzenosulfonate, brombenzoate, Cl ⁻ , SO ₄ ²⁻ , Ca ²⁺ , Mg ²⁺ , procaine, tyramine, ephedrine, codeine/infusion solution	CZE 20 mM boric acid, 10 mM LiOH, pH 9.20 20 mM citric acid, 10 mM LiOH, pH 2.95	Photometric-contactless FS: 75/375 µm $\lambda = 200$ nm	Tightly winding a Cu wire around capillary connected with all coils together, 3 mm long, detection gap 2 mm, f = 200 kHz, amplitude ± 10 V _{p-p} .	[42]
K ⁺ (10 µM), Na ⁺ , Ca ²⁺ , Mg ²⁺ , tyramine, His, pyridoxine, sorbic, butyric acid, gallic acid, Ca pantothenate, 20 mM boric acid, 10 mM LiOH, ascorbic acid, citric acid/multivitamin preparation	CZE 20 mM AcOH, 10 mM LiOH, pH 4.5 20 mM citric acid, 10 mM Tris, pH 9.20	Photometric-contactless FS: 75/375 µm $\lambda = 210$ nm	Semitubular from Al foil, 2 strips around capillary fixed to block, 3 mm long, detection gap 2 mm, f = 200 kHz, amplitude ± 10 V _{p-p} .	[43, 44]
Li ⁺ , Na ⁺ , K ⁺ , Mg ²⁺ , Ba ²⁺ , Ca ²⁺ (0.2–3 µM)	CZE 10 mM MES, 10 mM His, pH 6.0	Fluorescent-contactless FS: 75/375 µm $\lambda_{\text{exc}} = 470$ nm, $\lambda_{\text{em}} = 530$ nm	Two stainless-steel tubes, thin-grounded Cu plane, 5 mm long, detection gap 2 mm	[45]
FITC-labeled AAs: Phe, Gly, Trp, Lys (0.4–2.5 µM)	10 mM Tris, 5 mM MES, pH 8.2			
FITC-labeled peptides: Pro-Met, Gly-Tyr, Gly-Thr (0.4–0.6 µg/mL)	10 mM Tris, 5 mM MES, pH 8.2			

^a) Matrix is specified for real world samples only, in the other cases model analyte mixtures are involved.

was prolonged to 2 mm. A thin-grounded copper plane was placed between the electrodes to minimize the coupling capacitance between the electrodes in the air. For fluorescent detection the light emitting diode ($\lambda = 470$ nm) was used as the excitation source. An optical fiber was fixed perpendicularly against the window by a

methacrylate plate with T-shape groove to collect the emitting fluorescence, passing through two pieces of interference filters ($\lambda = 530$ nm).

The applications of CLCD combined with optical detectors in CE methods are summarized in Table 3.

3.4 Contactless conductivity detectors in chip-based systems

In 2001 Laugere *et al.* [11] developed the first CLCD with two- and four-electrode arrangement for microchip CE. After several experimental optimizations, a two-electrode detector was found unsuitable for miniaturization, so a four-electrode detector was chosen for further experiments in microchip CE. The silver electrodes with sizes $10\text{ mm} \times 0.5\text{ mm}$ were covered by silicon nitride on the glass chip and the basic physical characteristics, such as impedance, frequency, potential and current of the detector have been determined [46–50].

Another four-electrode CLCD for microchip CE was suggested by Lichtenberg *et al.* [51]. The detector was integrated inside the chip. All the four platinum electrodes were $100\text{ }\mu\text{m}$ long, two inner $30\text{ }\mu\text{m}$ and two outer $20\text{ }\mu\text{m}$ wide. Operating electrodes were placed $400\text{ }\mu\text{m}$ from each other, positioned perpendicular to the channel. The amplitude was $15\text{ V}_{\text{p-p}}$, and the operating frequency was $10\text{--}100\text{ kHz}$.

New types of CLCDs for microchip CE were developed by Wang and coworkers [52, 53]. Electrodes have rectangular shape made of aluminum foil and were placed 1.3 mm from each other. Their arrangement was antiparallel. The operating frequency was set at 200 kHz and the amplitude at $5\text{ V}_{\text{p-p}}$. The electric circuit was designed according to da Silva [24]. In another design, the CLCD was integrated into the microchip in combination with the amperometric detector [54]. Do Lago *et al.* [55] have also adapted his original CLCD design for application in a microchip. The electrodes were made of copper strips, detector operated at frequency 530 kHz and amplitude $10\text{ V}_{\text{p-p}}$. A new movable CLCD for microchip CE was introduced by Wang *et al.* [56], the detector could be placed at different points along the separation channel due to the electrodes holder. It was tested on CE separations of low explosive ionic compounds. The same authors designed a microchip with dual-opposite injection for simultaneous measurement of cations and anions with movable CLCD [57]. Similar construction of movable CLCD with small difference in electrode length was designed by Chen *et al.* [58].

Hauser and coworkers [59, 60] integrated the CLCD into a commercially available chip. The detector consisted of two silver parallel electrodes placed 1 mm from each other. They were 0.5 mm wide and 5 mm long. The amplitude was $500\text{ V}_{\text{p-p}}$ and the operating frequency 100 kHz . Special papers were dedicated to studies of the cell geometry and frequency behavior of the CLCD [61] and to the investigation of the analytical performance of the detector, mainly with regard to the S/N ratio, as well as investigation of the effects of a Faraday shielding between the electrodes and of different internal dia-

meters of electrodes on the S/N ratio [62] for better understanding of the characteristics of the axial contactless conductivity cell. On the chip, the electrodes consisted of two parallel selfadhesive copper strips of 1 mm width and 5 mm length and separated by a gap of 1 mm .

