

SEPARACE A KVANTIFIKACE NOVĚ SYNTETIZOVANÝCH THIODERIVÁTŮ AKRIDINU POMOCÍ KAPILÁRNÍ KAPALINOVÉ CHROMATOGRRAFIE

ZUZANA BOSÁKOVÁ^a, EVA TESAŘOVÁ^b,
PAVEL COUFAL^a, BOŽENA KAFKOVÁ^a
a JACQUES BARBE^c

^aKatedra analytické chemie, Přírodovědecká fakulta, e-mail: bosakova@natur.cuni.cz, ^bKatedra fyzikální a makromolekulární chemie, Přírodovědecká fakulta, Univerzita Karlova, Albertov 2030, 128 43 Praha 2, ^cGERCTOP UPRES A CNRS 6009, Faculty of Pharmacy, 27 Bd Jean-Moulin, 133 85 Marseille, France

Došlo dne 6.II.2001

Klíčová slova: kapilární kapalinová chromatografie, CLC, thioderiváty akridinu, separace, optimalizace, kvantifikace

Úvod

Deriváty akridinu patří do skupiny farmaceuticky významných chemoterapeutik, u nichž byl prokázán antibakteriální, antimykotický, a především antimalarický účinek¹. Řadu let se používají k léčbě protozoálního onemocnění, vyvolaného různými druhy mikroorganismů rodu *Plasmodium*². V posledních letech nabývají na významu i deriváty s antineoplastickými účinky³. Narůstající rezistence patogenů vůči dnes používaným chemoterapeutikům vede ke snaze produkovat nové deriváty se specifitějšími účinky. Současně roste i poptávka po citlivých a selektivních metodách určených k ověření čistoty léčiv a pro jejich detekci a stanovení v biologických matricích.

Z chemického hlediska jsou akridiny látky odvozené od struktury dibenzopyridinu, nesoucí ve své molekule minimálně jeden dusíkový atom jako součást aromatického cyklu. Vlastní akridin se chová ve vodném roztoku jako slabá báze (pK_b 8,4). Zachování bazických vlastností lze předpokládat i u syntetických derivátů a jejich degradačních produktů, s pouze malou změnou disociační konstanty, danou povahou a polohou substituentů⁴.

Při analýze akridinových derivátů se nejčastěji využívá technika vysokoúčinné kapalinové chromatografie, zejména pro zkoušky totožnosti a kontrolu čistoty akridinových substancí⁵ a pro farmakologické a farmakokinetické studie⁶. Díky zmíněné bazicitě těchto látek byla úspěšně provedena jejich separace i metodou kapilární elektroforézy⁷. Elektrochemicky byl oxidován pouze nesubstituovaný akridin⁸. Metodou kapilární kapalinové chromatografie (CLC) nebyly deriváty akridinu dosud analyzovány.

Výhodou CLC oproti klasické HPLC je menší spotřeba organických rozpouštědel (velmi malý průtok mobilní fáze ~ jednotky $\mu\text{l}\cdot\text{min}^{-1}$) a mnohem menší nároky na množství požadovaného vzorku⁹⁻¹⁰ (desítky nl). To je důležité např. při sledování metabolických produktů v tělních tekutinách nebo při určování obsahu hlavní komponenty a doprovodných nečistot

ze syntézy. Má-li se navíc zabezpečit věrohodnost analytických výsledků a dostát pravidlům správné laboratorní praxe (GLP), je třeba analýzu několikrát opakovat. Nezanedbatelnou výhodou CLC je i snazší spojení s detekcí hmotovou spektrometrií.

Z výše uvedených důvodů byla v této práci věnována pozornost vypracování analytické metody pro separaci a kvantifikaci nově syntetizovaných thioderivátů akridinu pomocí metody kapilární kapalinové chromatografie. Současně byla posouzena i možnost uplatnění této techniky pro kontrolu čistoty připravených substancí.

Experimentální část

Použité přístroje a chemikálie

Měření byla provedena na přístroji sestávajícím z ISCO pumpy 100 DM (Lincoln, NE, USA), LINEAR UV/VIS 205 fotometrického detektoru (San Jose, CA, USA) s CE detekční celou a dávkovacího ventilu Valco se 60 nl smyčkou (Schenk, Švýcarsko). Získaná experimentální data byla zpracována na počítači Pentium III s CSW 1.7 programem se čtyřkanálovou kartou pro sběr dat od fy DataApex (Praha, Česká republika).

Látky byly separovány na křemenné kapilární koloně (vnitřní průměr 320 μm) naplněné v délce 360 μm stacionární fází Nucleosil 100-5 C18 o průměru zrna 5 μm . Kolona byla na výstupním konci opatřena fritou ze skelné vaty, kterou uvnitř kolony držela křemenná kapilára o vnitřním průměru 75 μm a vnějším průměru 280 μm a délce 55 μm , která byla vlepena epoxidovým lepidlem do kapilární kolony se stacionární fází. Druhý konec této křemenné kapiláry byl napojen pomocí převlečné teflonové trubičky na křemennou kapiláru o vnitřním průměru 220 μm a vnějším průměru 340 μm , na níž bylo vypáleno detekční okénko ve vzdálenosti 70 mm od spoje obou kapilár.

K měření pH vodné složky mobilní fáze byl použit pH-metr PHM 64 Radiometer (Copenhagen, Dánsko) s kombinovanou skleněnou elektrodou. Detekce probíhala současně při dvou vlnových délkách 214 nm a 230 nm, vybraných na základě proměřených UV spekter jednotlivých derivátů. Pracovalo se za laboratorní teploty 22 °C. Mrtvý čas byl určen pomocí vodného roztoku uracilu o koncentraci $1\cdot 10^{-3}\text{ mol}\cdot\text{dm}^{-3}$ a činil 5,1 min.

Pro přípravu mobilní fáze byly použity tyto chemikálie: acetonitril LiChrosolv, čistota pro gradientovou eluci, Merck (Darmstadt, SRN), kyselina octová a hydroxid sodný, čistota p.a. Lachema (Brno, Česká republika), deionizovaná voda (Milli-Q). Složení binární mobilní fáze bylo: acetonitril/deionizovaná voda s proměnlivým obsahem acetonitrilu nebo acetonitril/0,2 $\text{mol}\cdot\text{dm}^{-3}$ octanový pufr o pH 3,5 (90:10 v/v). Mobilní fáze byly odplyněny pomocí ultrazvuku.

Strukturální vzorce a kódová označení pěti analyzovaných derivátů akridinu, syntetizovaných v miligramových množstvích na Farmaceutické fakultě v Marseille (Francie), jsou uvedeny na obr. 1. Zásobní roztoky všech studovaných látek o koncentraci $1\cdot 10^{-3}\text{ mol}\cdot\text{dm}^{-3}$, byly připraveny rozpuštěním příslušného množství látky v acetonitrilu.

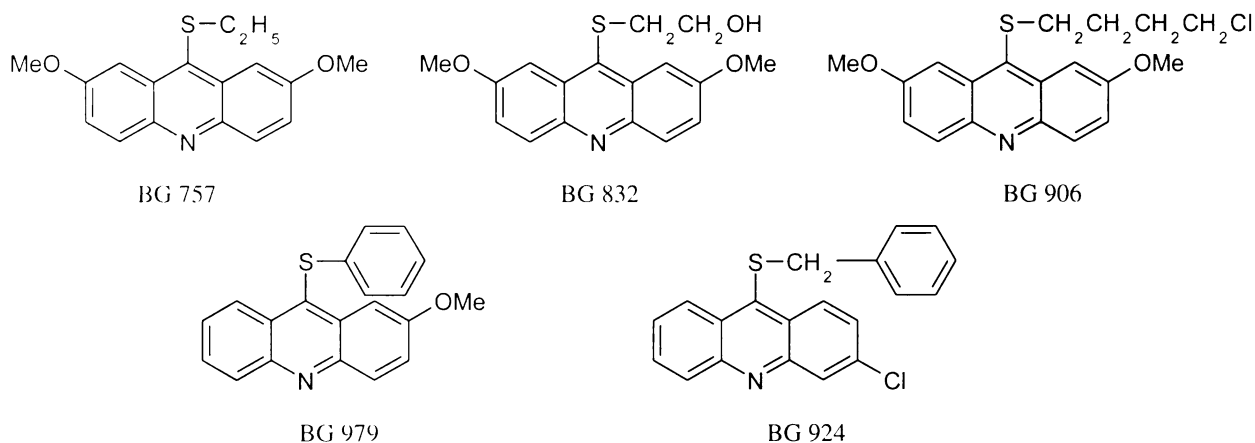
Všechny studované thioderiváty byly připraveny analogickým způsobem na Farmaceutické fakultě v Marseille v od-

dělení prof. J. Barbeho¹¹⁻¹³. Prvním krokem je příprava difenylaminokarboxylové kyseliny z aromatické halogenkarboxylové kyseliny a anilinu metodou podle Ullmanna¹¹. Ta se pomocí oxychloridu fosforečného zcyklizuje a získá se akridon. Ten se pomocí sulfidu fosforečného v pyridinu převede na thioakridon¹². Alkylací (arylací)¹³ thioakridonu se získá akridinový thioderivát (obr. 2).

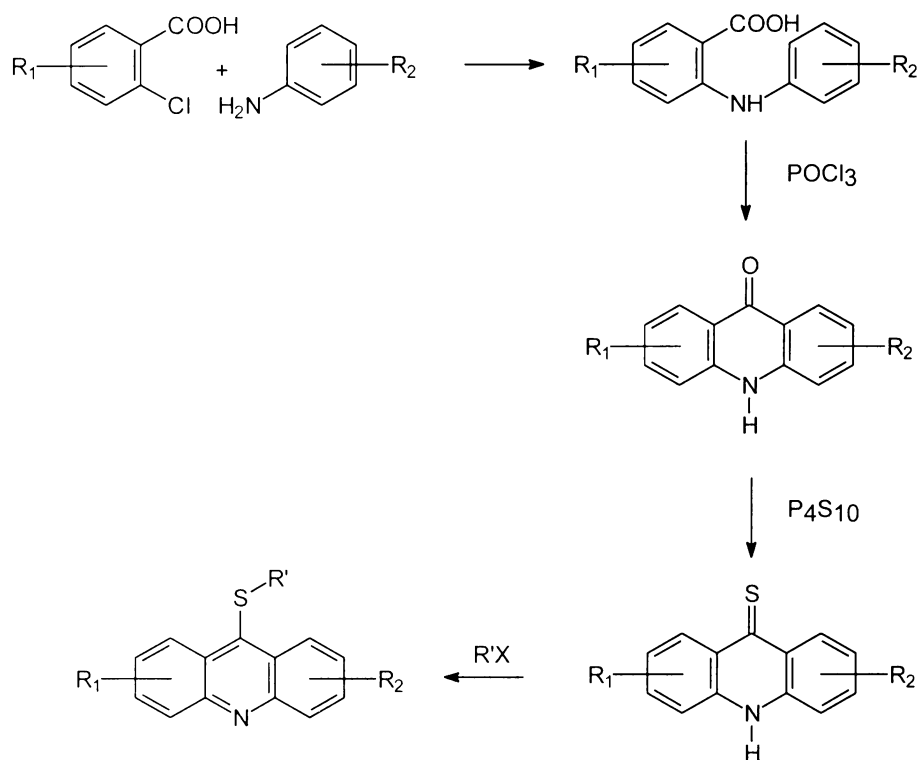
neměly deklarovanou čistotu, hledaly se nejprve vhodné separační podmínky pro získání dostatečného rozlišení mezi hlavní komponentou a doprovodnými nečistotami ze syntézy. Byl proměřen vliv obsahu acetonitrilu na retenční chování jednotlivých derivátů v mobilní fázi acetonitril/voda s proměnlivým obsahem acetonitrilu v rozmezí 100 % až 60 % (v/v). Na obr. 3 je patrné, že už v mobilní fázi s 90 % acetonitrilem došlo téměř u všech derivátů k separaci hlavní složky od doprovodných nečistot. Další snižování obsahu acetonitrilu mělo za následek zvyšování hodnot retenčního (k) a asymetrického (A_s) faktoru a ani při nejnižším proměřovaném obsahu acetonitrilu nedošlo k oddělení dalších nečistot od majoritního píku. Účinnost se-

Výsledky a diskuse

Vzhledem k tomu, že studované thioderiváty akridinu



Obr. 1. Strukturální vzorce a kódová označení analyzovaných derivátů akridinu



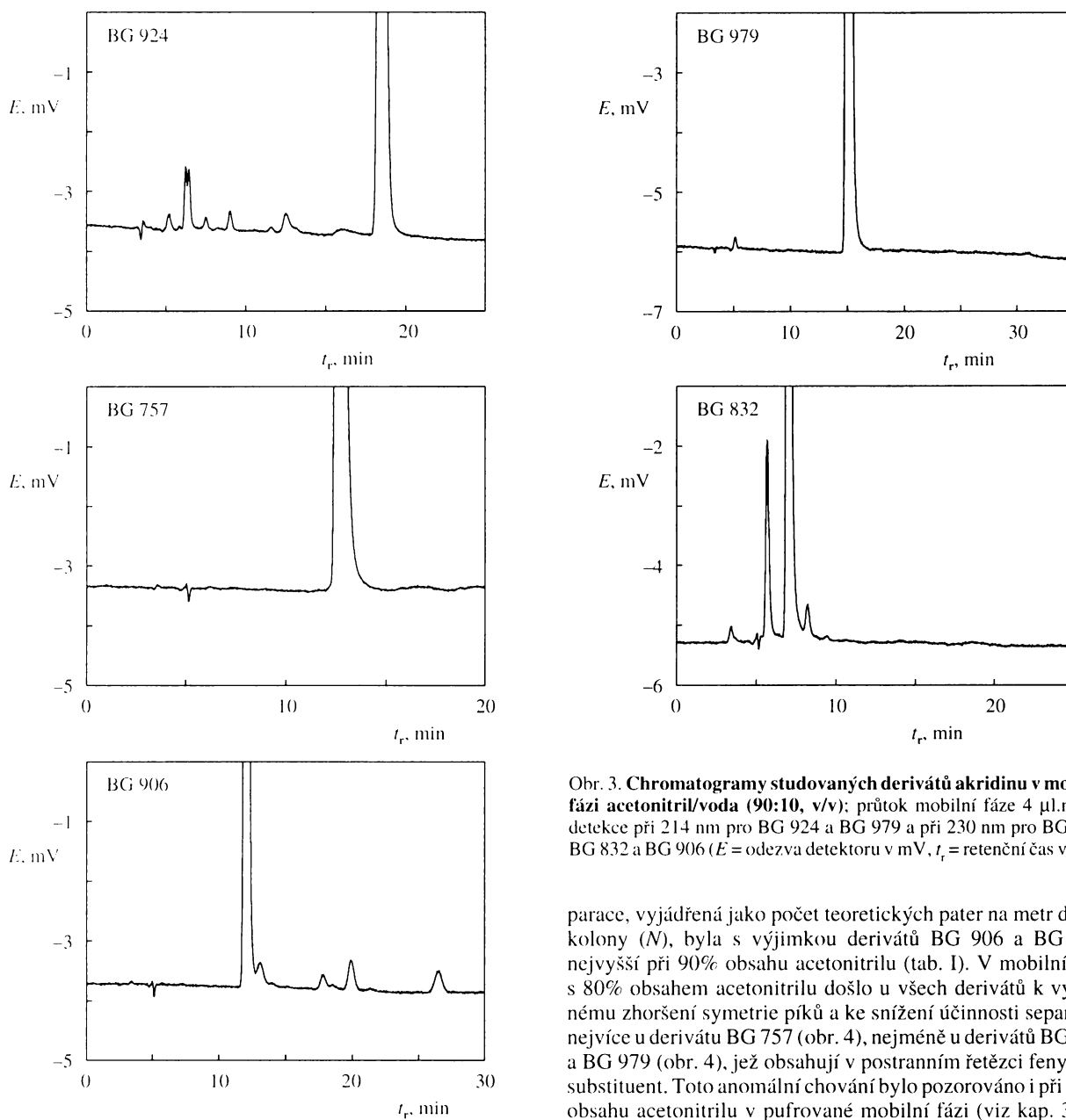
Obr. 2. Postup přípravy thioderivátů akridinu

Tabulka I

Retenční faktory (k), asymetrické faktory (As) a počet teoretických pater (N) studovaných derivátů v binární mobilní fázi acetonitril/voda s různým obsahem acetonitrilu, průtoková rychlost $4 \mu\text{l}\cdot\text{min}^{-1}$, detekce při 230 nm

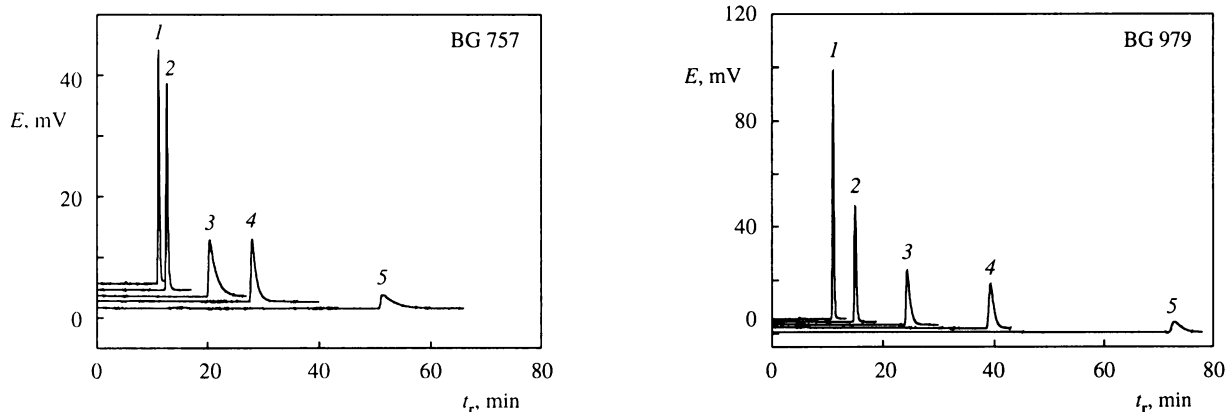
Derivát	90 %			80 %			70 %			60 %		
	k	As	N	k	As	N	k	As	N	k	As	N
BG 757	1,4	2,22	18800	2,8	7,9	4400	4,4	5,15	17100	8,8	8,23	8500
BG 832	0,3	1,63	12100	0,5	2,65	5600	0,7	1,88	10200	1,2	2,93	7700
BG 906	1,3	1,46	20400	2,8	3,72	14700	5,2	2,51	24200	10,9	5,56	21200
BG 924	2,5	1,36	29800	5,2	1,60	29200	11,6	1,51	26900	– ^a	– ^a	– ^a
BG 979	1,8	1,74	22800	3,5	3,77	17100	6,6	2,67	26900	13,0	3,75	26400

^a Neeluoval do 100 min



Obr. 3. Chromatogramy studovaných derivátů akridinu v mobilní fázi acetonitril/voda (90:10, v/v); průtok mobilní fáze $4 \mu\text{l}\cdot\text{min}^{-1}$, detekce při 214 nm pro BG 924 a BG 979 a při 230 nm pro BG 757, BG 832 a BG 906 (E = odezva detektoru v mV, t_r = retenční čas v min)

parace, vyjádřená jako počet teoretických pater na metr délky kolony (N), byla s výjimkou derivátů BG 906 a BG 979 nejvyšší při 90% obsahu acetonitrilu (tab. I). V mobilní fázi s 80% obsahem acetonitrilu došlo u všech derivátů k výraznému zhoršení symetrie píků a ke snížení účinnosti separace; nejvíce u derivátu BG 757 (obr. 4), nejméně u derivátů BG 924 a BG 979 (obr. 4), jež obsahují v postranním řetězci fenylový substituent. Toto anomální chování bylo pozorováno i při 80% obsahu acetonitrilu v pufrované mobilní fázi (viz kap. 3.1.).



Obr. 4. Vliv obsahu organického modifikátoru v binární mobilní fázi acetonitril/voda na retenční chování vybraných derivátů BG 757 a BG 979; průtok mobilní fáze $4 \mu\text{l}\cdot\text{min}^{-1}$, detekce při 230 nm, obsah acetonitrilu v mobilní fázi 1 – 100 %, 2 – 90 %, 3 – 80 %, 4 – 70 % a 5 – 60 % (E a t_r viz obr. 3)

Tabulka II

Retenční faktory (k), asymetrické faktory (A_s) a počet teoretických pater (N) studovaných derivátů v binární mobilní fázi (90:10 v/v) acetonitril/voda, průtoková rychlost $4 \mu\text{l}\cdot\text{min}^{-1}$ a acetonitril/0,2 mol. dm^{-3} octanový pufr o pH 3,5, průtoková rychlost $3 \mu\text{l}\cdot\text{min}^{-1}$, detekce 230 nm

Derivát	Mobilní fáze					
	nepufrovaná ^a			pufrovaná ^b		
	k	A_s	$N\cdot\text{m}^{-1}$	k	A_s	$N\cdot\text{m}^{-1}$
BG757	1,4	2,22	18800	1,5	1,86	25138
BG832	0,3	1,63	12100	0,4	1,33	16422
BG906	1,3	1,46	20400	1,5	1,30	26162
BG924	2,5	1,36	29800	2,5	1,19	36683
BG979	1,8	1,74	22800	2,3	1,37	33041

Průtok ^a $4 \mu\text{l}\cdot\text{min}^{-1}$, ^b $3 \mu\text{l}\cdot\text{min}^{-1}$

Z tabulky I je zřejmé, že změna obsahu acetonitrilu měla podstatně menší vliv na retenční chování derivátu BG 832 než na ostatní deriváty, což je důsledek přítomnosti polární hydroxyskupiny v postranním alkylovém řetězci derivátu BG 832.

Nízké hodnoty retenčních faktorů doprovodných nečistot (s výjimkou nečistot u derivátu BG 906) ukazují na jejich značně polární charakter, patrně odpovídající výchozím složkám syntézy derivátu 9-(alkylthio)akridinu (obr. 1). Ze získaných experimentálních dat je patrné, že separace v mobilní fázi acetonitril/voda (90:10 v/v) z hlediska kontroly čistoty jednotlivých derivátů byla dostačující. Vzhledem k absenci standardů (pro majoritní složky i pro doprovodné nečistoty) byla čistota jednotlivých derivátů (prekurzorů léčiv) určena metodou vnitřní normalizace. Za daných experimentálních podmínek (detekce při 230 nm, průtoková rychlost $4 \mu\text{l}\cdot\text{min}^{-1}$) byla stanovena čistota 100,0 % pro derivát BG 757, 90,2 % pro derivát BG 832, 92,9 % pro derivát BG 906, 92,3 % pro derivát 924 a 100,0 % pro derivát 979.

Optimalizace separačních podmínek za účelem kvantitativní analýzy

Z důvodu zvýšení citlivosti (zlepšení symetrie píků) při stanovení thioakridinových derivátů byl proměřen vliv acetátového pufru o pH 3,5 na separační chování jednotlivých derivátů. V mobilních fázích – acetonitril/0,2 mol. dm^{-3} octanový pufr o pH 3,5 s proměnlivým obsahem acetonitrilu došlo oproti vodně-acetonitrilovým mobilním fázím pouze k velmi malým změnám retence. Srovnávací měření byla prováděna při stejném průtoku mobilní fáze – tj. $4 \mu\text{l}\cdot\text{min}^{-1}$.

Proměřením van Deemterovy křivky ($2\text{--}5 \mu\text{l}\cdot\text{min}^{-1}$) v pufrované mobilní fázi s 90% obsahem acetonitrilu pro všechny deriváty bylo zjištěno, že účinnost kolony roste s klesajícím průtokem mobilní fáze. Pro měření kalibračních křivek byla tedy zvolena průtoková rychlost $3 \mu\text{l}\cdot\text{min}^{-1}$ jako výsledek kompromisu mezi vyšší účinností a únosností doby analýzy, která nepřesáhla u žádného derivátu dobu 22 min (tab. II).

V mobilní fázi (90:10 v/v) acetonitril/0,2 mol. dm^{-3} octanový pufr o pH 3,5 a průtokové rychlosti $3 \mu\text{l}\cdot\text{min}^{-1}$ byly pro všechny deriváty proměřeny kalibrační křivky při dvou vlnových délkách (214 a 230 nm) v koncentračním rozsahu $1\cdot 10^{-6}$ mol. dm^{-3} až $1\cdot 10^{-3}$ mol. dm^{-3} . Metodou lineární regrese byla vyhodnocována závislost jak plochy píku vs. molární koncentrace, tak výšky píku vs. molární koncentrace. Každá koncentrace příslušného derivátu byla proměřena 4x. Do kalibračních grafů byla vynášena průměrná hodnota plochy a výšky ze čtyř měření. V tabulce III a IV jsou uvedeny získané úseky, směrnice, korelační koeficienty (r) a lineární rozsahy kalibračních přímků (plocha vs. koncentrace) pro obě vlnové délky. Na obr. 5 je pro názornost uveden průběh závislosti log plochy píku na log molární koncentrace pro derivát BG 924 pro detekci při 214 nm.

Ze získaných parametrů kalibračních přímků je patrné, že detekce pro deriváty BG 757, BG 832 a BG 906 je citlivější při 230 nm, zatímco pro deriváty BG 924 a BG 979 je citlivější při 214 nm, což je v souladu s jejich strukturou. Nejvyšší hodnota směrnice, a tedy největší citlivost byla pozorována pro derivát BG 979 při 214 nm (viz tab. III). Úseky všech kalibračních přímků se neliší statisticky významně od nuly, a tudíž všechny tyto kalibrační přímků prochází nulou. U všech získa-

Tabulka III

Parametry kalibračních přímek a lineární rozsahy studovaných derivátů detegovaných při vlnové délce 214 nm

Derivát	Úsek ^a (SD ^b)	Směrnice ^c (SD ^b)	<i>r</i>	Lin. rozsah ^d
BG 757	0,034 (0,512)	280167 (1465)	0,9998	1.10 ⁻⁵ –8.10 ⁻⁴
BG 832	-0,001 (0,661)	182613 (2072)	0,9993	6.10 ⁻⁶ –8.10 ⁻⁴
BG 906	0,466 (1,738)	231656 (4973)	0,9981	1.10 ⁻⁵ –8.10 ⁻⁴
BG 924	1,306 (1,515)	447871 (3520)	0,9996	8.10 ⁻⁶ –1.10 ⁻³
BG 979	-1,351 (2,141)	600936 (5374)	0,9995	4.10 ⁻⁶ –1.10 ⁻³

^a mV.s., ^b SD – odhad směrodatné odchylky, ^c mV.s.dm³.mol⁻¹, ^d mol.dm⁻³

Tabulka IV

Parametry kalibračních přímek a lineární rozsahy studovaných derivátů detegovaných při vlnové délce 230 nm

Derivát	Úsek ^a (SD ^b)	Směrnice ^c (SD ^b)	<i>r</i>	Lin. rozsah ^d
BG 757	0,096 (0,725)	491191 (2074)	0,9999	1.10 ⁻⁵ –8.10 ⁻⁴
BG 832	-0,044 (0,838)	322043 (2626)	0,9996	6.10 ⁻⁶ –8.10 ⁻⁴
BG 906	0,658 (1,375)	405172 (4124)	0,9995	8.10 ⁻⁶ –8.10 ⁻⁴
BG 924	0,683 (1,198)	322749 (2784)	0,9996	8.10 ⁻⁶ –1.10 ⁻³
BG 979	-0,920 (1,629)	540494 (4089)	0,9996	4.10 ⁻⁶ –1.10 ⁻³

^a mV.s., ^b SD – odhad směrodatné odchylky, ^c mV.s.dm³.mol⁻¹, ^d mol.dm⁻³

Tabulka V

Hodnoty detekčních limitů (LOD) a limitů stanovení (LOS) měřených derivátů

Derivát	214 nm		230 nm	
	LOD (M) ^a	LOS (M) ^a	LOD (M) ^a	LOS (M) ^a
BG 757	7,8.10 ⁻⁶	2,6.10 ⁻⁵	5,9.10 ⁻⁶	2,0.10 ⁻⁵
BG 832	9,5.10 ⁻⁶	3,2.10 ⁻⁵	7,1.10 ⁻⁶	2,4.10 ⁻⁵
BG 906	7,6.10 ⁻⁶	2,5.10 ⁻⁵	5,4.10 ⁻⁶	1,8.10 ⁻⁵
BG 924	6,3.10 ⁻⁶	2,1.10 ⁻⁵	8,6.10 ⁻⁶	2,9.10 ⁻⁵
BG 979	5,3.10 ⁻⁶	1,8.10 ⁻⁵	6,0.10 ⁻⁶	2,0.10 ⁻⁵

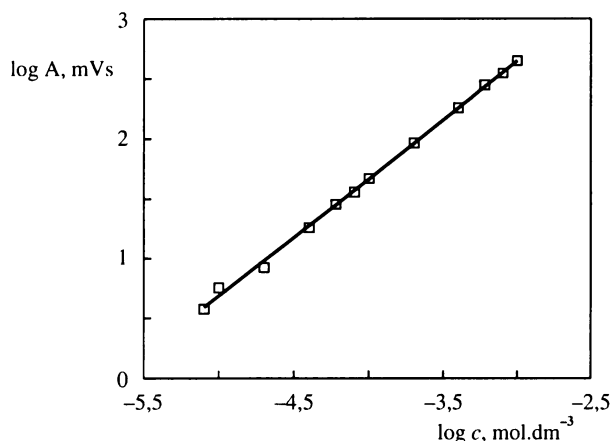
^a M – mol.dm⁻³

ných kalibračních přímek byl pozorován korelační koeficient velmi blízký 1,0000.

Detekční limit a limit stanovení byl vyhodnocován z regresních parametrů kalibračních přímek závislostí výšky píku na koncentraci. Pro detekci při 214 nm i při 230 nm se korelační koeficienty, získané pro jednotlivé deriváty, pohybovaly v rozsahu 0,9997 až 0,9998. Detekční limity (LOD) byly vypočítány jako trojnásobek absolutní hodnoty šumu, limity stanovení (LOS) byly vypočítány jako desetinásobek absolutní hodnoty šumu a jejich hodnoty jsou uvedeny v tabulce V.

Závěr

Byla vyvinuta metoda pro separaci a kvantifikaci pěti nových, farmaceuticky významných thioderivátů akridinu



Obr. 5. Závislost logaritmu plochy píku na logaritmu molární koncentrace derivátu BG 924 v koncentračním rozsahu 8.10⁻⁶–1.10⁻³ mol.dm⁻³ při 214 nm

pomocí kapilární kapalinové chromatografie. Byly nalezeny experimentální podmínky, zajišťující dobrou separaci vlastních látek a doprovodných nečistot. Pro všechny deriváty byly proměřeny kalibrační křivky při dvou vlnových délkách (214 a 230 nm). Lineární rozsah kalibračních přímek se pohyboval v oblasti 10⁻⁶ až 10⁻³ mol.dm⁻³ a detekční limity se pro jednotlivé deriváty pohybovaly v jednotkách až desítkách μmol.dm⁻³.

Práce na tomto projektu byla finančně podporována grantem GA ČR 203/00/1569 a byla součástí projektu COST Action

B 16. Autoři děkují paní Marcele Aussenbergové za pomoc při experimentální práci na tomto projektu.

LITERATURA

1. Modr Z.: *Remedia* 2, 821 (1994).
2. Barbe J.: *Bulletin 1st Acrival Meeting on Chemotherapy of Trypanosomatidae*, str. 63. Marseille 1992.
3. Schofield P. C., Robertson I. G. C., Paxton J. W.: *Cancer Chemother. Pharmacol.* 44, 51 (1999).
4. Kováč J., Kováč Š.: *Organická chemia*. SNTL, Praha 1977.
5. Jurlina J. L., Paxton J. W.: *J. Chromatogr.* 342, 431 (1985).
6. Jayewardene A. L., Santoro J. E., Gambertoglio J. G.: *J. Chromatogr.*, B 702, 203 (1997).
7. Košťálová I., Jelínek I., Zimová N., Barbe J.: *Chem. Listy* 91, 369 (1997).
8. Yakosouchi K., Taniguchi I., Yamaguchi H., Aekawa K.: *J. Electroanal. Chem.* 121, 230 (1981).
9. Vissers J. P. C., Claessens H. A., Cramers C. A.: *J. Chromatogr.*, A 779, 1 (1997).
10. Vissers J. P. C.: *J. Chromatogr.*, A 856, 117 (1999).
11. Ulmann F.: *Liebigs Ann. Chem.* 355, 327 (1907).
12. Smolders R. R., Hanuise J., Coonians R., Proietto V., Voglet N., Waefelaer A.: *Synthesis* 1982, 493.
13. Bisiri N., Johnsos C., Kayiréné M.: *Ann. Pharm. Fr.* 54, 27 (1996).

Z. Bosáková^a, E. Tesařová^b, P. Coufal^a, B. Kafková^a, and J. Barbe^c (^a*Department of Analytical Chemistry, ^bDepartment of Physical and Macromolecular Chemistry, Faculty of Science, Charles University, Prague, ^cFaculty of Pharmacy, Méditerranée Université, Marseilles, France*): **Separation and Quantification of Acridine Thio Derivatives by Capillary Liquid Chromatography**

An analytical method for separation and quantification of five acridine thio derivatives by capillary liquid chromatography has been developed. A capillary column, 320 μm I.D., filled with the Nucleosil 100-5 C 18 stationary phase, particle size 5 μm , was used. The mobile-phase composition was optimized in order to obtain a reasonable separation of the main component of each reaction mixture from impurities from the synthesis and a good quantification. It was shown that all the derivatives studied were well separated in binary mobile phases, acetonitrile/water or acetonitrile/0.2 mol.dm⁻³ acetate buffer of pH 3.5, with high acetonitrile contents (90 %). Calibration curves for all the derivatives were measured at wavelengths of 214 and 230 nm in the mobile phase composed of acetonitrile/0.2 mol.dm⁻³ acetate buffer of pH 3.5 (90:10, v/v). The linear dynamic ranges of the calibration curves were between 1.0 \times 10⁻⁶ and 1.0 \times 10⁻³ mol.dm⁻³ and the detection limits for the compounds were in the $\mu\text{mol.dm}^{-3}$ range.



ELSEVIER

Journal of Chromatography B, 770 (2002) 183–189

JOURNAL OF
CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Quantification and purity determination of newly synthesized thioacridines by capillary liquid chromatography

Pavel Coufal^{a,*}, Zuzana Bosáková^a, Eva Tesařová^b, Božena Kafková^a, Jana Suchánková^a, Jacques Barbe^c

^aDepartment of Analytical Chemistry, Faculty of Science, Charles University, Albertov 2030, 128 43 Prague 2, Czech Republic

^bDepartment of Physical and Macromolecular Chemistry, Faculty of Science, Charles University, Albertov 2030, 128 43 Prague 2, Czech Republic

^cGerctop Upres A CNRS 6009, Faculty of Pharmacy, 27 Bd Jean-Moulin, 133 85 Marseilles, France

Abstract

Capillary liquid chromatography (CLC) was applied for quantification and impurity profile determination of ten newly synthesized acridine thioderivatives. A reversed-phase CLC system employing two different stationary phases, Nucleosil C₁₈ and LiChrosorb RP-select B, was used. The mobile phase composition was optimized to get a satisfactory separation of impurities from the main acridine component in a reasonable analysis time. Significant differences in the chromatographic behavior between acridine derivatives containing and lacking amino groups were observed. Optimized separation conditions were used in CLC to measure the calibration curves of the acridine derivatives in a concentration range from $1.0 \cdot 10^{-6}$ to $1.0 \cdot 10^{-3}$ M at two different detector wavelengths (214 and 230 nm). Limits of detection and quantification of all the substances were determined. The detection limits went down to units of μM for most of the derivatives. CLC was also demonstrated to be a suitable method for the purity determination of test batches of the acridine thioderivatives. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Quantification; Thioacridines

1. Introduction

Some acridine derivatives are considered to be important chemotherapeutics because they show bactericidal, fungicidal, and especially, anti-malarial effects [1]. Derivatives of acridine have been applied for the treatment of protozoal infections caused by Plasmodium microorganisms for many years [2]. Additionally, there is the growing importance of acridine derivatives with antineoplastic properties, which have been identified recently [3]. An increas-

ing number of unhealthy factors in our environment, a decreasing immunity of all human beings to diseases and an increasing resistance of pathogens to chemotherapeutics used at present are the reasons why the pharmaceutical companies continue to develop and produce new derivatives with more efficient, effective and specific actions against pathogens. At the same time, analytical methods exhibiting higher selectivity and sensitivity are needed for purity determination of chemotherapeutics and their detection and quantification in biological materials.

Acridines are chemically derived from dibenzopyridine and contain at least one nitrogen atom in the aromatic cycle. Aqueous solutions of acridine show

*Corresponding author.

E-mail address: pcoufal@natur.cuni.cz (P. Coufal).

weak basic properties ($pK_b=8.4$). Derivatives and degradation products of acridine also behave as weak bases having altered dissociation constants determined through the character of substituents and their location in the acridine molecule [4].

High-performance liquid chromatography (HPLC) has most often been utilized to analyze acridine derivatives with respect to their identity, purity [5] and pharmacological and pharmacokinetic properties [6]. Capillary zone electrophoresis has been applied for the successful separation of acridines [7] as they can exhibit a positive charge in acidic solutions owing to their basic properties. Electrochemical oxidation of acridine without any substituents has also been studied [8]. However, capillary liquid chromatography (CLC) has not been employed for analysis of acridine and its derivatives to date.

CLC has some advantages, such as a low consumption of organic modifiers and a reduced sample amount (tens of nanoliters) compared to conventional HPLC [9,10]. These benefits of CLC can be especially advantageous for monitoring metabolites in body fluids and for determination of the main component and the impurities profile, since each analysis must be repeated several times to ensure reliability of the results in the frame work of good laboratory practice. Moreover, CLC seems to be more compatible with mass spectrometric detection than conventional HPLC.

Based on these facts, CLC was investigated in this work as a possible analytical technique for the quantification and the purity determination of ten newly synthesized acridine derivatives.

2. Experimental

2.1. Chemicals

Acetonitrile (gradient grade purity) for preparation of eluents was purchased from Merck (Darmstadt, Germany). Acetic acid (p.a.) and sodium hydroxide (p.a.) were supplied by Lachema (Brno, Czech Republic). Uracil (99%) for determination of the dead retention time was provided by Sigma (St. Louis, MO, USA). The water used for preparation of

all mobile phases and solutions was purified with a Milli-Q water purification system (Millipore, USA).

Acetonitrile–water and acetonitrile–0.2 M acetate buffer (pH 3.5) eluents containing various percentages of acetonitrile were used as mobile phases. The eluents were sonicated just before use for at least 10 min.

All the studied thioacridine derivatives, the structures and labels of which are given in Fig. 1, were synthesized at the Faculty of Pharmacy in Marseilles using the synthetic procedure depicted in Fig. 2. A substituted diphenylaminocarboxylic acid is formed by the reaction of a substituted *o*-chlorobenzoic acid and an aniline derivative in accordance to the Ullmann reaction [11]. The diphenylaminocarboxylic acid is cyclized to acridone using phosphoryl chloride and a substituted thioacridone is obtained by treating the acridone with phosphorus sulfide in

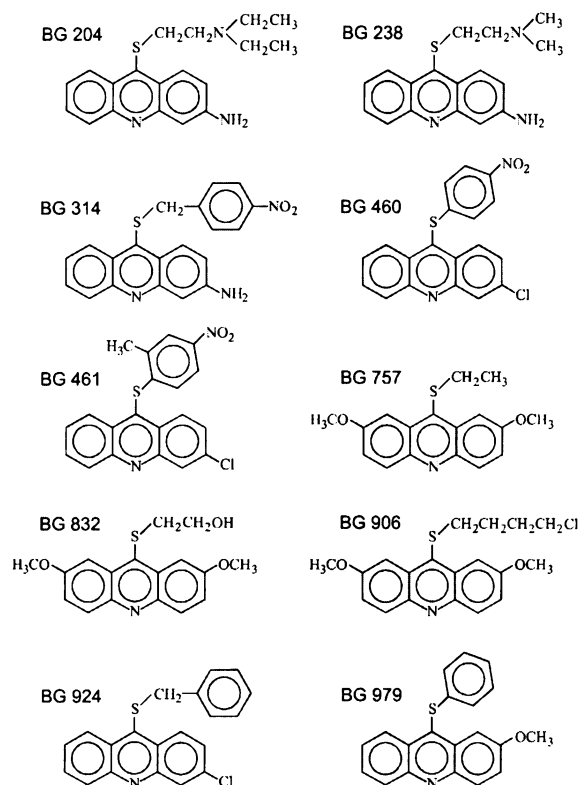


Fig. 1. Structures and labels of the acridine derivatives.