More detailed investigations of the effects of the cell geometry (orientation, length, width, distance from the channel of the electrodes, gap between them), and operating parameters (stray capacitance, excitation voltage, and feedback resistor) on the performance of the CLCD detector were reported by Kubáň and Hauser [63]. In this group two thin antiparallel adhesive copper strips were tested as electrodes with different width ($0.5\text{--}3\text{ mm}$) and length ($13.2\text{--}20\text{ mm}$), detection gap ($0.5\text{--}3\text{ mm}$), feedback resistor ($0.22\text{--}2.2\text{ MO}$) frequency ($100\text{--}800\text{ kHz}$), and amplitude ($25\text{--}100\text{ V}_{\text{p-p}}$) on the PMMA microchip. The optimal parameters of this construction (1.4 mm length, 1.0 mm width of the electrodes, detection gap 0.5 mm , frequency 600 kHz , and excitation voltage $20\text{ V}_{\text{p-p}}$) were used for detection of different types of analytes [64–66].

The applications of CLCD in chip CE are summarized in Table 4.

4 Applications of conductivity detectors

4.1 Model mixtures

It is natural that at the early days of the application of conductivity detection in CE methods, when the new detector designs were under development, mostly only model mixtures of the most suitable analytes, highly mobile small inorganic and organic ions, have been analyzed. The aim of the experiments was not only to separate and analyze these mixtures but also to test the characteristics of the newly developed detectors. Some representative analyses of model mixtures are described below, the others are presented in the Tables 1–4.

Many separations of small inorganic and/or organic cations [9, 23, 25, 32, 34, 36, 38, 45, 48–53, 55–57, 59, 67–69] and anions [9, 32, 36, 52, 53, 56, 57, 70] have been carried out by CZE in the BGEs composed of MES and histidine (His) at different concentrations and at pH around 6.0; sometimes the selectivity of cationic separations has been improved by the addition of a complexing agent, 18-crown-6-ether (18C6E), and the EOF has been reversed by the addition of CTAB for simultaneous [31, 34, 37] or divided [63, 70] separations of inorganic cations and anions. Examples of simultaneous determination of inorganic cations and anions in 20 mM MES/His with addition of 1.5 mM 18C6E and 10 (20) mM CTAB (pH 6.0) by CZE with dual injection from both capillary ends [34], are shown in Fig. 2. Other commonly used BGEs for determination of cations have been lactic acid (pH 4.9) [9], lactic

Table 4. Application of contactless conductivity detectors in chip CE

Analyte (LOD or concentration range)/matrix ^a	CE mode BGE composition	Microchip material Channel dimensions	Electrodes arrangement Frequency/excit. voltage	References
K ⁺ , Li ⁺ , Na ⁺ (5 µM) Peptides: Lys-Pro-His-Gly-Glu-Ala-Asp-Ser-Lys, Glu-Lys-Phe-Glu-Lys-Ser-Ala-Asn-Val-Asp-Gly (both 0.2 mM)	CZE 20 mM MES, 20 mM His, pH 6.0 50 mM phosphate buffer, 2 mM SDS, pH 2.5	Glass width/depth 150/70/20 µm	Two or four electrodes, Al foil, covered by silicon nitride or SiC, two outer electrodes 106 × 25 µm, two inner electrodes 106 × 35 µm (test device	[8, 46–50]
Ions of organic acids: fumaric, citric, succinic, acetic, pyruvic and lactic (all 100 µM)	20 mM MES, 20 mM His, 0.2 mM TTAB, pH 6.0		10 mm × 0.5 mm), f = 10 kHz–1 MHz	
Li ⁺ , Na ⁺ , K ⁺ (18 µM)	CZE 10 mM MES, 10 mM His, pH 6.0	Glass width/depth 50/12 µm	4 Pt electrodes, long 100 µm, [51] two inner 30 µm wide, 2 outer 20 µm wide, detection gap 400 µm, f = 10–100 kHz, amplitude 15 V _{p-p}	
K ⁺ (2.8 µM), Ba ²⁺ , Na ⁺ (5.8 µM), Li ⁺ , Ac ⁻ , SO ₄ ²⁻ (5.1 µM), Cl ⁻ (6.4 µM), PO ₄ ³⁻ , NO ₃ ⁻ (7.2 µM), F ⁻ , NH ₄ ⁺ (3.2 µM), perchlorate (6.2 µM), methylammonium (5.8 µM, 400–1600 µM)	CZE 20 mM MES, 20 mM His, pH 6.1	Glass PMMA width/depth 50/50 µm (squared cross section)	Rectangular-shaped from Ag foil strip, 0.8 mm × 10 mm, 100 µm wide, placed in antiparallel arrangement 1 mm from each other, f = 50–1000 kHz, amplitude ± 5 V _{p-p}	[52, 53, 67]
Alkyl derivatives methyl-phosphate acid (MPA): ethyl-, pinacolyl-, isopropyl-MPA, (48–86 µg/L, 0.3–100 mg/L) river water	5 (20) mM MES, 5 (20) mM His, pH 6.1 20 mM Tris, adj. 20 mM boric acid, pH 6.1 20 mM borate, adj. 20 mM boric acid, pH 6.1			
Aliphatic amines: tetramethylammonium, N,N-dimethyldodecylamine, tetrapropylammonium ion, tetrabutylammonium ion	CZE-NACE 5 mM 2,6-dihydroxybenzoic acid, 5 mM tetraethylammonium 2,6-dihydroxy-benzoate in DMF, DMAA, DMSO, PC			
Na ⁺ , NH ₄ ⁺ , methylammonium (all 2 mM)	CZE 20 mM MES, 20 mM His, 15 mM lithium dodecylsulfate (LiDS), pH 6.1	Glass Combined CLCD and amperometric detection	Rectangular shaped from Ag foil, 0.8 mm × 10 mm, 4 mm wide, antiparallel orientation 1.3 mm gap, f = 200 kHz, amplitude ± 5 V _{p-p}	[54]
Amperometric: trinitrotoluene (85 ppm), di-, trinitrobenzene (2.4 ppm), trinitrobenzene, o-, m-, p-aminophenol (0.3–0.9 mM)	20 mM sodium acetate, 20 mM AcOH, 7:3, pH 5.0			
K ⁺ , Na ⁺ , Li ⁺	CZE 30 mM MES, 30 mM His, pH 6.1	Laminated polyester chip width/depth 150/15 µm	Two adhesive Cu strips, antiparallel orientation, gap 1 mm, width 1 mm, length 5 mm, f = 600 kHz, amplitude ± 20 V _{p-p}	[55]
Methylammonium, NH ₄ ⁺ (80 µM), Na ⁺ (70 µM), K ⁺ , Cl ⁻ (150 µM), NO ₃ ⁻ , perchlorate (130 µM), methyl phosphonic acid (MPA), pinacolyl-, isopropyl-MPA	CE-DOI 20 mM MES, 20 mM His, pH 6.1	PMMA chip width/depth 50/50 µm	Rectangular shaped from Ag foil, 0.8 mm × 24 mm, electrodes fixed to detection holder moved along channel, antiparallel orientation, gap 0.8 mm, f = 200 kHz, amplitude ± 5 V _{p-p}	[56, 57, 95]
Methyl parathion (3 mg/L), para-thion, paraoxon (5 mg/L) Na ⁺ , K ⁺ , Li ⁺ /sea water	CZE 5 mM MES, 5 mM His, pH 6.1 CZE 20 mM boric acid, 20 mM Tris, pH 8.0	PMMA chip width/depth 50/40 µm	Rectangular-shaped Al electrodes, antiparallel orientation, shielding, width 0.8 mm, length 15 mm, gap 0.8 mm, f = 200 kHz, amplitude ± 5 V _{p-p}	[58]
NH ₄ ⁺ , Na ⁺ (0.41 µM), Mg ²⁺ (0.35 µM), K ⁺ (0.49 µM), Sr ²⁺ (1.4 µM), Mn ²⁺ (2.1 µM), Zn ²⁺ (2.8 µM), Cr ³⁺ (6.8 µM)	CZE 10 mM MES, 10 mM His, 2 mM 18C6E, pH 6.0	Glass chip width/depth 50/20 µm	Two Ag electrodes, 0.5 mm × 5 mm, detection gap 1 mm, f = 100 kHz, amplitude 500 V _s	[60, 70, 72, 90]
Citrate (2.3 µM), lactate (5.5 µM) 4-acetamidophenol, ibuprofen, salicylic acid (5–10 µM)	5 mM MES, 3 mM HIBA, pH 4.5 10 mM CHES, 6 mM Arg, pH 9.0 10 mM CAPS, 10 mM Arg, pH 10			
Na ⁺ , K ⁺ , Li ⁺ , Rb ⁺ (1.5 µM), Fe ³⁺ (3.5 µM), Cd ²⁺ (8 µM), Co ²⁺ (2 µM), Cl ⁻ (2.5 µM), NO ₃ ⁻ (3 µM), ClO ₄ ⁻ (2 µM)	CZE 10 mM MES, 10 mM His, 4 mM 18C6E, pH 6.0	PMMA chip width/depth 50/50 µm	Two thin parallel selfadhesive Cu strips of 1 (2) mm width and 5 (4) mm length placed in 90°angle across channel, detection gap 1 (2.5) mm, shielding, f = 100 kHz, amplitude 400 (300, 500) V _{p-p}	[59]
Oxalate (4.4 µM), tartrate (7 µM), succinate (10 µM), acetate (25 µM), lactate (30 µM)	5 mM His, 3 mM HIBA, pH 4.5 10 mM MES, 10 mM His, pH 6.0 10 mM MES, 10 mM His, 0.2 mM CTAB, pH 6.0	Glass chip		