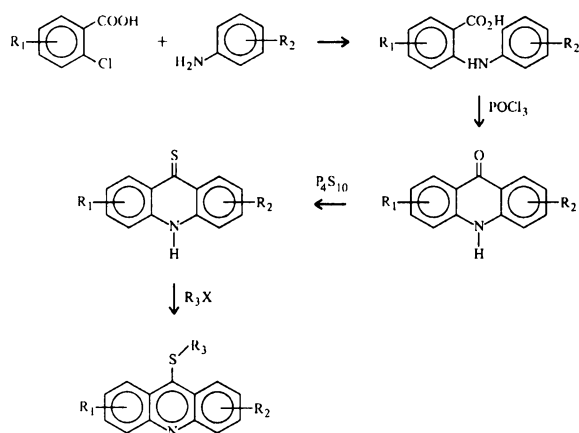


Fig. 2. Reaction scheme of the synthesis of acridine derivatives.

pyridine [12]. An alkylation or arylation of the thioacridone leads to a thioacridine derivative [13].

2.2. Equipment

An ISCO syringe pump model 100 DM (Lincoln, NE, USA), a Valco injection valve with a 60-nl internal loop (Schenkon, Switzerland) and a Linear UV-VIS 205 dual absorbance detector equipped with a CE on-column flow cell (San Jose, CA, USA) were applied for the CLC experiments. A fused-silica capillary column of 36 cm × 320 μm I.D. packed with 5 μm Nucleosil 100-5 C₁₈ was prepared at the Laboratory of Instrumental Analysis in Eindhoven Technical University, The Netherlands [14]. A stainless steel capillary column of 25 cm × 300 μm I.D. packed with 5 μm LiChrosorb RP-select B was purchased from Grom (Herrenberg, Germany). The column inlet was installed in the injection valve using a 5-cm polyether ether ketone (PEEK) sleeve (500 μm I.D.) and a PEEK finger-tight fitting and the column outlet was connected by PTFE tubing to a 220 μm I.D. fused-silica capillary with detection window located 7 cm from the column outlet. This capillary was placed into the absorbance detector operated at wavelengths of 214 and 230 nm. Chromatograms were recorded and evaluated employing CSW 1.7 computer software provided by DataApex (Prague, Czech Republic).

3. Results and discussion

3.1. Investigation of the separation conditions

The influence of the acetonitrile (ACN) content in the ACN–water eluent on retention of the studied acridine derivatives was investigated within the range 60–100% of ACN on the Nucleosil C₁₈ column. Thioacridines without any amino group (i.e. BG 460, BG 461, BG 757, BG 832, BG 906, BG 924 and BG 979, see Fig. 1) were eluted using all the mobile phases, however, long analysis times (over 50 min) were observed if eluents with a low ACN content were applied. When the ACN–water mobile phases were replaced with ACN–0.2 M acetate buffer (pH 3.5) eluents, only a negligible change in retention behavior and a small improvement considering the separation efficiency and peak symmetry were observed for acridine derivatives without amino groups [15,16].

On the other hand, thioacridines containing amino groups (i.e. BG 204, BG 238 and BG 314, see Fig. 1) were not eluted in a reasonable analysis time of 60 min on the Nucleosil C₁₈ column, not even with the ACN–0.2 M acetate buffer eluents. The high silanol activity of this stationary phase induces its affinity to basic compounds which can be retained strongly or even irreversibly in the Nucleosil C₁₈ column. Based on these results and experience, the LiChrosorb RP-select B column was chosen for the analysis of the acridine derivatives with amino groups. The latter type of stationary phase is generally recommended for separation of basic compounds since the silanol activity of the silica gel support is considerably diminished. Indeed, the basic thioacridines eluted from the LiChrosorb RP-select B column in acceptable analysis times using the ACN–0.2 M acetate buffer mobile phases.

To find separation conditions under which impurities from synthesis can effectively be separated from the main acridine derivative, the ACN content in the mobile phases was tuned within the range 60–100% (v/v). The ACN–0.2 M acetate buffer, pH 3.5 (90:10, v/v) eluent was able to resolve, in a reasonable time, the impurities from the main component on the Nucleosil C₁₈ column and on the LiChrosorb RP-select B column in the case of

Table 1

Parameters of the calibration curves (including standard deviations in parentheses) and linear dynamic ranges of acridines containing amino groups. Stationary phase, LiChrosorb RP-select B; mobile phase, ACN–0.2 M acetate buffer, pH 3.5 (90:10, v/v); flow-rate, 3 $\mu\text{l}/\text{min}$; detection wavelength, 214 nm

Derivative	Intercept (SD) (mV s)	Slope (SD) (mV s l/mol)	<i>r</i>	Linear dynamic range (M)
BG 204	6.973 (3.567)	152 000 (4400)	0.9995	$4.0 \cdot 10^{-5}$ – $1.0 \cdot 10^{-3}$
BG 238	–5.114 (4.737)	120 000 (13 500)	0.9877	$8.0 \cdot 10^{-5}$ – $1.0 \cdot 10^{-3}$
BG 314	–4.047 (4.403)	253 000 (14 300)	0.9952	$2.0 \cdot 10^{-5}$ – $1.0 \cdot 10^{-3}$

thioacridines without and with amino groups, respectively. The decrease of ACN content in the mobile phases did not lead to a separation of further impurities from the main component, but a longer analysis time and a greater peak asymmetry of the acridine derivatives were observed under these conditions. Van Deemter curves for some of the acridine derivatives were measured in an interval from 2 to 5 $\mu\text{l}/\text{min}$ for optimization of the eluent flow-rate [16]; e.g. with the derivative BG 461, HETP values of 26, 31, 35 and 41 μm were reached for the flow-rates of 2, 3, 4 and 5 $\mu\text{l}/\text{min}$, respectively. The linear dependences of the height equivalent to a theoretical plate on the flow-rate obtained within this interval indicated that the resistance to mass transfer was the major mechanism of band broadening of the analyte zones. A mobile phase flow-rate of 3 $\mu\text{l}/\text{min}$ was selected as a compromise between the separation efficiency and the analysis time.

The results showed that the ACN–0.2 M acetate buffer, pH 3.5 (90:10, v/v) eluent at a flow-rate of 3 $\mu\text{l}/\text{min}$ can be applied for separation and quantification of impurities from the main acridine component in an analysis time not exceeding 30 min.

3.2. Quantification of the thioacridine derivatives

Calibration curves for all the studied thioacridine derivatives were measured in the concentration inter-

val from $1.0 \cdot 10^{-6}$ to $1.0 \cdot 10^{-3}$ M at two different wavelengths, 214 and 230 nm. The optimized separation systems composed of the Nucleosil C₁₈ column (for the seven derivatives lacking amino groups) and on the LiChrosorb RP-select B column (for the three thioacridines containing amino groups) and the ACN–0.2 M acetate buffer, pH 3.5 (90:10, v/v) mobile phase at the flow-rate of 3 $\mu\text{l}/\text{min}$ were applied. Measurements at each concentration level were repeated four times and average values of the peak area were subjected to linear regressions. Intercepts, slopes and correlation coefficients of the calibration curves (peak area versus molar concentration) and linear dynamic ranges for acridines containing amino groups determined at the wavelengths of 214 and 230 nm are summarized in Tables 1 and 2, respectively. Parameters of the calibration curves and linear dynamic ranges for acridines lacking amino groups obtained at the same two wavelengths are listed in Tables 3 and 4.

As an example, calibration curves in logarithmic coordinates for the acridine derivative BG 832 within the linear concentration range $6.0 \cdot 10^{-6}$ – $8.0 \cdot 10^{-4}$ M are depicted in Fig. 3. An excellent linearity was observed for the calibration curves of thioacridine BG 832 at both wavelengths over the three concentration orders. A very good agreement between the experimental points and the linear calibration curves was indicated through the correlation co-

Table 2

Parameters of the calibration curves (including standard deviations in parentheses) and linear dynamic ranges of acridines with amino groups. Detection wavelength, 230 nm; for other conditions see Table 1

Derivative	Intercept (SD) (mV s)	Slope (SD) (mV s l/mol)	<i>r</i>	Linear dynamic range (M)
BG 204	–4.043 (7.915)	275 000 (9700)	0.9993	$4.0 \cdot 10^{-5}$ – $1.0 \cdot 10^{-3}$
BG 238	–4.898 (9.065)	170 000 (13 400)	0.9938	$8.0 \cdot 10^{-5}$ – $1.0 \cdot 10^{-3}$
BG 314	–4.428 (5.241)	309 000 (17 000)	0.9954	$2.0 \cdot 10^{-5}$ – $1.0 \cdot 10^{-3}$

Table 3

Parameters of the calibration curves (including standard deviations in parentheses) and linear dynamic ranges of acridines without amino groups. Stationary phase, Nucleosil C₁₈; mobile phase, ACN–0.2 M acetate buffer, pH 3.5 (90:10, v/v); flow-rate, 3 µl/min; detection wavelength, 214 nm

Derivative	Intercept (SD) (mV s)	Slope (SD) (mV s l/mol)	<i>r</i>	Linear dynamic range (<i>M</i>)
BG 460	0.003 (0.689)	353 000 (13 100)	0.9959	6.0·10 ⁻⁶ –1.0·10 ⁻⁴
BG 461	-0.551 (0.232)	633 000 (4700)	0.9998	4.0·10 ⁻⁶ –1.0·10 ⁻⁴
BG 757	0.034 (0.512)	280 000 (1500)	0.9998	1.0·10 ⁻⁵ –8.0·10 ⁻⁴
BG 832	-0.001 (0.661)	183 000 (2100)	0.9993	6.0·10 ⁻⁶ –8.0·10 ⁻⁴
BG 906	0.466 (1.738)	232 000 (5000)	0.9981	1.0·10 ⁻⁵ –8.0·10 ⁻⁴
BG 924	1.306 (1.515)	448 000 (3500)	0.9996	8.0·10 ⁻⁶ –1.0·10 ⁻³
BG 979	-1.351 (2.141)	601 000 (5400)	0.9995	4.0·10 ⁻⁶ –1.0·10 ⁻³

efficients for all the calibrations. The worst value of the correlation coefficient (i.e. $r=0.9877$) was obtained for acridine BG 238 at 214 nm, the variation of experimental points was explained from 97.6% (i.e. $100r^2$) by the calibration curve variation.

Based on the results summarized in Tables 1–4, a more sensitive detection for all the acridine derivatives with amino groups was obtained at 230 nm compared to 214 nm. The most sensitive detection was observed for the thioacridines BG 461 and BG 979 at 214 nm. The lowest slope values were obtained for the calibration curves measured with derivatives BG 204 and BG 238 at the same detection wavelength. None of the intercepts of the calibration curves in Tables 1–4 were found to be significantly different from zero at a significance level of $\alpha=0.01$ applying the *t*-test for intercepts, $t = \alpha/SD$.

The dependences of the peak height on the molar concentration of the studied acridines were plotted for determination of the limits of detection (LOD)

and the limits of quantification (LOQ) under the experimental conditions described previously. The LOD and LOQ values were obtained from these dependences as concentrations corresponding to the three-multiple and ten-multiple of root mean squared baseline noise, respectively [17]. The obtained values are summarized in Table 5. The detection limits for most of the acridine derivatives were as low as units of µM.

3.3. Impurity profile of test batches of the acridine derivatives

The optimized separation conditions were used to analyze the impurity profiles of test batches of the thioacridine derivatives containing various impurities from the synthetic procedure. The profiles of test batches of derivatives BG 314 and BG 832 are presented in Fig. 4. Since the chemical structures of the impurities were not known and therefore any standards for the impurities were not available, an

Table 4

Parameters of the calibration curves (including standard deviations in parentheses) and linear dynamic ranges of acridines lacking amino groups. Detection wavelength, 230 nm; for other conditions see the caption to Table 3

Derivative	Intercept (SD) (mV s)	Slope (SD) (mV s l/mol)	<i>r</i>	Linear dynamic range (<i>M</i>)
BG 460	0.065 (0.578)	321 000 (11 000)	0.9965	6.0·10 ⁻⁶ –1.0·10 ⁻⁴
BG 461	-0.098 (0.206)	510 000 (4100)	0.9997	4.0·10 ⁻⁶ –1.0·10 ⁻⁴
BG 757	0.096 (0.725)	491 000 (2100)	0.9999	1.0·10 ⁻⁵ –8.0·10 ⁻⁴
BG 832	-0.044 (0.838)	322 000 (2600)	0.9996	6.0·10 ⁻⁶ –8.0·10 ⁻⁴
BG 906	0.658 (1.375)	405 000 (4100)	0.9995	8.0·10 ⁻⁶ –8.0·10 ⁻⁴
BG 924	0.683 (1.198)	323 000 (2800)	0.9996	8.0·10 ⁻⁶ –1.0·10 ⁻³
BG 979	-0.920 (1.629)	540 000 (4100)	0.9996	4.0·10 ⁻⁶ –1.0·10 ⁻³

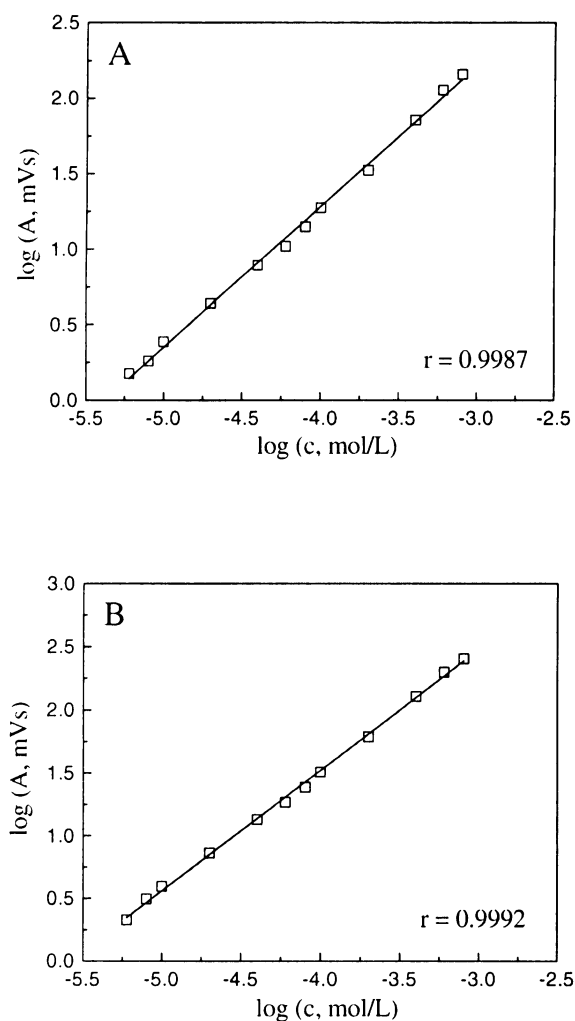


Fig. 3. Dependencies of the decadic logarithm of the peak area on the decadic logarithm of the molar concentration of the acridine derivative BG 832 in the concentration range $6.0 \cdot 10^{-6}$ – $8.0 \cdot 10^{-4}$ M measured at detection wavelengths of 214 (A) and 230 nm (B).

internal normalization evaluation method was applied to quantify the percentage of impurities in the test batches. Based on this evaluation method the following contents of the main components were determined in the corresponding test batches of thioacridines: 95.9% for BG 204, 96.7% for BG 238, 96.5% for BG 314, 100.0% for BG 460, 100.0% for BG 461, 100.0% for BG 757, 90.2% for BG 832, 92.9% for BG 906, 92.3% for BG 924 and 100.0% for BG 979 of the main component was determined in the corresponding test batch of thioacridine.

Table 5

Limits of detection (LOD) and limits of quantification (LOQ) of the thioacridines expressed in *M*. Stationary phase, Nucleosil C_{18} or LiChrosorb RP-select B; mobile phase, ACN–0.2 M acetate buffer, pH 3.5 (90:10, v/v); flow-rate, 3 μ l/min; detection wavelengths, 214 and 230 nm

Derivative	214 nm		230 nm	
	LOD	LOQ	LOD	LOQ
BG 204 ^a	$4.9 \cdot 10^{-5}$	$1.6 \cdot 10^{-4}$	$2.8 \cdot 10^{-5}$	$9.4 \cdot 10^{-5}$
BG 238 ^a	$3.6 \cdot 10^{-5}$	$2.1 \cdot 10^{-4}$	$2.7 \cdot 10^{-6}$	$1.1 \cdot 10^{-4}$
BG 314 ^a	$8.0 \cdot 10^{-6}$	$2.7 \cdot 10^{-5}$	$6.5 \cdot 10^{-6}$	$2.2 \cdot 10^{-5}$
BG 460	$9.1 \cdot 10^{-6}$	$3.1 \cdot 10^{-5}$	$1.0 \cdot 10^{-5}$	$3.5 \cdot 10^{-5}$
BG 461	$6.2 \cdot 10^{-6}$	$2.1 \cdot 10^{-5}$	$7.3 \cdot 10^{-6}$	$2.5 \cdot 10^{-5}$
BG 757	$7.8 \cdot 10^{-6}$	$2.6 \cdot 10^{-5}$	$5.9 \cdot 10^{-6}$	$2.0 \cdot 10^{-5}$
BG 832	$9.5 \cdot 10^{-6}$	$3.2 \cdot 10^{-5}$	$7.1 \cdot 10^{-6}$	$2.4 \cdot 10^{-5}$
BG 906	$7.6 \cdot 10^{-6}$	$2.5 \cdot 10^{-5}$	$5.4 \cdot 10^{-6}$	$1.8 \cdot 10^{-5}$
BG 924	$6.3 \cdot 10^{-6}$	$2.1 \cdot 10^{-5}$	$8.6 \cdot 10^{-6}$	$2.9 \cdot 10^{-5}$
BG 979	$5.3 \cdot 10^{-6}$	$1.8 \cdot 10^{-5}$	$6.0 \cdot 10^{-6}$	$2.0 \cdot 10^{-5}$

^a Determined on LiChrosorb RP-select B.

4. Conclusion

An analytical method for quantification and impurity profile determination of ten acridine thio derivatives by CLC has been developed. An ACN–0.2 M acetate buffer, pH 3.5 (90:10, v/v) eluent was found to sufficiently separate acridine impurities from the main component on the Nucleosil C_{18} column in the case of thioacridines without amino groups or on the LiChrosorb RP-select B column in the case of all ten acridine derivatives. These separation conditions were applied to measure the calibration curves for the thioacridines at two different wavelengths (214 and 230 nm) with linear dynamic ranges from $1.0 \cdot 10^{-6}$ to $1.0 \cdot 10^{-3}$ M and with detection and quantification limits reaching units and tens of μ M, respectively. Additionally, the content of impurities in the test batches of the thioacridine derivatives was determined by CLC under the same separation conditions. CLC was demonstrated to be a powerful separation technique for pharmaceutical applications, moreover, it guarantees a considerable saving in analysis time, organic modifiers and the use of mobile phases.

5. Nomenclature

A absorbance

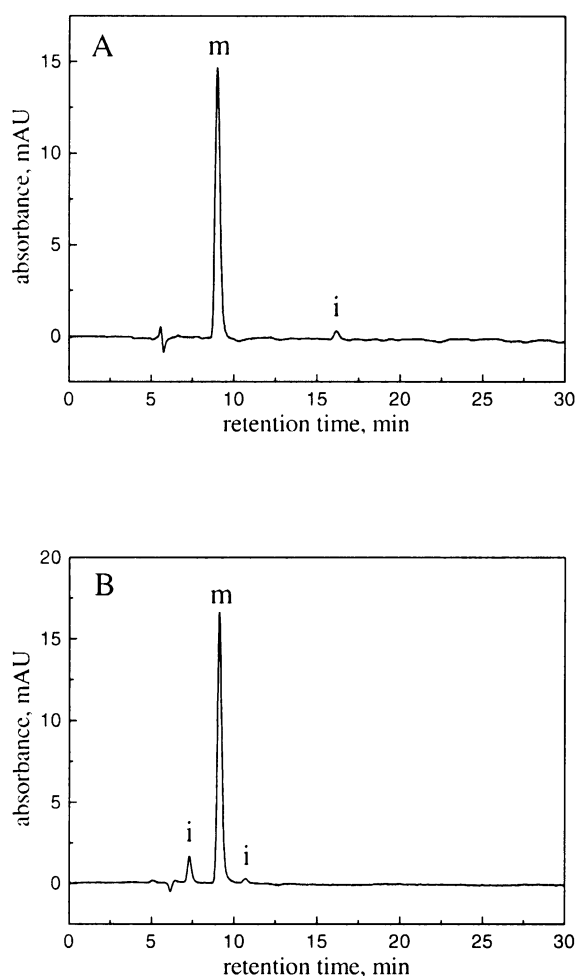


Fig. 4. Chromatograms of the impurity profile of the test batches of the derivatives BG 314 (A) and BG 832 (B). Stationary phases, LiChrosorb RP-select B (A) or Nucleosil C₁₈ (B); mobile phase, acetonitrile–0.2 M acetate buffer, pH 3.5 (90:10, v/v); flow-rate, 3 μ l/min; concentration, $1.0 \cdot 10^{-3}$ M; injection volume, 60 nl; detection wavelength, 230 nm; m, main component; i, impurity.