Table 4. Continued ...

Analyte (LOD or concentration range)/matrix ^{a)}	CE mode BGE composition	Microchip material Channel dimensions	Electrodes arrangement Frequency/excit. voltage	References
4-acetamidophenol, ibuprofen, salicylic acid (5–10 µM), alkyl sulfonates (6 µM): ethane sulfonate, heptane sulfonate	CZE 25 mM MOPS, 15 mM Arg, 0.1 mM CTAB, pH 7.0			
Carboxylic acids: methanoic (3.3 µM), ethanoic (5 µM), butanoic (10 µM), hexanoic (30 µM), ethanedioic (1.5 µM), malic (2.7 µM), hexanedioic (12 µM), hydroxybutanedioic, octanedioic	20 mM MES, 20 mM His, 0.2 mM CTAB, pH 6.020 mM MES, 20 mM His, 0.1 mM CTAB, pH 6.0			
AAs: Trp, Glu, Asp, Phe, Thr, Tyr	CZE 50 mM AMP, 10 mM CAPS, pH 10.8			
Artificial sweeteners (9 µM): acesulfame-K, cyclamate	20 mM MES, 20 mM His, 0.1 mM CTAB, pH 6.0			
Sugars: sucrose (10 µM), fructose (30 µM)	10 mM NaOH, pH 12.0 100 mM triethylamine, pH 12.0			
Drugs: dopamine, ephedrine, methamphetamine	10 mM MES, 0.2% HEC			
Human immunoglobulin M (IgM) (34 mg/mL), antihuman IgM	20 mM TAPS, 20 mM AMPD, pH 8.7, 0.01% Tween 20			
Amines: octopamine, adrenaline, noradrenaline, isoproterenol, doxylamine, pindolol, atenolol, acebutolol	100 mM AcOH, 1 mM His 10 mM citric acid, pH 3.0			
NH ₄ ⁺ (150 µg/L), Na ⁺ (150 µg/L), Mg ²⁺ (90 µg/L), K ⁺ (200 µg/L), Ca ²⁺ (150 µg/L), Li ⁺ (150 µg/L)/rain, tap, mineral waters; red, white wines; fruit juice, beer, milk	CZE 10.5 mM His, 50 mM AcOH, 2 mM 18C6E, pH 4.1 30 mM MES, 30 mM His, 2.5 mM 18C6E, pH 6.0	PMMA chip Cross injection configuration width/depth 50/50 µm (squared cross section)	Two thin antiparallel adhesive Cu strips of 1 (0.5–3) mm width and 14 (13.2–20) mm length, detection gap 0.5 (0.5–3) mm, f = 600 (tested 100–800) kHz, amplitude 20 (25–100) V _{p-p}	[63–66]
Cl ⁻ (125 µg/L), NO ₃ ⁻ (250 µg/L), SO ₄ ²⁻ (200 µg/L)/Tap, mineral waters; red, white wines	20 mM MES, 20 mM His, pH 6.0			
Oxalate, tartrate, malate, citrate, succinate, acetate, lactate, formate, pyruvate, pyroglutamate/ red, white wines; fruit juice, beer, milk	10 mM His, 7 mM Glu, pH 5.75			
Citrate, malate, acetate (3 mg/L), lactate (3 mg/L), benzoate (3 mg/L), sorbate (3 mg/L), ascorbate (10 mg/L)/vitamin C tablet, soft drink	Tartaric acid, His, pH 6.5, 0.06% HP-β-CD, 0.125 mM CTAB			
AAs: Lys, Arg, Gly, Ala, Val, Ile, Ser, Thr, Met, Gln, Phe, Pro (all 50–12 µM)	2.3 M AcOH, pH 2.1, 0.05% Tween 20			
Peptides: [Leu]enkephalin (5.2 µM), bradykinin (11.7 µM), oxytocin (6.0 µM), angiotensin II (11.0 µM), TRH (11.0 µM)				
Proteins: cytochrome c (6.0 µM), HSA (0.6 µM), myoglobin (1.4 µM), immunoglobulin G				
DNA fragment				

^{a)} Matrix is specified for real world samples only, in the other cases model analyte mixtures are involved.

acid/His (pH 4.9) [24, 68], citric acid (pH 2.95) [26, 42], and acetic acid (AcOH)/LiOH (pH 4.7) [29]. An alkaline sorbate/Arg (pH 8.9) [9] or borate buffer (pH 7.5) [14, 40] has been used as alternative BGEs for separation of anions or cations. Inorganic anions were also separated in succinate/1,3-bis[tris(hydroxymethyl)-methylamino]propane (Bis-Tris propane, BTP) (pH 3.55) and polyethyleneglycol dicarboxylic acid (PEG-DC)/BTP (pH 3.5) [20]. Potassium acetate BGE proved to be suitable for detection of alkyl-

ammonium cations [10] and ammonium acetate BGE for separation of catecholamines [38]. Simultaneous determination of organic and alkali and alkaline earth ions was performed in His/α-hydroxyisobutyric (HIBA)/18C6E BGE (pH 6.0) and His/lactic acid/18C6E (pH 4.25) [35]. In that study the effect of the properties of BGEs (pH, concentration of BGE, and concentration of the complexation additive 18C6E in BGE) was examined. Selected BGE, His/HIBA/18C6E BGE (pH 6.0) was used for simultaneous

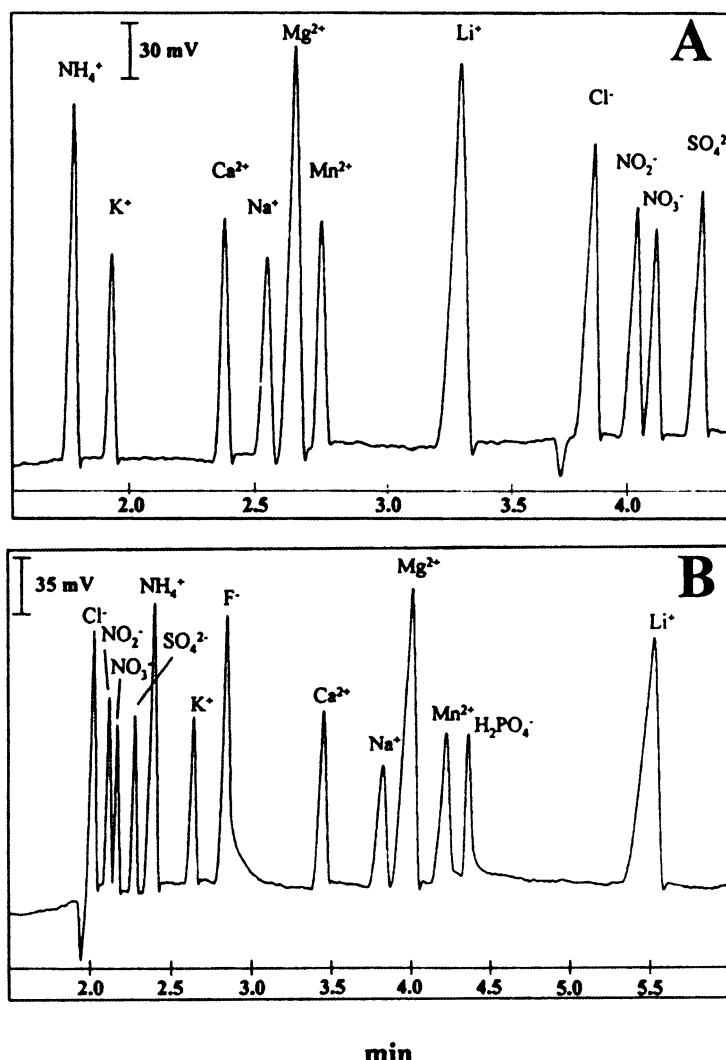


Figure 2. Simultaneous determination of inorganic cations and anions in a standard solution containing 1 mg/L of each ion (except for F^- 2 mg/L and $H_2PO_4^-$ 3 mg/L). Experimental conditions: (A) BGE: 20 mM MES/His, 1.5 mM 18-crown-6-ether, 10 μ M CTAB, pH 6.0; $T = 23^\circ\text{C}$, separation voltage –20 kV, hydrodynamic injection with capillary ends elevated to a height of 30 cm for 30 s (cathodic end) and 10 cm for 30 s (anodic end), time between injection 35 s, effective capillary length (anions) 46 cm and (cations) 21 cm (total length 67 cm); (B) BGE: 20 mM MES, 20 mM His, 1.5 mM 18-crown-6-ether, 20 μ M CTAB, pH 6.0; $T = 23^\circ\text{C}$, voltage –22.5 kV, hydrodynamic injection with capillary ends elevated to a height of 30 cm for 20 s (cathodic end) and 20 cm for 30 s (anodic end), time between injections 35 s, effective capillary length (anions) 41 cm and (cations) 26 cm (total length 67 cm). Reprinted with permission from [34].