α	intercept of calibration curve
ACN	acetonitrile
CLC	capillary liquid chromatography
HETP	height equivalent to a theoretical plate
HPLC	high-performance liquid chromatography
i	impurity
I.D.	inner diameter
LOD	limit of detection
LOQ	limit of quantification

m	main component
PEEK	polyether ether ketone
RP	reversed-phase
r	correlation coefficient
SD	standard deviation
t	Student distribution

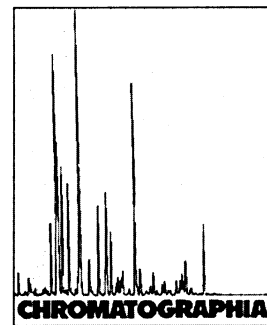
Acknowledgements

This work was financially supported by grant number 203/00/1569 of the Grant Agency of the Czech Republic. The study is a part of the international project COST Action B 16. The authors would like to thank Mrs. Marcela Aussenbergová for her technical assistance.

References

- [1] Z. Moder, *Remedia* 2 (1994) 821.
- [2] J. Barbe, in: *Bulletin 1st Acrival Meeting on Chemotherapy of Trypanosomatidae*, Marseilles, France, 1992, p. 63.
- [3] P.C. Schofield, I.G.C. Robertson, J.W. Paxton, *Cancer Chemother. Pharmacol.* 44 (1999) 51.
- [4] J. Kovác, Š. Kovác, *Org. Chem.*, SNTL, Prague, 1977.
- [5] J.L. Jurlina, J.W. Paxton, *J. Chromatogr.* 342 (1985) 431.
- [6] A.L. Jayewardene, J.E. Santoro, J.G. Gambertoglio, *J. Chromatogr. B* 702 (1997) 203.
- [7] I. Košťálová, I. Jelínek, N. Zimová, J. Barbe, *Chem. Listy* 91 (1997) 369.
- [8] K. Yakosouchi, I. Taniguchi, H. Yamaguchi, K. Aeakawa, *J. Electroanal. Chem.* 121 (1981) 230.
- [9] J.P.C. Vissers, H.A. Claessens, C.A. Cramers, *J. Chromatogr. A* 779 (1997) 1.
- [10] J.P.C. Vissers, *J. Chromatogr. A* 856 (1999) 117.
- [11] F. Ullmann, *Liebigs Ann. Chem.* 355 (1907) 327.
- [12] R.R. Smolders, J. Hanuise, R. Coonians, V. Proietto, N. Voglet, A. Waefelaer, *Synthesis* (1982) 493.
- [13] N. Bisiri, C. Johnsos, M. Kayiréné, *Ann. Pharm. Fr.* 54 (1996) 27.
- [14] J.P.C. Vissers, H.A. Claessens, J. Laven, C.A. Cramers, *Anal. Chem.* 67 (1995) 2103.
- [15] P. Coufal, E. Tesařová, Z. Bosáková, B. Kafková, J. Barbe, in: *Proceedings of the ITP 2000 Symposium*, Bratislava, September, 2000, p. B26.
- [16] Z. Bosáková, E. Tesařová, P. Coufal, B. Kafková, J. Barbe, *Chem. Listy* 95 (2001) 569.
- [17] R.C. Graham, in: *Data Analysis for the Chemical Sciences — A Guide To Statistical Techniques*, VCH, Weinheim, 1993, p. 161, 269.

System Peaks Observed in Capillary Liquid Chromatography with Eluents Containing Triethylamine



2002, 56, 445–447

B. Kafková¹ / Z. Bosáková¹ / E. Tesařová² / J. Suchánková¹ / P. Coufal^{1*} / K. Štulík¹

¹ Department of Analytical Chemistry, Faculty of Science, Charles University, Albertov 2030, 128 43 Prague 2, Czech Republic; E-Mail: pcoufal@natur.cuni.cz

² Department of Physical and Macromolecular Chemistry, Faculty of Science, Charles University, Albertov 2030, 128 43 Prague 2, Czech Republic

Key Words

Column liquid chromatography
Capillary liquid chromatography
Vacancy chromatography
System peaks
Triethylamine eluents

Summary

Triethylamine is often added to mobile phases in reversed-phase liquid chromatography for dynamic deactivation of free silanol groups of the stationary phase. It has been observed that eluents composed of methanol and triethylamine generate two system peaks in chromatograms obtained with LiChrosorb RP-select B stationary phase, whose retention times correspond to the dead time and to the retention time of triethylamine. It has been demonstrated that the system peaks can be positive or negative depending on the experimental conditions and may be incorrectly interpreted as peaks corresponding to sample components. An approach is outlined to unambiguously identify these system peaks in chromatograms of practical samples.

Introduction

When basic analytes are separated in reversed-phase liquid chromatography (RPLC), triethylamine (TEA) is often added to the mobile phase because TEA strongly interacts with the acidic free silanol groups of the stationary phase and thus deactivates them [1]. TEA is strongly adsorbed on any acidic RP stationary phase, substantially suppressing the interaction of basic analytes with the stationary phase surface, significantly reducing retention times and improving the peak

symmetry. TEA is, therefore, distributed between the stationary and the mobile phase during the RPLC separation and, because it strongly absorbs UV radiation, may give rise to pronounced system peaks also called vacancy peaks, ghost peaks, split peaks, pseudo peaks, induced peaks or eigenpeaks [2–4]. The system peaks of various mobile phase additives have been thoroughly studied, both theoretically [5, 6] and experimentally [7–9]. These peaks have also been utilized for indirect detection [10, 11] and for determination of adsorption isotherms and interaction constants of various analytes [12–16].

Levin and Grushka [3] have pointed out that system peaks are frequently misinterpreted and, therefore, the present work studies the character of the TEA-induced system peaks, explains their origin and draws conclusions on their interpretation.

Experimental

Methanol, acetonitrile (both of gradient grade purity) and triethylamine (99%) were supplied by Merck (Darmstadt, Germany). Thiourea (p.a.) and uracil (99%) were purchased from Lachema (Brno, Czech Republic) and Sigma (St. Louis, USA), respectively. The analyte, 3-amino-9-ethylthioacridine (BG 203), was synthesized at Gerctop Upres A CNRS 6009, Faculty of Pharmacy, Marseilles, France [17, 18]. Water was purified with a Milli-Q Water Purification System (Millipore Corp., USA).

An ISCO syringe pump 100 DM (Lincoln, NE, USA), a Valco injection valve with a 60 nL internal loop (Schenkon, Switzerland) and a Linear UV-vis-205 absorbance detector equipped with an on-column flow cell (San Jose, CA, USA) were used for the capillary liquid chromatography experiments. A stainless steel capillary column of 250 × 0.30 mm I.D. packed with 5 μm LiChrosorb RP-select B was purchased from Grom (Herrenberg, Germany). The column inlet was installed in the injection valve using a polyetheretherketone (PEEK) finger-tight fitting and the column outlet was directly connected by a piece of polytetrafluoroethy-

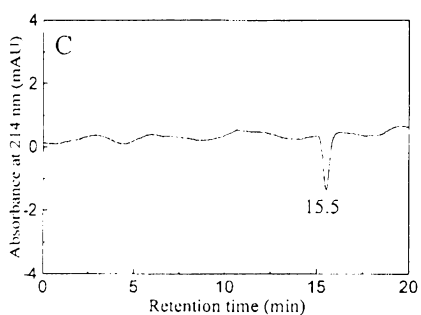
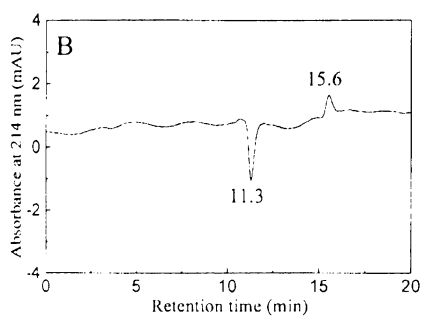
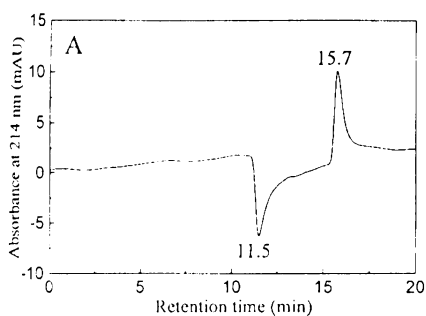


Figure 1. Chromatograms obtained with the methanol-triethylamine 99.9:0.1 (v/v) mobile phase on the LiChrosorb RP-select B capillary column injecting 60 nL of (A) water, (B) acetonitrile and (C) methanol. The system peaks are characterized by their retention times expressed in minutes. For the other conditions see Experimental.

lene (PTFE) tubing to a 0.22 mm I.D. fused-silica capillary with a detection window located 70 mm from the column outlet. This capillary was placed in the absorbance detector operated at wavelengths of 214 or 230 nm. A mobile phase flow-rate of $1 \mu\text{L} \cdot \text{min}^{-1}$ was maintained in all the experiments. The chromatograms were recorded and evaluated employing the CSW 1.7 computer software provided by DataApex (Prague, Czech Republic). All the measurements were carried out in triplicate.

Results and Discussion

In the first set of experiments, the methanol-triethylamine 99.9:0.1 (v/v) mobile

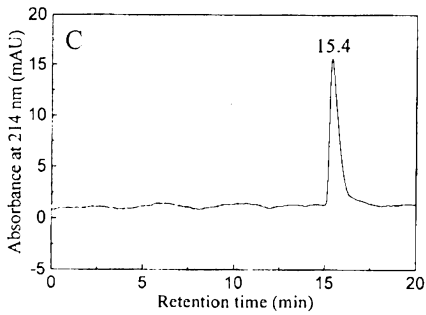
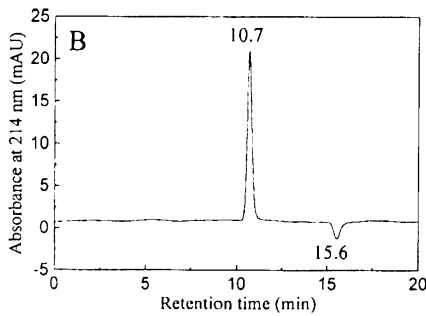
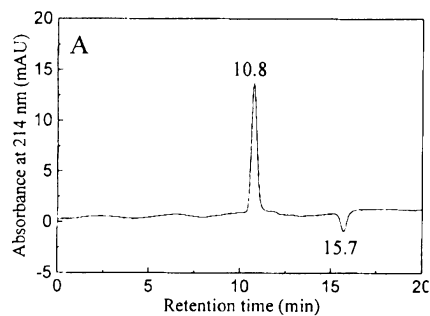


Figure 2. Chromatograms obtained with the methanol-triethylamine 99.9:0.1 (v/v) mobile phase on the LiChrosorb RP-select B capillary column injecting 60 nL of (A) uracil, (B) thiourea and (C) triethylamine, all dissolved in methanol. The system peaks are characterized by their retention times expressed in minutes. For the other conditions see Experimental.

phase was pumped through the LiChrosorb RP-select B column at a flow-rate of $1 \mu\text{L} \cdot \text{min}^{-1}$ and 60 nL samples of deionized water, acetonitrile and methanol were injected onto the column. For water (A) and acetonitrile (B) samples, the chromatograms contained a negative peak at 11.5 and 11.3 min and a positive peak at 15.7 and 15.6 min, respectively. The resulting chromatograms containing system peaks are depicted in Figure 1. The chromatogram with the methanol sample (C) contained only one negative peak at 15.5 min.

Considering the literature [2–4, 12], we assumed that the first, negative system peak could be attributed to a local deficiency of triethylamine in the mobile

phase caused by injection of the sample lacking TEA. This system peak might also be induced by a methanol misbalance between the stationary and mobile phases. Therefore, the first system peak would correspond to the injection peak [4] indicating the dead time. The second system peak, positive for water and acetonitrile samples and negative for the methanol sample was considered to be a local excess and deficiency, respectively, of TEA on the stationary phase [2]. The second peak would thus represent the retention time of TEA in the given chromatographic system.

To confirm these assumptions, a second set of experiments was performed. Three samples containing uracil, thiourea and TEA dissolved in methanol were injected into the same chromatographic system (Figure 2). The chromatograms with the uracil (A) and thiourea (B) samples contained two peaks with the retention times of 10.8 and 15.7 min (A), and 10.7 and 15.6 min (B), while that with the TEA sample (C) exhibited only one peak at 15.4 min. Uracil and thiourea, as dead time markers, proved that the negative system peaks observed in Figure 1 were real injection peaks generated by a local decrease in the TEA concentration in the injection zone that moved down the column with the eluent velocity. The retention time of the TEA peak in Figure 2 confirmed that the second system peak, positive or negative, was caused by a local increase or decrease, respectively, of the TEA concentration at the stationary phase surface. The zone corresponding to the second system peak moved with the velocity of TEA as the analyte along the column.

When summarizing the results described above, the following general explanation can be formulated. The first system peak with the retention time $t_1 = (11.1 \pm 0.6) \text{ min}$ ($\alpha = 0.05$) is caused by a local deficiency of TEA in the mobile phase because the injected zones of water and acetonitrile contain no TEA and proceed down the column with the eluent velocity, indicating the system dead time. The second system peak with the retention time $t_2 = (15.6 \pm 0.1) \text{ min}$ ($\alpha = 0.05$) has a more complex origin and its character is determined by the actual distribution of TEA between the stationary and mobile phases which is primarily affected by the differences in the polarities of water, acetonitrile and methanol. TEA is very poorly miscible with highly polar water,

its miscibility with substantially less polar acetonitrile is much higher and that with the least polar methanol virtually unlimited under the present conditions. Therefore, the zone of water shifts the distribution of TEA between the mobile and stationary phases strongly in favour of the stationary phase (the high positive peak in Fig. 1A), this effect is much less pronounced with acetonitrile (the small positive peak in Fig. 1B) and reversed with methanol (the negative (vacancy) peak in Fig. 1C). This desorption of TEA from the stationary phase in the presence of a pure methanol zone compensates the TEA deficiency in the mobile phase and thus the first peak observed with water and acetonitrile does not appear in Fig. 1C. In all these cases, the retention time of the system peak is determined by the TEA affinity to the stationary phase as with any other analyte. This interpretation of the experimental observations is in agreement with the conclusions published earlier [2, 3, 12].

To test these conclusions, a practical analysis has been carried out. Figure 3 gives chromatograms of a BG 203 sample at two different concentration levels detected at two wavelengths. The positive system peak, generated by the injection of water as a component of the sample solution into the eluent containing TEA, may be misinterpreted. Chromatograms A and B represent analyses of 1 mM BG 203 dissolved in water with detection at 214 and 230 nm, respectively, and chromatograms C and D correspond to the same analysis at a BG 203 concentration one order of magnitude lower. The first positive peak corresponds to BG 203 and the second one is the system peak. It can be seen that the identification of the peaks can be quite difficult in more complex samples: e.g., the system peak observed in chromatogram C is even higher than the analyte peak as the absorption coefficient of TEA at 214 nm is much higher than that at 230 nm.

Conclusion

The results described and discussed above stress the necessity of careful testing of separation systems selected for particular analyses for the presence and character of system peaks. It seems that the best approach is injection of several compounds of different polarities whose behaviour in the given separation system is well known, which can affect the equilibria between

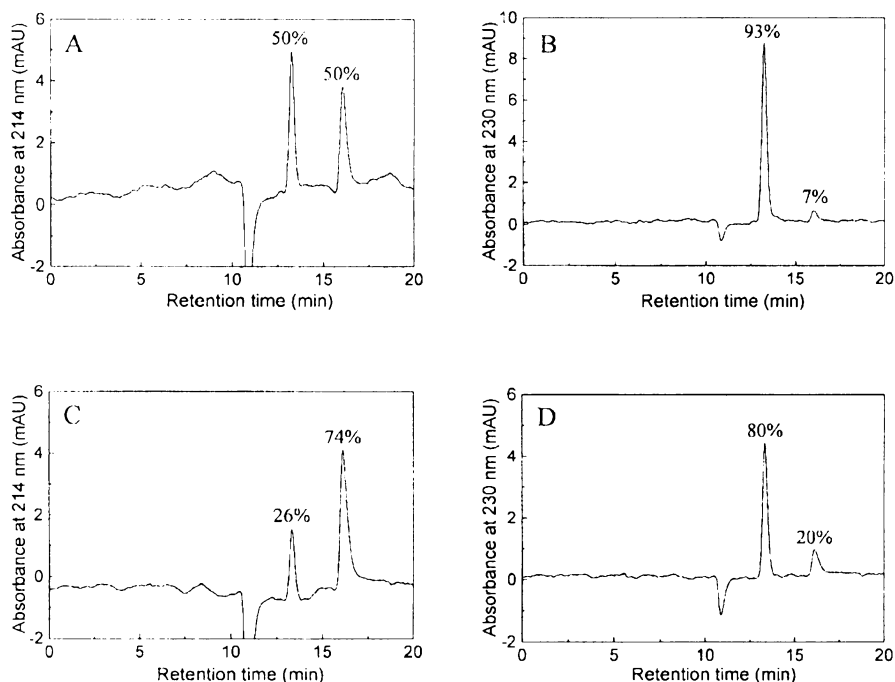


Figure 3. Chromatograms obtained with the methanol-triethylamine 99.9:0.1 (*v/v*) mobile phase on the LiChrosorb RP-select B capillary column injecting 60 nL of (A, B) 1 mM and (C, D) 0.1 mM solution of BG 203 in water. Detector wavelengths were 214 (A, C) and 230 nm (B, D). The first positive peak represents BG 203 and the second one is the system peak. The peak areas are expressed in percentage. For the other conditions see Experimental.

the stationary and mobile phases in a well-defined way and which are likely to be present in the practical samples, e.g., as their solvents.

Acknowledgments

This work was financially supported by Grant No. 203/00/1569 of the Grant Agency of the Czech Republic and the salaries of the scientists were supported by research projects No. J13/98:113100002 and J13/98:113100001 of the Charles University. The authors would like to thank Prof. Jacques Barbe of Geretop Upres A CNRS 6009, Faculty of Pharmacy, Marseilles for his kind providing of the BG 203 sample.

References

- [1] Vervoort, R.J.M.; Maris, F.A.; Hindriks, H. *J. Chromatogr.* **1992**, *623*, 207–220.
- [2] Stranahan, J.J.; Deming, S.N. *Anal. Chem.* **1982**, *54*, 1540–1546.
- [3] Levin, S.; Grushka, E. *Anal. Chem.* **1986**, *58*, 1602–1607.
- [4] Gennaro, M.C. *J. Liq. Chromatogr.* **1987**, *10*, 3347–3375.
- [5] Golshan-Shirazi, S.; Guiochon, G. *Anal. Chem.* **1990**, *62*, 923–932.
- [6] Zhong, G.; Fornstedt, T.; Guiochon, G. *J. Chromatogr. A* **1996**, *734*, 63–74.