determination of inorganic ions by flow injection-CE system (FI-CE) with dual opposite end injection [71]. His/HIBA BGE without any additives was utilized for separation of heavy metal cations determined by microchip CE [59]. Separation and CLCD detection of inorganic or carboxylic acids and artificial sweeteners were also performed in MES/His BGE on a chip [72]. Alkyl methylphosphonic acids (breakdown products of nerve agents) were separated in MES/His (pH 6.0) with high sensitivity, fast

response, high precision, and good linearity [67]. Separation of 20 underivatized amino acids (AAs) was carried out in acetate buffer in 35 min [73]. Later 12 underivatized AAs were separated in three acidic BGEs, composed of glycolic acid, HIBA and lactic acid, and in two basic BGEs, containing 2-amino-2-methyl-1-propanol (AMP) or 2-amino-2-methyl-1,3-propanediol (AMPD and Ammediol) (pH 10.8) and the best separation was achieved in lactic acid [74]. AAs (Trp, Phe, Thr, Tyr, Glu, and Asp) were

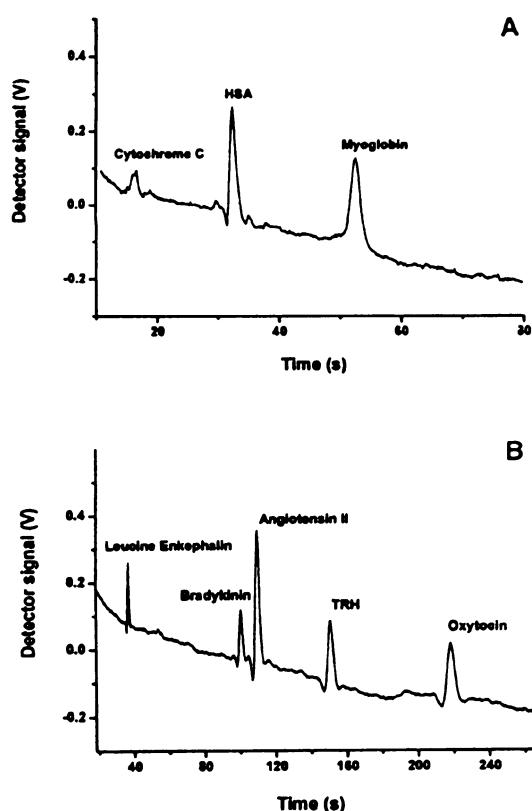


Figure 3. Electrophoretic separation of (A) proteins, cytochrome c ($40 \mu\text{M}$), HSA ($15 \mu\text{M}$), and myoglobin ($30 \mu\text{M}$), and (B) peptides, Leu-enkephalin ($1000 \mu\text{M}$), bradykinin ($190 \mu\text{M}$), angiotensin II ($640 \mu\text{M}$), thyrotropin releasing hormone (TRH) ($1186 \mu\text{M}$), and oxytocin ($109 \mu\text{M}$), on microchip CE. Experimental conditions: BGE: $2.3 \text{ M AcOH}, 0.05\%$ Tween, pH 2.1; $T = 24 \pm 1^\circ\text{C}$, separation voltage 6 kV (A) and 4 kV (B), total/effective length of the separation channel $75/85 \text{ mm}$. Reprinted with permission from [66].

separated in alkaline BGE 3-(cyclohexylamino)-1-propan-sulfonic acid (CAPS) and AMP (pH 10.8) [36, 72] and another separation of a set of 12 AAs (Lys, Arg, Gly, Ala, Val, Ile, Ser, Thr, Met, Gln, Phe, and Pro) was carried out in AcOH (pH 2.1) containing 0.05% polyoxyethylene sorbitan monolaurate (Tween 20) [66]. A mixture of four FITC-labeled AAs (Phe, Gly, Trp, and Lys) and a mixture of three FITC-labeled peptides (Pro-Met, Gly-Tyr, and Gly-Thr) were separated in Tris/MES (pH 8.2) using the combined CLCD and fluorescent detection [45]. A mixture of four carbohydrates and three AAs was successfully analyzed in BGE composed of NaOH/ Na_2HPO_4 /CTAB mixture (pH > 12) [75]. Human immunoglobulin (IgM) without labeling was determined as a model analyte of large molecules in N-tris(hydroxymethyl)-methyl-3-aminopropanesulfonic acid (TAPS)/AMPD in capillaries as well as on chips [60] and similarly monoclonal immunoglobulin

G (IgG) in 2.3 M AcOH containing 0.05% Tween 20 on a chip [66]. The same BGE has been employed also for CLCD detection of CE separation of other medium- and high-molecular-mass compounds, such as DNA-fragment (1652 bp), proteins (cytochrome c, HSA, and myoglobin), and peptides (Leu-enkephalin, bradykinin, angiotensin II, thyrotropin releasing hormone, and oxytocin) as demonstrated in Fig. 3 [66]. The model mixture of low mobility anions lactate and citrate and another model mixture of pharmaceutically relevant compounds were successfully analyzed in Good's buffers containing CHES/Arg (pH 9.0) and CAPS/Arg (pH 10) [59]. Sulfonates were analyzed in neutral BGE composed of 3-morpholino-2-hydroxypropane sulfonic acid (MOPSO)/Arg [72]. Highly alkaline BGEs, composed of NaOH and triethylamine, were used for the separation of sucrose and fructose [72]. A set of nine peptides were separated in conventional phosphate buffer (pH 2.5) [11, 40]. The successful separations of different UV-light nonabsorbing organic amines, such as short-chained aliphatic primary, secondary and tertiary amines, branched aliphatic amines, diamines, and hydroxyl-substituted amines were achieved in five different BGEs: Arg/AcOH (pH 8.3), MES/His (pH 6.0), AcOH/Bis-Tris (pH 4.0), 25 mM AcOH (pH 3.0) and 0.5 M AcOH (pH 2.5) [76]. Complete separation of *cis*- and *trans*-1,2-diaminocyclohexane was achieved in 0.5 M AcOH (pH 2.5) with $18\text{C}6\text{E}$ as a modifier. Nine phenolic acids, derivatives of benzoic, and cinnamic acids, were separated in 150 mM AMP -based BGE (pH 11.6) and CLCD was compared with UV-absorption detection [77]. After sample stacking procedure, CLCD was found to be more sensitive than UV detection.

4.2 Real samples

As the sensitivity and reproducibility of the conductivity detectors in CE methods have been improved they have started being employed in the CE analysis of the real world samples of various origins. Some representative examples are described in this section, the others are presented in the Tables 1–4.

Very frequent applications concern determination of inorganic and organic ions in water samples of different sources, such as, e.g., in mineral waters [15, 31, 65, 71, 78], sea water [58], tap water [39, 63, 65, 71], surface water [41], rinse water [79], rain water collected in São Paulo [80, 81], and many different types of water samples collected in different areas of the Czech Republic [34, 82]. The example of determination of inorganic cations in mineral water Mattoni by CE with CLCD in His/MES/tetradecyltrimethylammonium bromide (TTAB) BGE [78] is shown in Fig. 4. Different samples of drainage water were quantitatively analyzed by miniaturized and traditional FI-CE systems with flow injection sample introduction in MES/His/ $18\text{C}6\text{E}$ [41]; inorganic anions in these

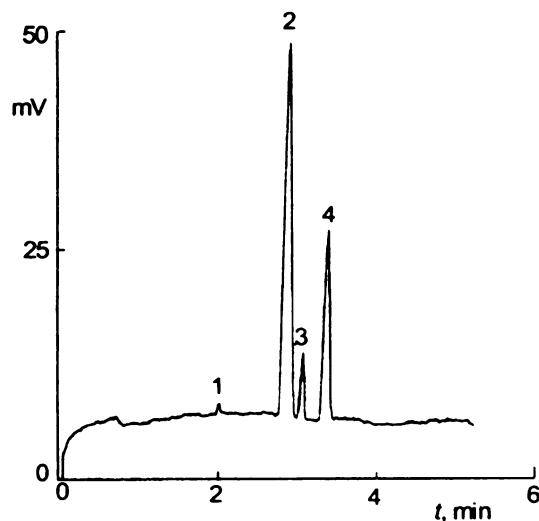


Figure 4. Electrophoregram of determination of inorganic cations in mineral water Mattoni. Experimental conditions: BGE: 20 mM His, 20 mM MES, 0.1 mM TTAB, pH 6.0; $T = 30^\circ\text{C}$; separation voltage 20 kV, effective/total capillary length 45/60 cm, id/od 75/375 μm , hydrodynamic injection at 20 mbar for 6 s. Peak identification: 1, K^+ ; 2, Ca^{2+} ; 3, Na^+ ; 4, Mg^{2+} . Reprinted with permission from [78].

waters were determined in His/AcOH (pH 4.0) [71] or in His/HIBA/18C6E/AcOH (pH 2.8) [83]. This method with the same BGE was used for the fast and sensitive speciation of Se^{4+} and Se^{6+} in soil samples [84]. CZE with field-amplified sample injection (FASI) in BGE composed of MES/His (pH 4.9) in hydro-organic water/ACN solvent (85/45 v/v) has been used for the analysis of heavy metal ions (Zn^{2+} , Co^{2+} , Cu^{2+} , and Ni^{2+}) and for the determination of Mg^{2+} ions in tap water [39]. Another rapid method was used for quantification of inorganic cations and anions in ethanol fuel [85] using MES/His/cetyltrimethylammonium hydroxide (CTAH) for separation of anions and His/lactic acid (pH 4.9) for separation of cations. Determination of metal cations (I.A and II.A groups) in biological matrices with high sodium concentration, was carried out utilizing the reduction of mobilities of Mg^{2+} and Ca^{2+} by addition of methanol (MeOH) and lactate in His/MES BGE [68]. The content of inorganic cations and anions was determined in human blood serum and urine in maleic acid/Arg (pH 5.5) with addition of 18-crown-6-ether for separation of cations [86]. Successful separation of inorganic cations in red and white wines, fruit juice, beer, and milk was obtained by microchip CE in His/AcOH/18C6E (pH 4.1), the inorganic anions in red and white wines were analyzed in MES/His (pH 6.0), and both inorganic and small organic anions in red and white wines, fruit juices, beer, and milk were determined in His/Glu (pH 5.75) [65]. The content of small organic and inorganic anions in vitamin C tablets and soft drinks was

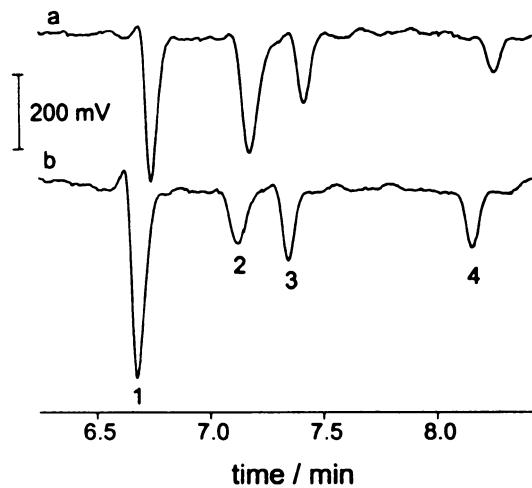


Figure 5. Electrophoretic separation of fructose, glucose and sucrose using galactose as internal standard in unprocessed (a) and processed (b) coconut water. Experimental conditions: BGE, 10 mM NaOH, 4.5 mM Na_2HPO_4 , 0.2 mM CTAH, pH 11.5; voltage, 15 kV; effective/total capillary length 41/50 cm, id 50 μm , gravity injection at 100 mm for 10 s. Peak identification: 1, fructose; 2, glucose; 3, galactose; 4, sucrose. Reprinted with permission from [87].