- [7] Ye, M.; Ding, Y.; Mao, J.; Shi, L. *J. Chromatogr.* **1990**, *518*, 238–241.
- [8] Sajonz, P.; Yun, T.; Zhong, G.; Fornstedt, T.; Guiochon, G. *J. Chromatogr. A* **1996**, *734*, 75–81.
- [9] Abreu, O.; Lawrence, G.D. *Anal. Chem.* **2000**, *72*, 1749–1753.
- [10] Herne, P.; Renson, M.; Crommen, J. *Chromatographia* **1984**, *19*, 274–279.
- [11] Shill, G.; Arvidsson, E. *J. Chromatogr.* **1989**, *492*, 299–318.
- [12] Levin, S.; Grushka, E. *Anal. Chem.* **1987**, *59*, 1157–1164.
- [13] Levin, S.; Abu-Lafi, S. *J. Chromatogr.* **1991**, *556*, 277–285.
- [14] Porcar, I.; García, R.; Campos, A.; Soria, V. *J. Chromatogr. A* **1994**, *673*, 65–76.
- [15] Hage D.S.; Tweed S.A. *J. Chromatogr. B* **1997**, *699*, 499–525.
- [16] Hage, D.S. *J. Chromatogr. A* **2001**, *906*, 459–481.
- [17] Bsiri, N.; Johnson, C.; Kayirere, M.; Galy, A.M.; Galy, J.P.; Barbe, J.; Osuna, A.; Mesa-Valle, C.; Castilla-Calvente, J.J.; Rodrigues-Cabezas, M.N. *Ann. Pharm. Fr.* **1996**, *54*, 27.
- [18] Coufal, P.; Bosáková, Z.; Tesařová, E.; Kačková, B.; Suchánková, J.; Barbe, J. *J. Chromatogr. B* **2002**, *770*, 183–189.

Received: Feb 25, 2002
Revised manuscript received: Jun 4, 2002
Accepted: Jun 14, 2002

Božena Kafková^{a)},
Eva Tesařová^{b)},
Jana Suchánková^{a)},
Zuzana Bosáková^{a)},
Pavel Coufal^{a)}

Capillary liquid chromatography as a tool for separation of hydrophobic basic drugs. Relation between tests for column characterization and real analysis

^{a)} Department of Analytical Chemistry, Faculty of Science, Charles University, Albertov 2030, 128 43 Prague 2, Czech Republic

^{b)} Department of Physical and Macromolecular Chemistry, Faculty of Science, Charles University, Albertov 2030, 128 43 Prague 2, Czech Republic

Two capillary columns for reversed phase (RP) capillary liquid chromatography (CLC), viz. Nucleosil 100–5 C18 and LiChrosorb RP-select B, were characterized by the Walters test, i.e. the chromatographic test proposed for RP stationary phases. Hydrophobicity indices were determined not only in acetonitrile/water mobile phase, as proposed in the test, but they were also measured in buffered systems. This approach was used to quantify the influence of mobile phase composition on the modification of the surface of the stationary phases. In the next step, small basic compounds differing in their hydrophobicity and basicity were selected and their retention on the stationary phases in mobile phases of the same composition as used for column testing was examined. Furthermore, the retention of newly synthesized drugs, chemotherapeutics derived from thioacridine and pyridoquinoline, differing in their structures, basicity, and hydrophobicity, was also studied. The composition of the mobile phases had to be shifted to higher contents of organic modifiers – acetonitrile or methanol – in order to elute these hydrophobic compounds from the columns. The question we wanted to answer was: How is the method for testing of reversed phases related to retention, separation efficiency, and peak symmetry of various analytes?

Key Words: Capillary liquid chromatography; Reversed phase separation; Column testing; Thioacridines; Pyridoquinolines

Received: June 27, 2002; revised: December 12, 2002; accepted: December 23, 2002

DOI 10.1002/jssc.200301383

1 Introduction

Reversed phase capillary liquid chromatography (RP CLC) represents a modern variant of RP HPLC. Its advantages are low consumption of mobile phase and small sample volume without loss of separation efficiency. Reversed phase liquid chromatography almost completely covers the demand for separation in routine analysis. Complex mixtures of hydrophobic compounds can be separated with high efficiency in a short analysis time. Separation of basic compounds, on the other hand, can still cause serious problems. The right choice of reversed stationary phase is essential for successful analysis of basic analytes. A wide variety of reversed phases is nowa-

days available. Various tests or methods of characterization providing preliminary information about the properties of a given RP and about their potential suitability for separation of hydrophobic and basic compounds of stationary phases have been described [1–8]. Nevertheless, the final result of separation of a mixture of interest does not always match the requirements.

Selection and testing of columns is a complex process and is often carried out on the basis of subjective criteria and personal experience. Information on the physico-chemical properties and separation mechanisms of commercially available columns is limited, as manufacturers usually do not disclose fundamental information. Furthermore, materials that are nominally identical often exhibit manufacturer-to-manufacturer and even batch-to-batch differences in their behaviour. Many chromatographic procedures have been proposed for column testing but none of them has gained general acceptance [9]. Most of the tests have been designed for reversed stationary phases. It has been shown [10] that the column parameters such as hydrophobicity and hydrophilicity strongly depend on the nature of the test substances. It is thus of fundamental importance to choose test substances that are as similar

Correspondence: Eva Tesařová, Department of Physical and Macromolecular Chemistry, Faculty of Science, Charles University, Albertov 2030, 128 43 Prague 2, Czech Republic. Phone: +420 2 21952606. Fax: +420 2 24919752. E-mail: tesarove@natur.cuni.cz.

Abbreviations: As, asymmetry factor; CLC, capillary liquid chromatography; HI, hydrophobicity index; log *P*, common logarithm of the octanol/water partition coefficient; *N,N*-DMA, *N,N*-dimethylaniline; SI, silanol index; TEA, triethylamine; TEAA, triethylamine acetate.

the compounds to be separated as possible. This requirement is often difficult to fulfil. Recently, Lopez et al. [11] have shown different approaches to identify small subsets of solutes that can be used to predict retention factors of a broad range of compounds on different octadecyl silica columns.

An increasing number of unhealthy factors in our environment, a decreasing immunity of all human beings to diseases, and an increasing resistance of pathogens to chemotherapeutics in present use drive the efforts of pharmaceutical companies to continue developing and producing new derivatives with a more efficient, effective, and specific action against pathogens. Some acridine and pyridoquinoline derivatives can be considered as important chemotherapeutics because they show bactericidal, fungicidal, and especially anti-malarial effects [12–15]. Recent thioacridine and pyridoquinoline derivatives with promising antineoplastic properties have been identified [6, 17].

Acridines and pyridoquinolines are chemically derived from dibenzopyridine and benzopyridine, respectively, and contain at least one nitrogen atom in the aromatic cycle. Aqueous solutions of acridine ($pK_b = 8.4$) and pyridoquinoline ($pK_b = 9.2$) show weak basic properties. Their derivatives and degradation products also behave as weak bases having modified dissociation constants determined by the nature and location of substituents in their molecules [18].

Separation methods exhibiting a high selectivity and sensitivity are needed for purity control of chemotherapeutics and their quantification in biological materials. Since a suitable separation system is a prerequisite for successful analysis, our work had the goal of establishing whether any relations exist between the results of the Walters test, characterization of the stationary phase with small basic analytes differing in their hydrophobicity, and analysis of complex mixtures of hydrophobic precursors of drug with and without basic substituents in their molecules.

Experimental

1 Chemicals

Acetonitrile (ACN) and methanol (MeOH) (both in gradient grade purity) for preparation of eluents were purchased from Merck (Darmstadt, Germany). Acetic acid (p.a.) and sodium hydroxide (p.a.) were supplied by Lachema Brno, Czech Republic). Triethylamine (TEA) (99.6%) as obtained from Merck (Darmstadt, Germany). Uracil (99%) for determination of the dead retention time was provided by Sigma (St. Louis, MO, USA). Water used for preparation of all mobile phases and solutions was purified with a Milli-Q Water Purification System (Millipore Corp., MA, USA).

Acetonitrile/water, acetonitrile/0.2 mol/L acetate buffer (pH = 3.5) and acetonitrile/1% triethylamine acetate buffer (pH = 3.5) (TEAA) eluents containing 60 or 65% of acetonitrile were used as mobile phases. Also methanol, its mixture with triethylamine (99.5/0.5 v/v) or MeOH with 0.5% TEA in water (80/20 v/v) were used as mobile phases. The eluents were sonicated for at least 10 min just before use.

Small basic analytes, i.e., arginine, imidazole, creatinine, aniline, and *N,N*-dimethylaniline, were purchased from Sigma (St. Louis, MO, USA).

All the studied thioacridine and pyridoquinoline derivatives, structures and designations of which are given in **Figure 1**, were synthesized at the Faculty of Pharmacy in Marseilles by a synthetic procedure described in ref. [19]. The thioacridines and pyridoquinolines had already been studied in our previous work, in which the separation of the compounds of interest from impurities and quantification of the main products were performed [19, 20].

2.2 Instrumentation

An ISCO model 100 DM syringe pump (Lincoln, NE, USA), a Valco International injection valve with a 60 nL internal loop (Schenk, Switzerland), and a LINEAR UVIS-205 dual absorbance detector equipped with a CE on-column flow cell (San Jose, CA, USA) were applied for the CLC experiments. A fused silica capillary column of 320 μm ID and 36 cm length packed with 5 μm Nucleosil 100-5 C18 was prepared at the Laboratory of Instrumental Analysis of Eindhoven Technical University, the Netherlands [21]. A stainless steel capillary column of 300 μm ID and 25 cm length packed with 5 μm LiChrosorb RP-select B was purchased from Grom (Herrenberg, Germany). A home-made thermostat was used to control the column temperature. The absorbance detector was operated at wavelengths of 200, 214, 230, and 254 nm. Chromatograms were recorded and evaluated employing a CSW 1.7 computer software package provided by DataApex (Prague, Czech Republic).

Before measurements, the columns were equilibrated overnight with each new mobile phase. All the measurements were repeated three times.

3 Results and discussion

3.1 Column testing – The Walters test

Two capillary columns for RP CLC, viz. Nucleosil 100-5 C18 and LiChrosorb RP-select B, were characterized by the Walters test [2]. This relatively simple test procedure classifies reverse stationary phases based on the evaluation of two predominating retention mechanisms in RPLC, the hydrophobicity and silanophilicity of a stationary phase. Hydrophobic interactions, depending on the

Thioacridine derivatives

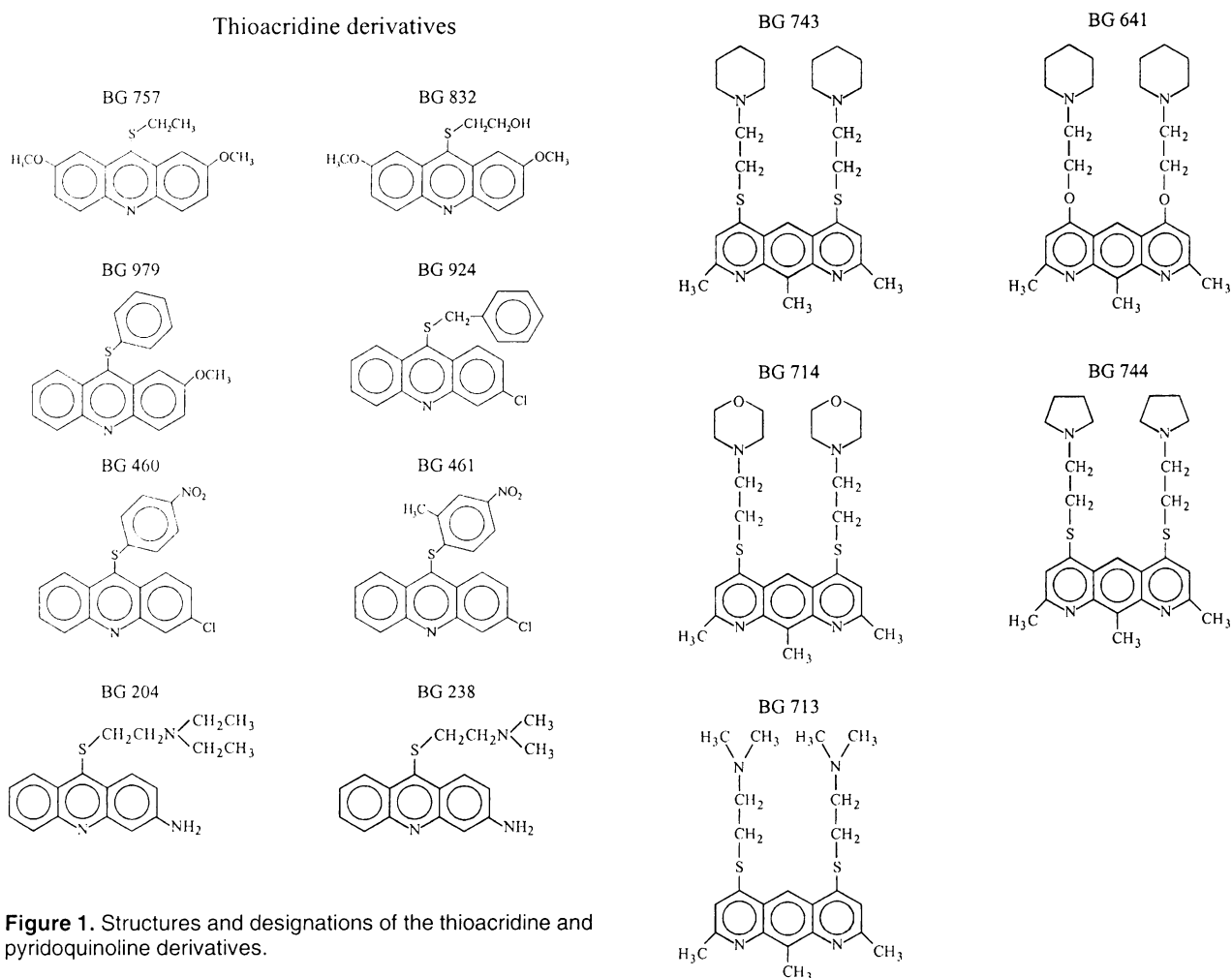


Figure 1. Structures and designations of the thioacridine and pyridoquinoline derivatives.

hydrocarbon content, are represented by the ratio of the retention factors of anthracene and benzene in an acetonitrile-water mobile phase and are expressed in terms of the *hydrophobicity index*. The silanophilic interactions of a column (i. e., the *silanol index*), depending on accessible active silanols, are determined by the ratio of the retention factor of *N,N*-diethyltoluamide (*N,N*-DETA) to that of anthracene in pure acetonitrile, as reversed-phase alkyl chains (i. e., C18, C8) are solvated well using pure acetonitrile. The retention of *N,N*-DETA is sensitive to the stationary phase silanol activity, while the anthracene retention behaviour is assumed to be exclusively determined by the hydrophobic interactions. Values of hydrophobicity and silanol indices of the studied columns are shown in **Table 1**. Hydrophobicity indices were determined not only in acetonitrile/water mobile phase, as proposed in the test, but they were also measured in buffered systems. This approach was used to quantify the influence of mobile phase composition on modification of the surface of stationary phases. The problems with relations between the test data in unbuffered systems and those of real analyses

Table 1. Characterization of the studied RP CLC stationary phases according to the Walters test.

	SI	HI	HI* _{acet}	HI* _{TEAA}
Nucleosil100-5 C18	0.46	4.60	4.94	2.61
LiChrosorb RP-select B	1.01	2.91	2.78	1.67

SI = silanol index. HI = hydrophobicity index.

Mobile phase compositions: SI, pure acetonitrile; HI, 65/35 (v/v) acetonitrile/water; HI*_{acet}, 65/35 (v/v) acetonitrile/0.2 mol/L acetate buffer, pH 3.5; HI*_{TEAA}, 65/35 (v/v) acetonitrile/1% triethylamine acetate buffer, pH 3.5.

usually performed in buffered systems were also mentioned by McCalley [22].

Nucleosil 100-5 C18 exhibited much higher hydrophobicity and lower silanol activity than the base deactivated stationary phase LiChrosorb RP-select B. The HI values were almost unaffected if water in the mobile phase was replaced with acetate buffer. On the other hand, the use of

Table 2. Retention factors, plate numbers per meter, and peak asymmetries of small basic analytes on the stationary phases studied.

Analyte	log <i>P</i>	<i>pK_a</i>	Nucleosil 100-5 C18								
			water			acet			TEAA		
			<i>k</i>	N/m	<i>As</i>	<i>k</i>	N/m	<i>As</i>	<i>k</i>	N/m	<i>As</i>
<i>N,N</i> -DMA	2.31	8.85	1.85	1240	7.80	5.21	3900	4.70	0.50	19800	1.18
niline	1.06	4.63	0.41	7440	2.35	0.57	6080	2.50	0.18	14800	1.56
midazole	−0.08	6.95	>	n.a.	n.a.	>	n.a.	n.a.	0.54	290	7.20
reatinine	−1.76	4.60	0.12	1200	4.67	0.80	1950	3.60	0.17	2730	2.60
rginine	−4.20	1.82/8.99	3.49	1100	1.68	0.45	4680	1.00	0.10	6640	1.23

Analyte	log <i>P</i>	<i>pK_a</i>	LiChrosorb RP-select B								
			water			acet			TEAA		
			<i>k</i>	N/m	<i>As</i>	<i>k</i>	N/m	<i>As</i>	<i>k</i>	N/m	<i>As</i>
<i>N,N</i> -DMA	2.31	8.85	1.31	40900	1.28	1.13	36970	1.70	0.40	23400	1.60
niline	1.06	4.63	0.47	23800	1.24	0.77	27560	1.32	0.23	21380	1.35
midazole	−0.08	6.95	0.85	1610	3.25	2.04	18900	1.55	0.73	3470	5.48
reatinine	−1.76	4.60	−	n.a.	n.a.	−	n.a.	n.a.	0.39	3980	3.17
rginine	−4.20	1.82/8.99	1.25	1780	1.85	0.02	2670	4.91	−	n.a.	n.a.

mobile phases: water, 65/35 (v/v) acetonitrile/water; acet, 65/35 (v/v) acetonitrile/0.2 mol/L acetate buffer, pH 3.5; TEAA 65/35 (v/v) acetonitrile/1% triethylamine acetate buffer, pH 3.5.

Notes: >, analyte does not elute within 100 min; −, analyte elutes with the dead volume marker; n.a., not applicable.

Formulae: $N/m = 5.54(t_R/w_{1/2})^2/L$.

N/m = number of theoretical plates per meter; t_R = retention time; $w_{1/2}$ = peak width at half height; L = column length [m]; $As = a/b$ as evaluated at 10% of the peak height; a = rear side of peak; b = front side of peak ($w_{0.1} = a + b$).

triethylamine acetate buffer in the mobile phases substantially reduced the indices of hydrophobicity on both columns. This result may be explained by the fact that TEAA not only masks active silanols on the silica gel surface but can also interact with certain analytes or test compounds in the mobile phase. Under specific circumstances, repulsive forces can be generated. Moreover, the Walters test is not designed for systems with TEAA. The high silanol index of LiChrosorb RP-select B was a surprising result as this RP was designed for separation of basic compounds whose strong interaction with the silica gel surface cause inferior separation and quantification.

Modification of the surface of the unshielded Nucleosil stationary phase was also observed after long-term use of the column for separation of basic hydrophobic compounds. A corresponding increase of HI (HI = 5.46) and decrease of SI (SI = 0.31), indicating irreversible sorption of these analytes, was obtained.

2 Retention of small basic compounds

Another possible method of checking the suitability of a reversed phase for separation of a mixture of compounds of interest uses a selected group of compounds differing in their hydrophobicity (expressed by log *P*) and basicity

(*pK_a*) [23]. As already mentioned above, the interaction mechanism on reversed phases is a combination of hydrophobic interactions of solutes with hydrophobic C18 (or some other) chains and ion exchange (or exclusion) with residual, more or less deprotonated, silanols [24]. Therefore the retention of a set of basic analytes on the stationary phases in mobile phases of the same composition as used for column testing according to Walters was examined. **Table 2** summarizes the results. The basicity of these analytes is the dominating factor that determines retention in a non-buffered mobile phase on both stationary phases. This is demonstrated by the highest retention factors of the most basic analytes of this series – *N,N*-dimethylaniline (*N,N*-DMA) and arginine (Arg). These two analytes represent the extreme log *P* values of the studied set of compounds (see Table 2). The retention factor of *N,N*-DMA with the highest hydrophobicity was enhanced in the mobile phase composed of acetonitrile/acetate buffer while the hydrophilic molecule of arginine was much less retained in this mobile phase on Nucleosil 100-5 C18. The steep increase of retention of *N,N*-DMA is also due to its increased cationic ionization. The decreased retention of Arg can be explained by partial repulsion of the negatively charged part of the molecule (*pK_{a1}* = 1.82) and a portion of dissociated silanol groups

on the stationary phase surface. The results correspond to a small change of hydrophobicity indices if acetate buffer was used instead of water in the mobile phase. On the Nucleosil column, the hydrophobicity increased slightly in the buffered mobile phase but was somewhat reduced on the LiChrosorb stationary phase. Obviously, the acidic buffer suppresses the dissociation of the accessible silanol groups on the silica carrier in the case of Nucleosil. Therefore the ion exchange interactions are reduced while the hydrophobic ones are enhanced. The different modification of the silanols on the used RPs by acetate can be explained by various properties of the silica support. Silanol groups are known to dissociate over a wide range of pH depending on the structural arrangement (isolated, geminal, or vicinal forms) [25]; in addition, the bonding chemistry can influence the accessibility of the silica surface to analytes. The purity of silica gel and the manner of its purification or synthesis also play a role here [24]. Triethylamine in the mobile phase reduced the retention independently of the column used and the compound measured because TEA modifies separation mechanisms by masking silanol groups and can also interact with solutes. This is again in accordance with the results obtained by the Walters test (see Table 1).