found by conventional CE as well as by chip CE [64]. Inorganic and small organic cations and sugars, such as fructose, glucose, and sucrose were also determined in five different samples of coconut water in MES/His/CTAH running buffer [87]. The separation of fructose, glucose, sucrose, and galactose as an internal standard in unprocessed and processed coconut water is shown in Fig. 5. Common mono- and disaccharides in soft drinks were separated and determined in high pH BGEs by CZE [88]. Inorganic anions present in milk were separated in BTP/succinate by a method based on complexation with α -CDs [22]. Simultaneous determination of Cr^{3+} and Cr^{6+} in rinse water from the galvanic industry was presented as an alternative method to standard validated methods [79]. The antibiotic fosfomycin in biological fluids was successfully determined in BGE consisting of benzoic acid/CTAB/Tris (pH 6.95) in human plasma and at pH 8.05 in microdialysis samples [89]. β -Blockers such as pindolol, propranolol, atenolol, metoprolol, labetalol, and acebutolol were separated in lactic acid and AcOH (pH 2.7) [90]. Optimized experimental conditions of model separation of AAs were applied for the determination of AAs content in budvar beer, human urine and saliva, panganin yeast, and extracts from herbs [73]. CZE separation of 18 proteinogenic AAs, three nonproteinogenic AAs (citrulline, ornithine, and methylhistidine) and creatinine in human plasma was carried out in 1.7 M AcOH with 0.1% m/v hydroxyethylcellulose (HEC) (pH 2.2) [91]. Advantageous usage of CLCD instead of UV-absorption

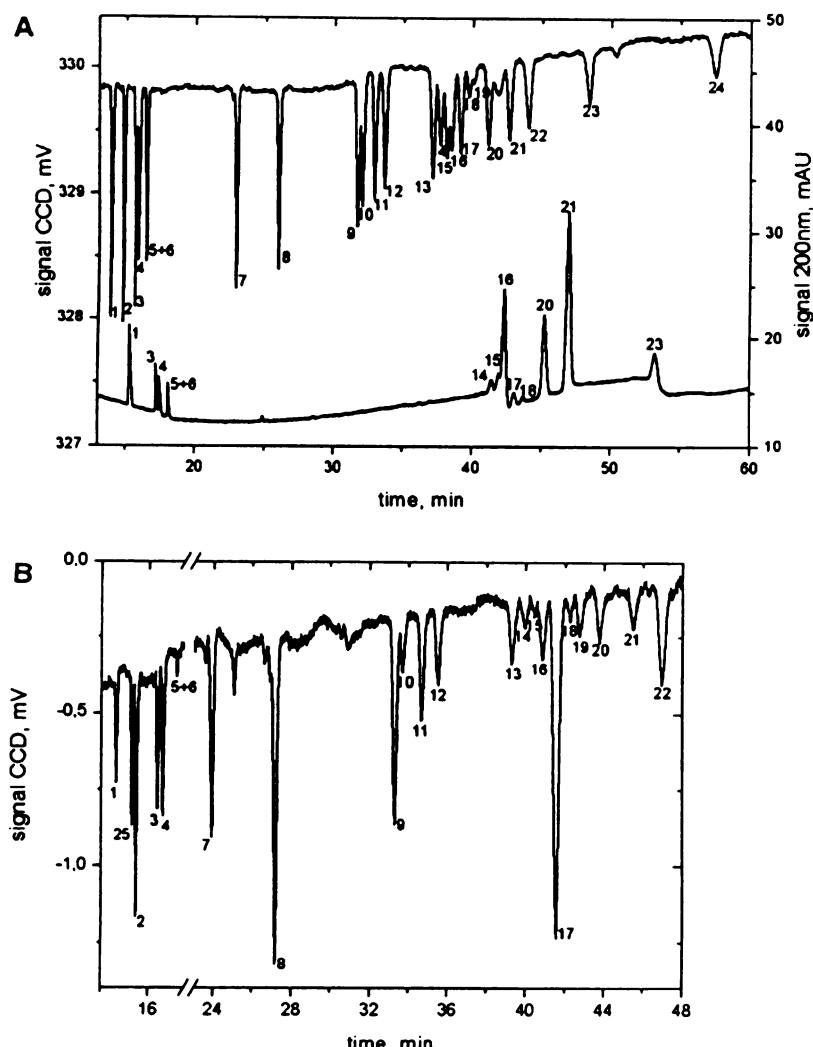


Figure 6. CZE separation of (A) a model mixture of AAs containing 24 mg/L of each (except for 1-methylhistidine, 12 mg/L, and 3-methylhistidine, 12 mg/L) with CLCD and diode array detection and (B) AAs in a human plasma with CLCD detection. Experimental conditions: BGE: 1.7 M AcOH, 0.1% HEC, pH 2.2; separation voltage 20 kV, electric current 20 μ A, $T = 25^\circ\text{C}$, effective capillary length 67 cm for CLCD and 72 cm for DAD, total capillary length 80 cm, id/od 50/375 μ m, hydrodynamic injection 300 mbar s. Peak identification: 1, Creatinine; 2, Lys; 3, Arg; 4, His; 5, 1-MeHis; 6, 3-MeHis; 7, Gly; 8, Ala; 9, Val; 10, Ile; 11, Leu; 12, Ser; 13, Thr; 14, Asn; 15, Met; 16, Trp; 17, Gln; 18, citrullin; 19, Glu; 20, Phe; 21, Tyr; 22, Pro; 23, Cys; 24, Asp; 25, Orn. Reprinted with permission from [91].

detection for these compounds is demonstrated on the CZE separation of a model mixture in Fig. 6A, and on the CZE separation of AAs in plasma in Fig. 6B. The CE method for identifying different species of proteinaceous binders – collagen, egg white, gum arabic, linseed oil, stand oil, and milk casein, on the basis of their AAs composition was developed by Kenndler and coworkers [92, 93], underivatized AAs were separated in chloroacetic acid/LiOH (pH 2.26). This method was used in the analysis of historic and artistic objects and it provided useful data for their restoration and conservation. For similar pur-

poses, long-chain fatty acids and dicarboxylic acids were identified in drying oils used as binding media for objects of art by CE with indirect UV and CLCD in two weakly acidic BGEs at pH 6.86 and 5.85, respectively [94]. On-chip separation of the phosphonic acid products of organophosphate nerve agent, paraoxon, and methyl parathion, in His/MES (pH 6.1) was presented as an enzymatic assay for screening organophosphate nerve agents, based on precolumn conversion by organophosphorus hydrolase [95]. Chemically similar alkyl methylphosphonic acids acting as warfare agent degradation products