As peak tailing is an indicator of ionic interactions, the interaction mechanism can also be deduced from asymmetry values (A_s). In addition, comparison of number of theoretical plates per meter (N/m) can reveal the effect of hydrophobic interactions. The values are summarized in Table 2. Although no clear trends were observed, some general conclusions can be formulated. In general, higher efficiency and better peak symmetry were observed for all the test compounds on LiChrosorb RP-select B. The plate number per meter increased and peak asymmetry decreased along the following sequence of aqueous components of mobile phases: water < acetate < TEA on the Nucleosil 100-5 C18 column. On LiChrosorb RP-select B neither separation efficiency nor peak shape were improved if water was replaced by acetate buffer or TEA. Due to the surface deactivation of the latter column ACN/water mobile phase already gave satisfactory results.

In the next step, the retention behaviour of two groups of larger basic compounds – potential drugs, differing in size of their molecules, hydrophobicity, and basicity – was evaluated using both the stationary phases studied. These two groups of analytes comprised selected derivatives of thioacridine, and some analogues of pyridoquinoline.

3.3 Retention of newly synthesized chemotherapeutics – thioacridines

Thioacridines exhibited much higher hydrophobicity than the small analytes. Therefore the composition of the

mobile phases had to be changed in order to elute these compounds from the columns. High contents of organic modifiers – acetonitrile or methanol – were needed for this purpose. Thioacridine derivatives without an amino group in their molecule showed very similar retention behaviour on both the stationary phases. The effect of different alkyl chain lengths (C8 vs. C18) was negligible. Separation efficiency and peak symmetry were slightly higher on LiChrosorb RP-select B, as demonstrated by the values of retention factors (k) and asymmetry factors (A_s) for the derivative BG 832 ($k = 0.3$, $N/m = 6800$, $A_s = 1.3$, and $k = 0.3$, $N/m = 7700$, $A_s = 1.2$ on Nucleosil 100-5 C18 and on LiChrosorb RP-select B, respectively). A similar effect was also noticed if the non-buffered mobile phase was replaced by the buffered one on the Nucleosil column. Again plate number and symmetry of peaks were improved while almost no change of retention was observed. Although the molecule size and hydrophobicity of these thioacridine derivatives were higher, their retention behaviour was similar to that of the small analytes. An illustrative chromatogram of a separation of a mixture of thioacridines without an amino group in their molecule on LiChrosorb RP-select B column is shown in **Figure 2**.

More basic thioacridines possessing an amino substituent exhibited unacceptable retention times (over 60 min) on Nucleosil 100-5 C18 using both organic modifiers – methanol or acetonitrile – in the mobile phase. The change of Nucleosil 100-5 C18 for LiChrosorb RP-select B caused a substantial decrease of retention time, as can be seen in **Figure 3.A,B**. On LiChrosorb RP-select B the use of mobile phase with acetate buffer led to a decrease of peak asymmetry and an increase of efficiency compared with ACN/water mobile phase while the retention was almost unaffected. As an example, the retention and asymmetry factors of the BG 204 derivative on LiChrosorb RP-select B stationary phase are given: $k = 1.4$, $A_s = 4.4$,

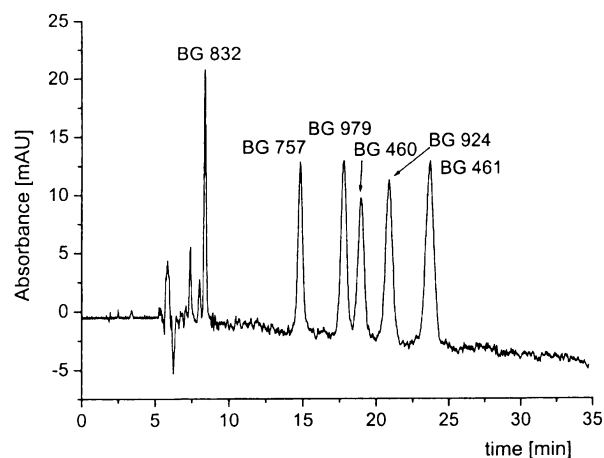


Figure 2. Separation of thioacridine derivatives on the LiChrosorb RP-select B column using the mobile phase 60/40 (v/v) acetonitrile/0.2 mol/L acetate buffer, pH 3.5.

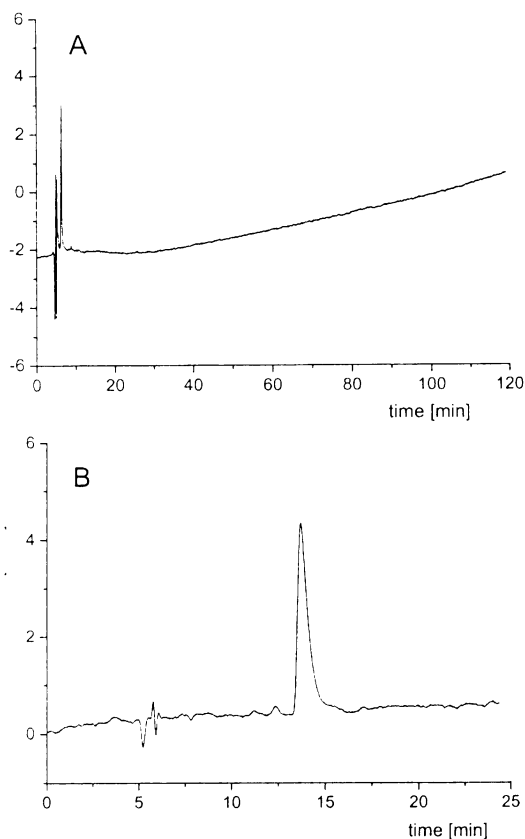


Figure 3. The influence of stationary phase nature on the separation of thioacridine derivative BG 204 possessing an amino group using the mobile phase 60/40 (v/v) acetonitrile/2 mol/L acetate buffer, pH 3.5. **A)** column Nucleosil 100–5 18; **B)** LiChrosorb RP-select B.

$k' = 10\ 300$ in the mobile phase composed of 90/10 (v/v) ACN/water, and $k = 1.5$, $A_s = 3.5$, $N/m = 12\ 500$ in 90/10 (v/v) ACN/0.2 mol/L acetate buffer.

4 Retention of newly synthesized chemotherapeutics – pyridoquinolines

Pyridoquinolines represented the most hydrophobic set of analytes. They could not be eluted from the studied stationary phases using any of the mobile phases mentioned above. Considering the lower solubility of pyridoquinolines in ACN we decided to use methanol as the mobile phase organic modifier instead of acetonitrile. Pyridoquinoline derivatives could be analysed on LiChrosorb RP select B column in pure methanol only and even then excessively long retention times, accompanied by bad peak shape and low efficiency, were obtained (**Figure 4.A**). In contrast to the retention behaviour of thioacridines, the length of the alkyl chain bonded to the silica surface plays a role in the retention of these hydrophobic compounds. To improve the peak shape and efficiency it was necessary to add small quantities of TEA (0.05–0.5%) to the mobile phase, as demonstrated in **Figure 4.B**. The concentration

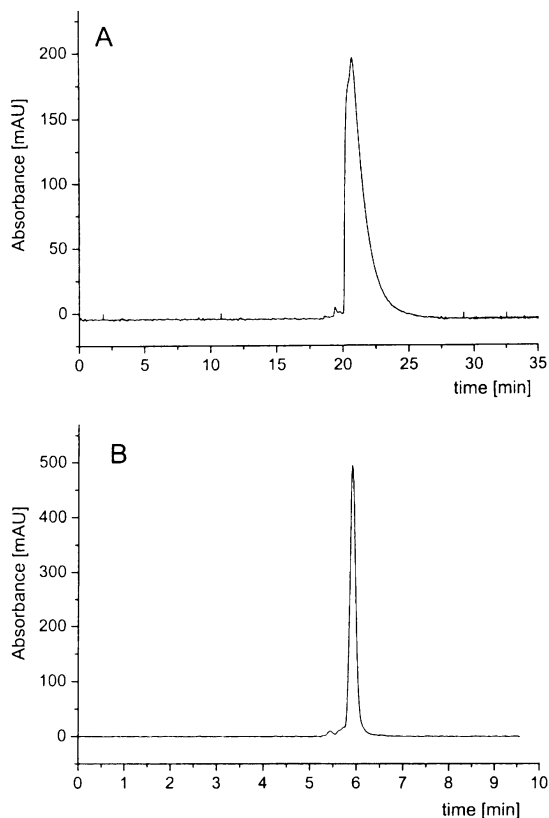


Figure 4. The influence of addition of TEA to the mobile phase on the retention behaviour of pyridoquinoline derivative BG 641 using the column LiChrosorb RP-select B. Mobile phase composition: **A)** 100% methanol; **B)** 99.5/0.5 (v/v) methanol/TEA.

of TEA in the mobile phase affected retention, peak symmetry and efficiency of pyridoquinolines in a similar way on both columns but the required amount of TEA was different. (While 0.05% of TEA was sufficient in the case of the LiChrosorb RP-select B, about ten times higher concentration was necessary with the Nucleosil C-18 column.) The first addition of TEA yielded significant reduction of retention and peak asymmetry while further increase of the concentration of TEA had a negligible effect on any of the retention characteristics.

These results are in accordance with the substantial decrease of the surface hydrophobicity (HI values) related to the addition of TEA (see Table 1). Separation of a mixture of pyridoquinoline derivatives under optimized conditions is illustrated in **Figure 5**. In order to achieve separation of a mixture of pyridoquinolines, 20% (v/v) of water had to be added to the mobile phase.

4 Concluding remarks

Compared to LiChrosorb RP-select B, the Nucleosil 100-5 C18 column showed a higher hydrophobicity but a lower

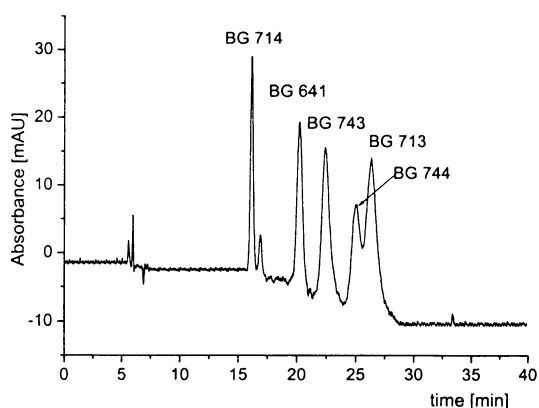


Figure 5. Separation of pyridoquinoline derivatives on the LiChrosorb RP-select B column using the mobile phase 80/20 (v/v) methanol/water with addition of 0.5% TEA.

silanol activity based on the Walters test. The same differences between these columns were found when low-molecular-weight compounds of various polarities were analysed. Strongly hydrophobic compounds of a large molecule size with or without amino group (thioacridines) were more successfully analysed on the LiChrosorb RP-select B column with the buffered mobile phases. The most hydrophobic compounds (pyridoquinolines) gave good separation results on the LiChrosorb RP-select B using TEA in the mobile phase.

Correlation between the results of column testing and real analysis of a mixture of hydrophobic basic compounds – newly synthesized potential drugs, derivatives of thioacridine and pyridoquinoline – could be found. For basic, rather hydrophobic compounds stationary phases with lower hydrophobicity and less deactivated surface with respect to residual silanol groups could be recommended. Variation of mobile phase composition has a substantial influence i) on the solvating/interaction of analytes in the environment of the mobile phase, but also, ii) on the modification of the surface of stationary phases. Both these factors subsequently affect the result of separation.

Acknowledgements

This work was financially supported by grant No. 245/2001/B-CH/PřF of the Grant Agency of the Charles University and by research projects J 13/98: 113100001 and J 13/98: 113100002 of the Charles University.

References

[1] H. Engelhardt, R. Grüner, *International Laboratory* **1999**, September, 34–42.

- [2] M.J. Walters, *J. Assoc. Off. Anal. Chem.* **1987**, *70*, 465–469.
- [3] K. Kimata, K. Iwaguchi, S. Onishi, K. Jinno, R. Eksteen, K. Hosoya, M. Araki, N. Tanaka, *J. Chromatogr. Sci.* **1989**, *27*, 721–728.
- [4] B.A. Bidlingmeyer, S.N. Deming, W.P. Price, B. Schoh, M. Petrussek, *J. Chromatogr.* **1979**, *186*, 419–434.
- [5] S.V. Galushko, A.A. Kamenchuk, G.L. Pit, *J. Chromatogr. A* **1994**, *660*, 47–59.
- [6] C. Horváth, W.R. Melander, J. Molnár, *Anal. Chem.* **1977**, *49*, 142–154.
- [7] P. Jandera, J. Rozkošná, *J. Chromatogr.* **1991**, *556*, 145–158.
- [8] L.C. Tan, P.W. Carr, M.H. Abraham, *J. Chromatogr. A* **1996**, *752*, 1–18.
- [9] H.A. Claessens, M.A. van Straten, C.A. Cramers, M. Jezierska, B. Buszewski, *J. Chromatogr. A* **1998**, *826*, 135–156.
- [10] G. Rippel, E. Alattyani, L. Szepeszy, *J. Chromatogr. A* **1994**, *668*, 301–311.
- [11] L.A. Lopez, S.C. Rutan, *J. Chromatogr. A* **2002**, *965*, 301–314.
- [12] J. Barbe, *Bulletin 1st Acrival Meeting on Chemotherapy of Trypanosomatidae*, France 1992.
- [13] J. Barbe, *The 2nd Congress of Cost Acrival Antiparasitic Chemotherapy*, Budapest 1993.
- [14] C. Matias, A. Mahamoud, J. Barbe, B. Pradines, J.C. Doury, *Heterocycles* **1996**, *43*, 1621–1632.
- [15] J. Singh, M. Seth, N. Fatma, S. Sharma, A.P. Bhaduri, R.K. Chatterjee, *Z. Naturforsch.* **1990**, *45*, 1210–1214.
- [16] P.C. Schofield, I.G.C. Robertson, J.W. Paxton, *Cancer Chemother. Pharmacol.* **1999**, *44*, 51–58.
- [17] C. Ordonez, *Ph.D. Thesis*, University of Leon, Spain, 2000.
- [18] J. Kováč, Š. Kováč, *Organická chemie*, SNTL, Praha 1977.
- [19] P. Coufal, Z. Bosáková, E. Tesařová, B. Kafková, J. Suchánková, J. Barbe, *J. Chromatogr. B* **2002**, *770*, 183–189.
- [20] J. Srbek, P. Coufal, E. Tesařová, Z. Bosáková, J. Suchánková, *J. Sep. Sci.*, in preparation.
- [21] J.P.C. Vissers, H.A. Claessens, J. Laven, C.A. Cramers, *Anal. Chem.* **1995**, *67*, 2103–2109.
- [22] D.V. McCalley, R.G. Bereton, *J. Chromatogr. A* **1998**, *828*, 407–420.
- [23] D. Sýkora, E. Tesařová, M. Popl, *J. Chromatogr. A* **1997**, *758*, 37–51.
- [24] U.D. Neue, C.H. Phoebe, K. Tran, Y.-F. Cheng, Z. Lu, *J. Chromatogr. A* **2001**, *925*, 49–67.
- [25] J. Nawrocki, *J. Chromatogr. A* **1997**, *779*, 29–71.

Chiral separation of beta-adrenergic antagonists, profen non-steroidal anti-inflammatory drugs and chlorophenoxypropionic acid herbicides using teicoplanin as the chiral selector in capillary liquid chromatography

B. Kafková^a, Z. Bosáková^{a,*}, E. Tesařová^b, P. Coufal^a

^a Department of Analytical Chemistry, Faculty of Science, Charles University, Albertov 2030, 128 40 Prague 2, Czech Republic

^b Department of Physical and Macromolecular Chemistry, Faculty of Science, Charles University, Albertov 2030, 128 40 Prague 2, Czech Republic

Available online 25 February 2005

Abstract

Three groups of structurally diverse chiral compounds were used to study the interaction mechanism responsible for stereoselective recognition with teicoplanin as chiral selector in capillary liquid chromatography. Teicoplanin-based chiral stationary phase (CSP) was used. The effect of the variation of mobile phase composition on retention and enantioselective separation was studied. The mobile phase composition suitable for enantioresolution of the various chiral compounds differed according to the interaction forces needed for chiral recognition. Mobile phases with high buffer portion (70–90 vol.%) were preferred for separation of enantiomers of profen non-steroidal anti-inflammatory drugs and chlorophenoxypropionic acid herbicides that require hydrophobic interactions, inclusion and π – π interactions for stereoselective recognition with teicoplanin. Higher concentration triethylamine in the buffer (0.5–1.0%) increased resolution of these acids. On the other hand, H-bonding and electrostatic interactions are important in stereoselective interaction mechanism of β -adrenergic antagonists with teicoplanin. These interaction types predominate in the reversed phase separation mode with high organic modifier content (95% methanol) and in polar organic mobile phases. For this reason β -adrenergic antagonists were best enantioresolved in the polar organic mode. The mobile phase composed of methanol/acetic acid/triethylamine, 100/0.01/0.01 (v/v/v), provided enantioresolution values of all the studied β -adrenergic antagonists in the range 1.1–1.9. Addition of teicoplanin to the mobile phase, which was suitable for enantioseparation of certain compounds on the CSP, was also investigated. This system was used to dispose of nonstereoselective interactions of analytes with silica gel support that often participate in the interaction with CSPs. Very low concentration of teicoplanin in the mobile phase (0.1 mM) resulted in enantioselective separation of 2,2- and 2,4-chlorophenoxypropionic acids.

© 2005 Elsevier B.V. All rights reserved.

Keywords: Chiral stationary phases; LC; Enantiomer separation; Teicoplanin; β -Adrenergic antagonists; Profen NSAIDs; Chlorophenoxypropionic acids

1. Introduction

Capillary liquid chromatography (cLC) can be considered a variant of high-performance liquid chromatography (HPLC). Miniaturization of separation columns in LC has some advantages such as small volumes of sample (tens of nanoliters), mobile and stationary phases. The benefits of cLC for enantioselective separations is related with the fact that it allows also using expensive mobile phase additives,

such as chiral selectors. The situation is more difficult with availability of CSPs for cLC. While a wide variety of ready-to-use chiral columns are produced for HPLC, chiral capillary columns must be prepared by packing a capillary with chiral stationary phase.

Macrocyclic antibiotics (MA) are a relatively new class of chiral selectors for chromatography and capillary electrophoresis (CE). The macrocyclic antibiotics have been used in HPLC [1,2], CE [3–5], capillary electrochromatography (CEC) [6], as reported in the review papers [1–6]. The most important selectors of this type are teicoplanin, vancomycin, ristocetin A and avoparcin. Teicoplanin (Fig. 1) is probably

* Corresponding author. Fax: +420 2 24 913 538.

E-mail address: bosakova@natur.cuni.cz (Z. Bosáková).

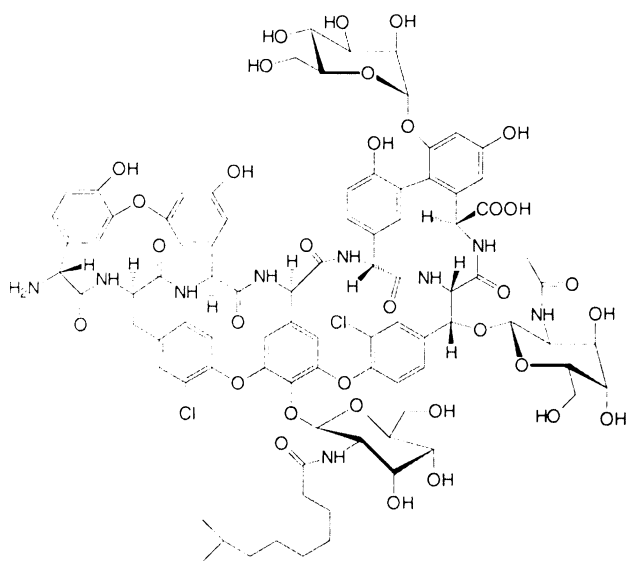


Fig. 1. Structure of teicoplanin used as the chiral selector in this study.

the most effective for enantioseparation of many structurally different compounds as amino acids, proteins and various drugs [1,2].

Three groups of chiral compounds with different chemical structures, namely β -adrenergic antagonists, profen NSAIDs and chlorophenoxypropionic acid (CPPA) herbicides, have been used to study their enantiomeric behavior in cLC with teicoplanin as the chiral selector. β -Adrenergic antagonists, compounds containing hydroxyl and amine groups and possessing at least one aromatic moiety in their molecules, are used in the treatment of some neurological, neuropsychiatric and cardiovascular disorders [7]. It is known that enantiomers of β -adrenergic antagonists have different therapeutic potencies and effects. For example, the *S*-enantiomer of propranolol is 100 times more potent as β -blocking agent than the *R*-enantiomer [8].

Various HPLC methods were proposed for the chiral separation of one or even a set of β -adrenergic antagonists [9–21]. Their enantiomers were separated using α -acid glycoprotein CSP (AGP) [9], polysaccharide [9–15] and cyclodextrin based [9,16] CSPs. Macrocyclic glycopeptides also contain in their structures peptides and carbohydrates. This indicates that all the typical interactions characteristic for cellulose (carbohydrates) and protein types of CSPs can be employed in the separation mechanism on the glycopeptide phases. Therefore, CSPs based on glycopeptides, namely on teicoplanin [17–21], teicoplanin aglycon [20] and vancomycin [17,21] were also tested for enantioseparation of several β -adrenergic antagonists. From the tested analytes atenolol and pindolol could not be baseline resolved on teicoplanin aglycone CSP [20].

Profen NSAIDs (2-arylpropionic acids) represent an important group of non-steroidal anti-inflammatory drugs, characterized by a chiral carbon atom near the carboxylic acid group. Several direct or indirect liquid chromatographic

methods involving a variety of CSPs were reported for their enantiomeric analysis [22–26]. Ibuprofen, fenoprofen, carprofen and flurbiprofen have been enantioseparated on cellulose tris-(4-methylbenzoate) CSP [22] and flurbiprofen on tris-(3,5-dimethylphenylcarbamate) of amylose CSP [23]. Native and derivatized β -cyclodextrins were used as chiral mobile phase additives to investigate the enantiomeric separation of ketoprofen, fenoprofen and ibuprofen [24]. Enantiomers of flurbiprofen and ketoprofen were resolved by using vancomycin CS [25]. Flurbiprofen could not be separated in the separation system when vancomycin is covalently bonded to silica gel support (column Chirobiotic V), but successful enantioresolution was obtained by addition of vancomycin to the mobile phase [26].

Phenoxypropionic acid (PPA) derivatives are widely used in agriculture as selective herbicides. Due to their solubility in water, they can easily move in agriculture ecosystems, causing surface and ground water pollution [27]. *R*-enantiomer of PPAs is known for its herbicidal activity while *S*-isomer is inactive as herbicidal agent [28]. Chiral separations of these herbicides are required in order to assess the enantiopurity and to optimize enantioselective production processes. The HPLC CSPs, which have been used to separate enantiomers of phenoxypropionic acid derivatives, included teicoplanin [20,29], cellulose derivatives [30,31], derivatized cyclodextrin [32] and some brush-type CSPs [33–35].

The aim of this work was to study the interaction of three structurally different groups of chiral compounds, i.e. β -adrenergic antagonists (Fig. 2), profen NSAIDs (Fig. 3) and chlorophenoxypropionic acids (Fig. 4), with teicoplanin-based CSP in capillary liquid chromatography. The influence of two separation modes, organic modifier content, concentration and pH of triethylamine acetate buffer on the chiral separation was investigated. Moreover, enantioseparation system with the chiral selector added to mobile phases was tested. Chromatographic systems with teicoplanin bonded to the silica gel support and free in solution were compared.

2. Experimental

2.1. Chemicals

The mobile phases were prepared from the following compounds and solvents: methanol, LiChrosolv, purity >99.8% (Merck, Darmstadt, Germany); triethylamine, purity >99.5% (Fluka, Buchs, Switzerland) and acetic acid (p.a. 99%) (Lachema, Brno, Czech Republic). The water used for preparation of all the mobile phases was purified with a Milli-Q water purification system (Millipore, USA).

Triethylamine acetate (TEAA) buffers, 0.1–1.0%, pH from 4.0 to 6.6, containing various percentages of methanol were used as mobile phases with a teicoplanin-based chiral stationary phase. Methanol/0.1% TEAA, pH 5.0 eluents

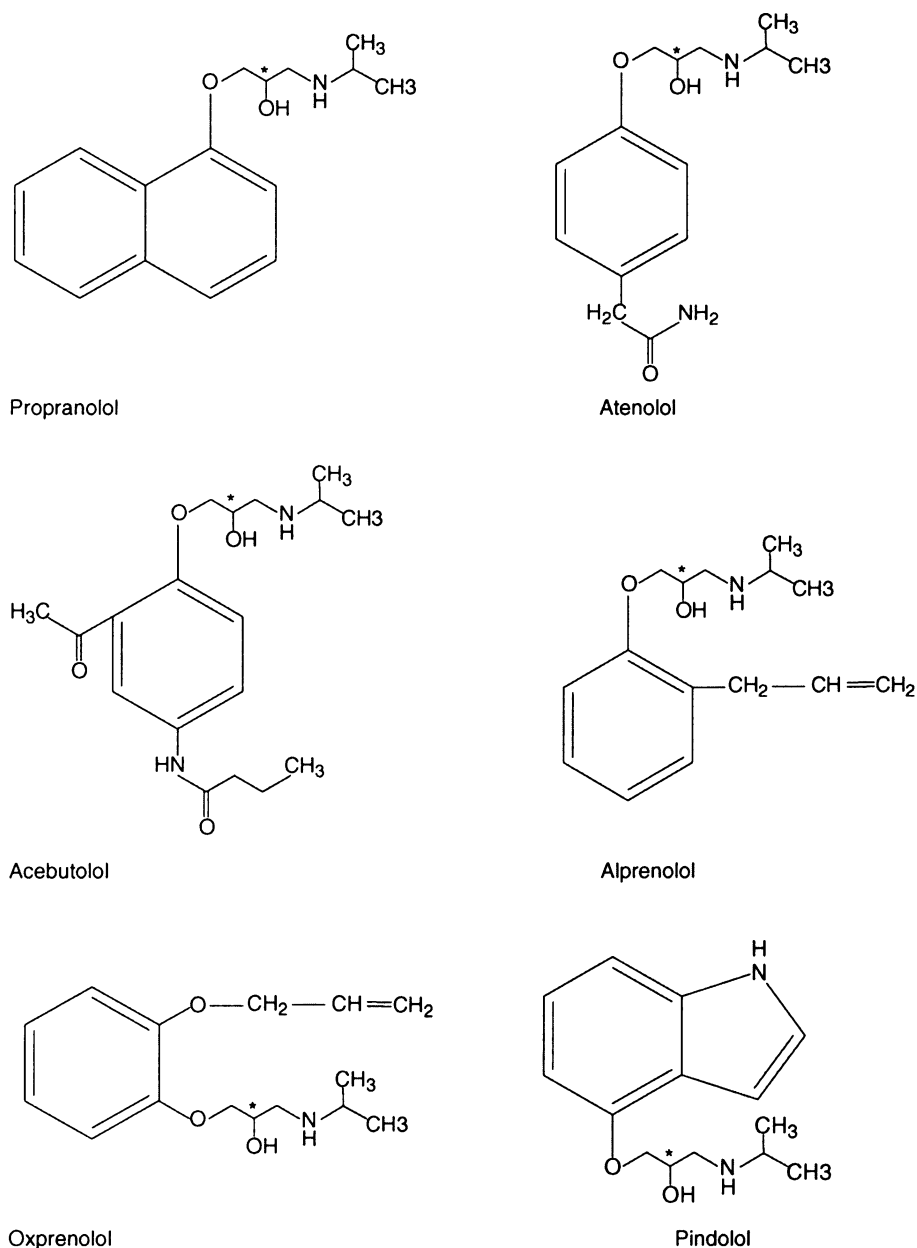


Fig. 2. Chemical structures of studied β -adrenergic antagonists.

containing various concentration of teicoplanin were used as mobile phases if an achiral stationary phase was employed. The eluents were sonicated just before use for at least 10 min.

Racemic profen NSAIDs, i.e. fenoprofen, carprofen, flurbiprofen, indoprofen, ibuprofen, flobufen, ketoprofen and suprofen; β -adrenergic antagonists, oxprenolol, alprenolol, propranolol, atenolol, acebutolol and pindolol; phenoxypropionic acid herbicides, 2-(2-chlorophenoxy) propionic acid (2,2-CPPA), 2-(3-chlorophenoxy) propionic acid (2,3-CPPA) and 2-(4-chlorophenoxy) propionic acid (2,4-CPPA), all p.a. purity, were obtained from (Sigma Aldrich, St. Louis, MO, USA). All the studied derivatives were injected as 0.2 mg ml⁻¹ methanolic solutions.

2.2. Equipment

An ISCO syringe pump model 100 DM (Lincoln, NE, USA), a Valco injection valve with a 60 nl internal loop (Schenkon, Switzerland) and a Linear UV–VIS 205 detector equipped with a CE on-column flow cell (San Jose, CA, USA) were applied for the cLC experiments. A fused-silica capillary column of 25 cm \times 300 μ m I.D. packed with 5 μ m Nucleosil 100 C₁₈ HD was purchased from GROM (Herrenberg-Kayh, Germany). Flow-rate of mobile phases was 5 μ l min⁻¹. Free teicoplanin chiral selector was provided by Astec (Whippany, NY, USA).

A capillary column of 22.5 cm \times 320 μ m I.D. packed with teicoplanin bonded to silica gel, particle size, 5 μ m (CHIRO-

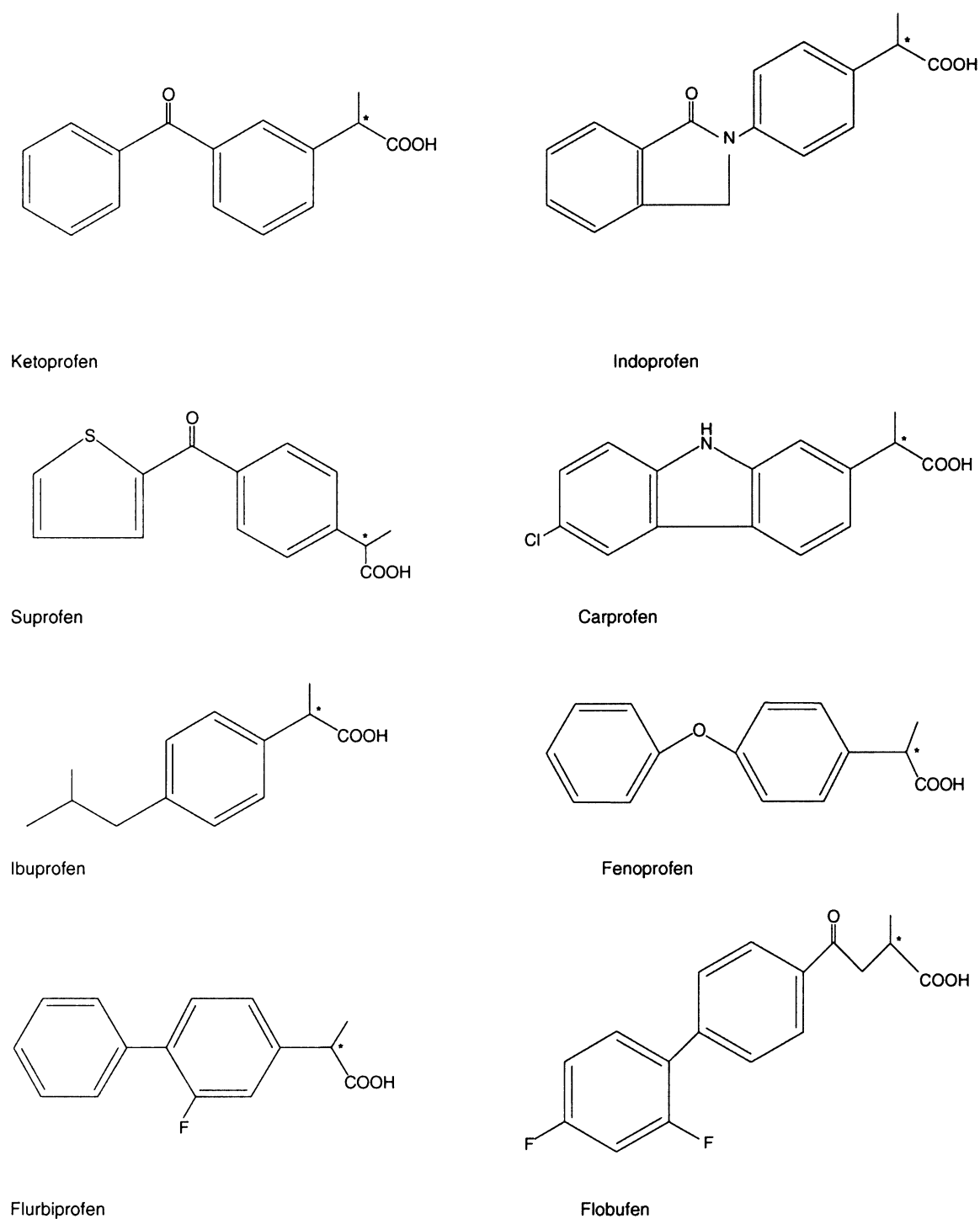


Fig. 3. Chemical structures of studied profen NSAIDs.

BIOTIC T, Astec, Whippany, NY, USA) was prepared by Ing. J. Planeta, Ph.D., from the Institute of Analytical Chemistry of the Czech Academy of Sciences, Brno, Czech Republic. Flow-rate of mobile phases was $4 \mu\text{l min}^{-1}$.

The column inlet was installed in the injection valve using a 5-cm polyether ether ketone (PEEK) sleeve ($500 \mu\text{m}$ I.D.) and a PEEK finger-tight fitting and the column outlet was connected by PTFE tubing to a $100 \mu\text{m}$ I.D. fused-silica cap-

illary with detection window located 7 cm from the column outlet.

Individual samples were detected at various wavelengths according to the absorption maxima elicited from their absorption spectra. The detection wavelengths for profen NSAIDs were 230 or 270 nm; β -adrenergic antagonists were detected at 230 or 254 nm and chlorophenoxypropionic acid derivatives also at 230 nm. Chromatograms were recorded

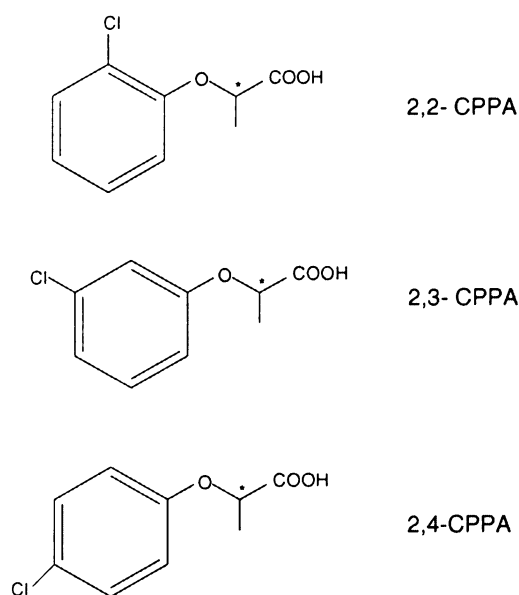


Fig. 4. Chemical structures of studied CPPA herbicides.

and evaluated employing CSW computer software provided by DataApex (Prague, Czech Republic).

3. Results and discussion

Separation systems composed of a teicoplanin-based chiral stationary phase and various mobile phases were studied for enantioseparation of β -adrenergic antagonists, profen NSAIDs and chlorophenoxypropionic acid herbicides in cLC. Reversed phase (RP) separation mode, as well as, polar organic (PO) mode were tested. Also teicoplanin was added to the mobile phase if an achiral capillary column Nucleosil 100 C₁₈ HD was used.

3.1. Enantioseparation of β -adrenergic antagonists

3.1.1. Reversed separation mode

With respect to the chemical structure of β -adrenergic antagonists (Fig. 2) we studied the influence of mobile phase

composition on their enantioseparation in two separation modes, i.e. reversed phase and polar organic ones. The effect of methanol (MeOH) contents in mobile phases on retention factors (k), selectivity factors (α) and enantioresolutions (R) in the reversed separation mode can be seen from Table 1. The retention of all β -adrenergic antagonists first decreased and at higher concentration of methanol (close to 95%) again substantially increased with increasing content of methanol. (At even lower percentages of the organic modifier in the mobile phase, the retention times were too long and no partial separation was observed, so we did not investigate the chromatographic behavior of β -adrenergic antagonists further to this region.) Such dependency is a typical indication for suitability to apply polar organic separation mode. The best enantioresolution and the highest values of selectivity of all the derivatives were obtained in the mobile phase containing 95% methanol. If the MeOH–buffer (v/v) ratio was shifted just to 90/10, the resolution markedly decreased or was even lost.

Effect of the buffer concentration on the chromatographic data of β -adrenergic antagonists is summarized in Table 2. Increasing concentration of TEAA decreased retention of all the derivatives, which was accompanied at 0.5% TEAA by higher resolution values (except of alprenolol). At the highest TEAA concentration tested (1.0%), all interaction types (stereoselective and nonstereoselective) were reduced and as the result enantioresolutions decreased again to almost the same values as at 0.1% buffer concentration. Too low concentration of TEAA (0.1%) cannot eliminate nonstereoselective interactions with the carrier of the CSP while at high concentration of the buffer (1.0%), there is no sufficient difference in the interaction of both enantiomers with the CSP to yield higher resolution values.

Assuming the structure of β -adrenergic antagonists significant influence of buffer pH on change of their dissociation/protonation in the “allowed” pH range could not be expected. On the other hand, pH can affect the polar groups of teicoplanin and in this way the stereoselective (but also nonstereoselective) interactions with the analytes. Enantioseparations of atenolol at three different pH values are compared in Fig. 5. It is obvious that higher pH value of the buffer elongates elution of analytes, which is accompanied by only

Table 1
Effect of methanol content in the mobile phase (0.1% aqueous TEAA, pH 5.0) on chromatographic data of β -adrenergic antagonists using the teicoplanin-based CSP

β -Adrenergic antagonists	Methanol (%)											
	40			50			90			95		
	k_1	α	R	k_1	α	R	k_1	α	R	k_1	α	R
Oxprenolol	6.78	1.00	0.00	5.45	1.00	0.00	2.98	1.00	0.00	8.64	1.04	0.75
Alprenolol	8.95	1.00	0.00	5.43	1.00	0.00	2.97	1.05	0.37	9.05	1.08	1.69
Propranolol	10.32	1.00	0.00	9.15	1.00	0.00	3.47	1.05	0.38	9.87	1.07	1.25
Atenolol	7.70	1.00	0.00	6.65	1.00	0.00	5.39	1.03	0.30	15.67	1.06	1.03
Acebutolol	8.95	1.00	0.00	6.88	1.00	0.00	3.98	1.00	0.00	13.22	1.04	0.59
Pindolol	6.15	1.00	0.00	6.72	1.00	0.00	3.24	1.03	0.21	9.07	1.06	0.79

Note: k_1 , retention factor of the first eluting enantiomer; α , selectivity factor; R , resolution.