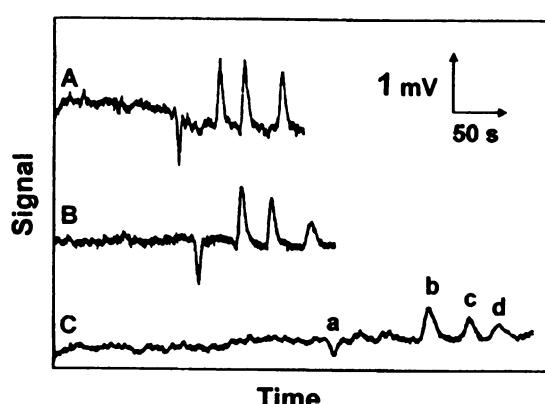


Figure 7. NACE separation of alkylamines in organic solvents, DMF (A), DMAA (B), and DMSO (C), analytes: tetramethylammonium (a), tetrapropylammonium (b), tetrabutylammonium (c), and *N,N*-dimethyldodecylamine (d), concentration of each 500 μ M. Experimental conditions: BGE: 5 mM 2,6-dihydroxybenzoic acid, 5 mM tetraethylammonium, 5 mM 2,6-dihydroxy benzoate, separation voltage 1.5 kV, effective/total length of chip separation channel 73/80 mm, injection 1.5 kV for 1 s (A, B) and 2 s (C). Reprinted with permission from [100].

were sensitively monitored by microchip electrophoresis in river water [67]. The antiseptics, chlorhexidine digluconate, and polyhexamethylene biguanide, were determined with high sensitivity in eye drops, using 2.3 M AcOH (pH 2.1) as BGE [96]. Pharmaceutical drug substances such as ephedrine, dopamine, metanephrine, octapamine, noradrenaline, adrenaline, isoproterenol, and doxylamine were analyzed in citric acid (pH 3.0) [72], MES/His (pH 6.2) [72, 75], and lactic acid (pH 2.83), BGE [90], then the content of real pharmaceutical preparation Ternormin, Sanalepsi N and Vick Medi-Nite were determined. L- and D-enantiomers of six of these lipophilic drug substances and pseudoephedrin were also separated in lactic acid/His BGE (pH 2.7–3.1) with chiral selector hydroxypropyl- β -CD (HP- β -CD) [97]. CLCD was also employed to monitor enantiomeric separation of AAs (Arg, Ser, Thr, Met, Trp, Asn, Val, Phe, and Tyr) in citric acid (pH 2.1) and/or citrate/Tris (pH 2.2) with 10 mM (+)-(18-crown-6-ether)-2,3,11,12-tetracarboxylic acid (18C6EH4) as chiral selector [97]. Another example of enantioseparation in citric acid (pH 2.4) was demonstrated on (R/S)-1-phenylethylamine and (R/S)-1-cyclohexyl ethylamine with high resolution 2.3 and 3.3. Combination of dimethyl- β -CD (DM- β -CD) and nonchiral crown ether 18C6EH4 was chosen as a chiral selector [98].

4.3 Other applications

In addition to analytical CE applications CLCD has been utilized also in CE separation used for the determination

of physico-chemical characteristics of compounds. Their examples are also included in the Tables 1–4. The haloacetic acids were separated in phosphate, citrate, and borate buffers [99] to compare their simulated separation predicted by the computer simulation program PeakMaster with real separation results. Another application concerns the determination of thermodynamic pK_a values of the carboxylic groups and limiting mobilities of cationic forms of underivatized AAs from the CZE measurement of the pH dependence of their effective electrophoretic mobilities; the pK_a values of carboxylic groups of AAs were found to be in the range 1.70–3.43 and the cationic mobilities were in the range 24.7×10^{-9} to $39.5 \times 10^{-9} \text{ m}^2\text{V}^{-1}\text{s}^{-1}$ [30]. Migration behavior of aliphatic amines in organic solvents was studied by nonaqueous CE (NACE) on a chip [100]. Effect of three different organic solvents upon the separation and detection of alkylamines is shown in Fig. 7. A series of papers from the Kenndler's group deal with the usage of organic solvents, such as formamide (FA) [101], propylene carbonate (PC) [102], and nitromethane [103] in NACE with CLCD to test the applicability of these solvents in CZE. Strongly UV-absorbing organic solvents, such as DMF, *N,N*-dimethylacetamide (DMAA), and PC were described and characterized as alternative mediums to aqueous BGEs in CZE with CLCD [28]; suitability of CLCD for NACE was demonstrated on separation of aliphatic amines and/or their ammonium ions and on the determination of their actual mobilities and pK_a values. A special study was focused on relationships between mobility and diffusion coefficient as a function of ionic strength in different organic solvents in NACE [104].

CLCD was capable of detecting even the zones of non-charged aliphatic alcohols separated by MEKC with a micellar pseudophase formed by sodium dodecylsulfate and the mobile phase composed of phosphate buffer with and without methanol added as a modifier of the mobile phase [105].

5 Conclusions

CLCD has proved to be a suitable detection technique for capillary and microchip electrophoresis. The mechanical constructions and electric circuitry of CLCD have been recently significantly improved, better characteristics such as sensitivity and reproducibility were obtained and the detectors started becoming commercially available. CLCD is becoming widely used for detection of inorganic ions and non-UV-absorbing as well as for UV-absorbing small organic ions and it is a promising method for the determination of biomolecules since successful application of CLCD for CE separations of AAs, peptides and proteins and for enzymatic assays and immunoassays on chip have been already demonstrated.

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