Table 2

Effect of the TEAA concentration in the mobile phase on chromatographic data of β -adrenergic antagonists using the teicoplanin-based CSP

β -Adrenergic antagonists	TEAA (%)								
	0.1			0.5			1.0		
	k_1	α	R	k_1	α	R	k_1	α	R
Oxprenolol	8.64	1.04	0.75	5.69	1.08	1.04	3.21	1.05	0.74
Alprenolol	9.05	1.08	1.69	4.66	1.12	1.42	2.89	1.08	1.09
Propranolol	9.87	1.07	1.25	6.27	1.10	1.30	3.52	1.08	1.00
Atenolol	15.67	1.06	1.03	10.12	1.09	1.13	5.79	1.07	1.00
Acebutolol	13.22	1.04	0.59	7.93	1.06	0.98	4.07	1.05	0.56
Pindolol	9.07	1.06	0.79	5.97	1.09	1.12	3.17	1.07	0.88

The mobile phase composition: 95/5 (v/v) methanol/TEAA, pH 5.0.

small change of enantioresolution. The higher retention can be attributed to stronger interaction of analytes with the dissociated carboxylic group of teicoplanin. The best chiral separation of β -adrenergic antagonists enantiomers was obtained in the mobile phase composed of methanol and 0.5% TEAA, pH 5.0 in the ratio 95/5. In the reversed phase separation mode, hydrophobic interactions and inclusion are favored but the importance of H-bonding in the interaction mechanism increases with increasing contents of methanol.

Due to solubility problems it was not possible to add teicoplanin to the mobile phase (with high content of methanol), which yielded the enantioseparation of β -adrenergic antagonists, and so to investigate a separation system with an achiral stationary phase.

3.1.2. Polar organic separation mode

Polar organic separation mode was developed for separation of enantiomer pairs possessing two functional groups capable of electrostatic interactions. These groups shall be located near the stereogenic center. Polar organic mobile phase is typically based on methanol (or acetonitrile/methanol) with very small amounts of acetic acid (HAc) and triethylamine (TEA). As enantioseparations of β -adrenergic antagonists were succeeded at high methanol contents in the RP

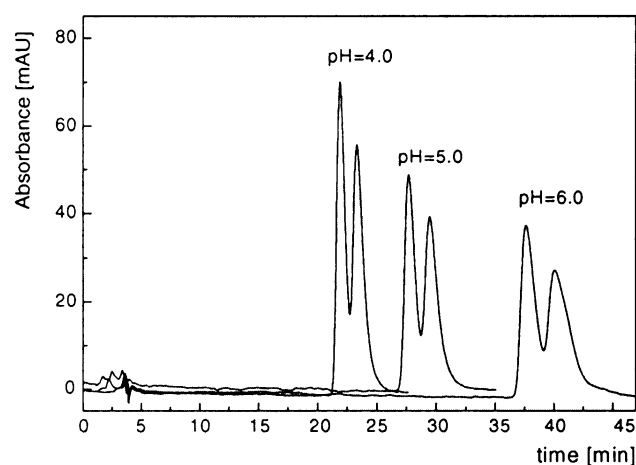


Fig. 5. Effect of TEAA buffer pH on cLC enantioseparation of atenolol using the teicoplanin-based CSP. Mobile phase composition: 95/5 (v/v) methanol/1.0% TEAA, pH 4.0–6.0.

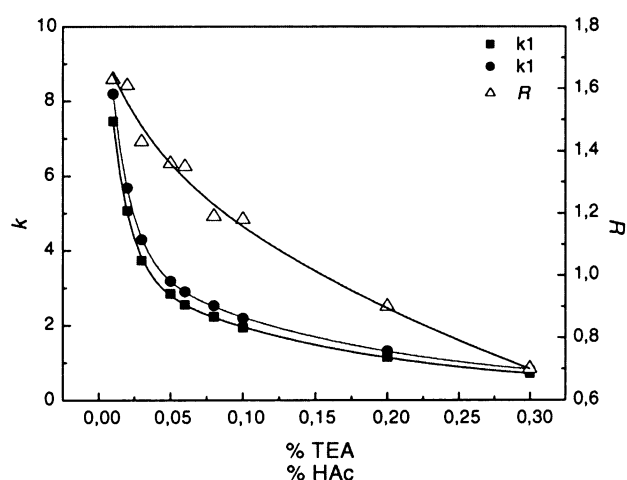


Fig. 6. Effect of the concentration of TEA and HAc in the mobile phase on the retention factors of the first (k_1) and second (k_2) enantiomer and the enantioresolution (R) of β -adrenergic antagonist (alprenolol) on the teicoplanin-based CSP. Mobile phase composition: methanol/HAc/TEA from 100/0.01/0.01 to 100/0.3/0.3 (v/v/v).

mode, the PO mode was applied in the following experiments. Fig. 6 shows that with increasing concentration of HAc and TEA, the retention and the enantioresolution of alprenolol significantly decreased. The same trend was observed for all β -adrenergic antagonists. From the tested concentrations of acetic acid and triethylamine (their volumes [ml] added to 100 ml MeOH) and their ratios, the maximum resolution was obtained at HAc/TEA = 0.01/0.01. Results summarized in Table 3 depicts that almost all the derivatives were baseline separated. If we compare this optimized mo-

Table 3

Enantioresolution of β -adrenergic antagonists in the polar organic separation mode on the teicoplanin-based CSP

β -Adrenergic antagonists	k_1	α	R
Oxprenolol	4.30	1.07	1.08
Alprenolol	4.11	1.10	1.63
Propranolol	5.34	1.10	1.85
Atenolol	10.02	1.09	1.58
Acebutolol	6.12	1.07	1.10
Pindolol	4.60	1.09	1.50

The mobile phase composition: methanol/HAc/TEA, 100/0.01/0.01 (v/v/v).

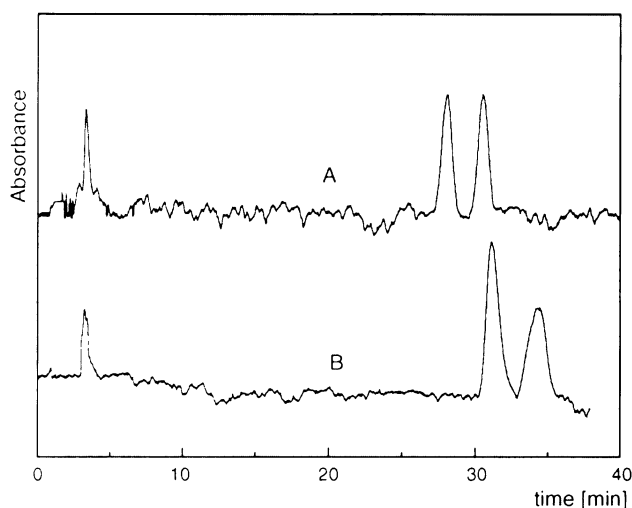


Fig. 7. Chromatograms showing the enantioseparation of alprenolol on the teicoplanin-based CSP. Mobile phases composition: (A) methanol/HAc/TEA, 100/0.01/0.01 (v/v/v) and (B) 95/5 (v/v) methanol 0.5% TEAA, pH 5.0.

mobile phase composition for enantioseparation of β -adrenergic antagonists with that published previously [36], i.e. acetonitrile/methanol/HAc/TEA = 55/45/0.3/0.2, it is obvious that the addition of acetonitrile, as a proton acceptor, requires an increase of the HAc/TEA ratio and concentration. This shows on the importance of a proton-donating agent in the mobile phase to ensure protonation of the amino groups of the analytes. Protonated β -adrenergic antagonists provide interaction possibilities for H-bonding with teicoplanin-based CSP.

3.1.3. Comparison of enantioseparation of β -adrenergic antagonists in PO and RP modes

Fig. 7 allows comparison of the enantioseparations of alprenolol on teicoplanin-based capillary column in PO and RP separation modes. The respective separation parameters of alprenolol in the polar organic and the reversed phase separation modes are: resolution 1.63 and 1.42, selectivity factor 1.12 and 1.10, efficiency of the first eluted peak 36530 and 23660 theoretical plates per meter of column, and asymmetry

of the second peak 1.00 and 0.70. Some β -adrenergic antagonists show much better enantioseparation parameters in the PO mode (the example of alprenolol, but also pindolol, propranolol and atenolol) while some others give similar results in both separation modes (for example acebutolol, and also oxprenolol). Generally said, selectivity is similar in both separation modes. The main contributors to the improved enantioresolution of β -adrenergic antagonists in the polar organic mobile phase are higher efficiency and better peak symmetry. These parameters also make the PO mode advantageous for practical purposes. The polar organic separation mode prefers electrostatic interactions and hydrogen bonding. The better enantioseparation in the polar organic mode shows the importance of the H-bonding and/or electrostatic interactions in the chiral recognition mechanism of β -adrenergic antagonists with teicoplanin. This is also the reason why no chiral resolution appears if mobile phases with lower amount of methanol are applied in the RP mode (see Table 1). In such separation systems, these electrostatic/H-bonding types of interactions are suppressed while hydrophobic and π - π interaction predominate. Due to the fact that the aromatic moiety is far away from the chiral center the latter interaction have no stereoselective significance.

3.2. Enantioseparation of profen NSAIDs

3.2.1. Reversed separation mode

Teicoplanin stationary phase shows affinity to compounds with a carboxylic group. It is important for enantioselective separations of amino acids and proteins [37,38]. The primary interaction of acids with teicoplanin is realized between their carboxyl group and amino group of the chiral selector [20]. Profen NSAIDs, as compounds with significant hydrophobic moiety close to the chiral carbon (Fig. 3), require for the enantioselective recognition also hydrophobic interaction or inclusion. These interaction types are favored if mobile phases with great aqueous portion are used. For this reason we examined the influence of methanol content in the mobile phase down to much lower values (in the range 10–30% of methanol) than in the case of β -adrenergic an-

Table 4
Effect of the TEAA in the mobile phase on chromatographic data of profen NSAIDs using the teicoplanin-based CSP; the mobile phase (aqueous TEAA, pH 5.0) and methanol

Profen NSAIDs	10% Methanol				20% Methanol				30% Methanol									
	0.1 ^a		0.5 ^a		1.0 ^a		0.1 ^a		0.5 ^a		1.0 ^a		0.1 ^a		0.5 ^a		1.0 ^a	
	k_1	R	k_1	R	k_1	R	k_1	R	k_1	R	k_1	R	k_1	R	k_1	R	k_1	R
Fenoprofen	1.29	0.00	2.57	0.00	2.87	0.00	0.59	0.00	1.90	0.00	1.96	0.00	0.00	0.00	0.94	0.00	1.19	0.00
Carprofen	5.56	0.00	9.37	0.00	9.45	0.00	3.22	0.00	6.75	0.00	6.89	0.00	0.41	0.46	2.34	0.26	3.22	0.26
Flurbiprofen	1.98	0.15	3.84	0.61	3.86	0.60	1.03	0.00	2.90	0.67	3.05	0.70	0.00	0.00	1.30	0.55	1.65	0.69
Flobufen	5.48	0.00	6.44	0.00	7.39	0.00	2.48	0.00	5.06	0.00	5.08	0.00	0.29	0.00	1.92	0.00	2.75	0.00
Ketoprofen	2.16	0.82	3.47	0.98	3.73	1.05	0.91	0.00	2.54	0.80	2.71	0.84	0.00	0.00	1.21	0.18	1.83	0.64
Suprofen	2.45	0.37	3.86	0.58	5.16	0.59	1.08	0.00	3.03	0.58	3.38	0.70	0.26	0.00	1.41	0.31	2.34	0.68
Indoprofen	4.26	0.50	6.46	0.70	7.39	0.72	2.10	0.65	4.95	0.64	4.98	0.64	0.51	0.00	2.18	0.51	2.82	0.59
Ibuprofen	1.17	0.00	1.82	0.00	2.06	0.00	0.53	0.00	1.61	0.00	1.66	0.00	0.10	0.00	0.84	0.00	0.98	0.00

^a TEAA (%).

Table 5
Effect of the buffer pH on cLC enantioseparation of profen NSAIDs using the teicoplanin-based CSP

Profen NSAIDs	k_1				α				R			
	4.0 ^a	5.0 ^a	6.0 ^a	6.6 ^a	4.0 ^a	5.0 ^a	6.0 ^a	6.6 ^a	4.0 ^a	5.0 ^a	6.0 ^a	6.6 ^a
Fenoprofen	2.15	1.96	1.89	1.22	1.00	1.00	1.00	1.00	0.00	0.00	0.00	0.00
Carprofen	6.19	6.89	5.46	3.20	1.00	1.00	1.04	1.05	0.00	0.00	0.15	0.18
Flurbiprofen	3.03	3.05	2.70	1.89	1.00	1.11	1.12	1.15	0.00	0.70	0.66	0.79
Flobufen	4.77	5.08	3.89	3.25	1.00	1.00	1.08	1.16	0.00	0.00	0.41	0.90
Ketoprofen	2.47	2.71	2.41	1.66	1.03	1.13	1.15	1.17	0.10	0.84	0.84	0.85
Suprofen	3.05	3.38	2.90	2.09	1.07	1.09	1.10	1.11	0.36	0.70	0.71	0.73
Indoprofen	4.96	4.98	5.02	2.98	1.06	1.08	1.08	1.08	0.47	0.64	0.52	0.64
Ibuprofen	1.68	1.66	1.35	0.86	1.00	1.00	1.00	1.00	0.00	0.00	0.00	0.00

The mobile phase composition: 20/80 (v/v) methanol/1.0% TEAA, pH 4.0–6.6.

^a pH values.

tagonists. The effect of the methanol content on retention factors and enantioresolution of profen NSAIDs was studied in the mobile phases composed of methanol and (0.1–1.0%) TEAA buffer, pH 5.0. Results obtained in the range 10–30% of methanol and different concentrations of TEAA are shown in Table 4. (In the range 30–90% of methanol, almost all profen NSAIDs were eluted at the death time.) Lower methanol contents resulted in higher retention and partial enantioresolution. In general, increasing concentration of TEAA in the mobile phase resulted in increased retention and improved enantioresolution of the profen NSAIDs. However, the difference between resolution values obtained using 0.5% and 1.0% TEAA buffer was not significant. Also the retention factors were almost not affected with this change of the buffer concentration. On contrary, increase of retention of profen NSAIDs was observed if TEAA concentration was raised from 0.1% to 0.5%. These results indicate that TEA forms ion-pairs with the analytes and in this way increase their hydrophobicity. Stronger hydrophobic interactions, which can be both enantioselective and nonenantioselective, between the analytes and CSP in higher polarity mobile phases are then responsible for higher retention and improved enantioseparation at 0.5% (or 1.0%) TEAA.

Based on the fact that 1.0% TEAA yielded the best results, the effect of the buffer pH was studied at this TEAA concentration. The results are shown in Table 5. The retention remained almost unchanged at pH 4.0 and 5.0, while it decreased more significantly towards pH 6.6. Selectivity and enantioresolution was markedly improved with increasing buffer pH only for flobufen. Considering the pK_a values of profen NSAIDs (3.9–5.0), chiral recognition interactions are favored if their carboxyl group is dissociated. The highest enantiomeric resolution was achieved for flurbiprofen, flobufen, ketoprofen, suprofen and indoprofen in the mobile phase composed of methanol/1.0% TEAA, pH 6.6, in the ratio 20/80. Fig. 8 shows enantioseparation of flobufen at different pH values of the TEAA buffer. The structure of flobufen differs from the other profen NSAIDs derivatives because the aromatic part is not attached directly to the asymmetric center. This is the reason why separation of flobufen enantiomers is more difficult to achieve. It is obvious from Fig. 8 that the best enantioresolution of flobufen can be reached in the

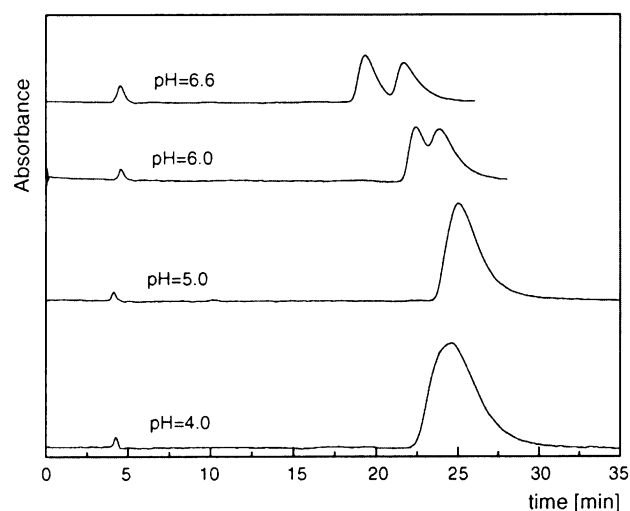


Fig. 8. Effect of the buffer pH on cLC enantioseparation of flobufen using the teicoplanin-based CSP. Mobile phase composition: 20/80 (v/v) methanol/1.0% TEAA, pH 4.0–6.6.

mobile phase composed of methanol/1.0% TEAA, pH 6.6, in the ratio 20/80 on the teicoplanin-based column in cLC.

Results obtained in the reversed phase mode in the mobile phases with higher methanol contents indicated that there is no need to investigate profen NSAIDs in the polar organic separation mode because they are not retained on the column under these conditions.

An attempt was made to use an achiral capillary column Nucleosil 100 C₁₈ HD and mobile phase composed of methanol and 0.1% TEAA, pH 5.0, in the range 10–30% of methanol. However, retention of profen NSAIDs in these mobile phases was too long (without the addition of teicoplanin). Higher concentration of methanol in the mobile phase did not allow the addition of teicoplanin for the solubility reasons. As a result, enantioseparation of profen NSAIDs with teicoplanin chiral additive to the mobile phase was not realized.

3.3. Enantioseparation of chlorophenoxypropionic acid derivatives

In the final part of this work, we wanted to compare the effects of separation conditions on the chromatographic

Table 6

Effect of the TEAA in the mobile phase on chromatographic data of CPPAs using the teicoplanin-based CSP; the mobile phase (aqueous TEAA, pH 5.0) and methanol

CPPA	10% Methanol						20% Methanol						30% Methanol					
	0.1 ^a		0.5 ^a		1.0 ^a		0.1 ^a		0.5 ^a		1.0 ^a		0.1 ^a		0.5 ^a		1.0 ^a	
	<i>k</i> ₁	<i>R</i>	<i>k</i> ₁	<i>R</i>	<i>k</i> ₁	<i>R</i>	<i>k</i> ₁	<i>R</i>	<i>k</i> ₁	<i>R</i>	<i>k</i> ₁	<i>R</i>	<i>k</i> ₁	<i>R</i>	<i>k</i> ₁	<i>R</i>	<i>k</i> ₁	<i>R</i>
2,2-CPPA	0.00	0.00	0.43	1.10	0.66	1.30	0.00	0.00	0.36	0.80	0.52	1.10	0.00	0.00	0.14	0.60	0.35	1.08
2,3-CPPA	0.00	0.00	0.60	0.00	0.78	0.62	0.00	0.00	0.48	0.15	0.62	0.56	0.00	0.00	0.29	0.00	0.44	0.54
2,4-CPPA	0.00	0.00	0.54	1.10	0.75	1.35	0.00	0.00	0.43	1.18	0.60	1.56	0.18	0.27	0.25	1.20	0.41	1.70

^a TEAA (%).

behavior of three racemates, position isomers of chlorophenoxypropionic acid herbicides (Fig. 4) in capillary separation systems with teicoplanin as a chiral selector. Table 6 summarizes the data measured in mobile phases composed of different concentrations of TEAA (0.1%, 0.5% and 1.0%), pH 5.0, and various contents of methanol in the range 10–30% (v/v). (At higher methanol contents the analytes eluted at the dead time.) Retention of all CPPAs decreased with increasing contents of methanol in the mobile phases at any concentration of TEAA. The data displayed in Table 6 show that the retention and enantioresolution values of CPPA derivatives had the same trend as had been observed with profen NSAIDs, i.e. *k*- and *R*-values increased with increasing concentration of TEAA. These stronger interactions between the analytes and CSP are clearly enantioselective. For 2,3-CPPA and 2,4-CPPA, similar retention factors were obtained in the mobile phase methanol/1.0% TEAA, pH 5.0 (in the range 10–30% of methanol). Lower retention of 2,2-CPPA in these mobile phases can be attributed to intramolecular interaction between chlorine and oxypropionic acid moiety in *ortho* position. Nevertheless, even this low retention was sufficient for the chiral separation of 2,2-CPPA. Enantioresolution values of 2,2-CPPA and 2,3-CPPA decreased with increasing content of methanol in the mobile phases composed of 1.0% or 0.5% aqueous TEAA, pH 5.0, while the resolution of 2,4-CPPA had the opposite trend. These results show on the impact of steric factors on stereoselective interaction.

The influence of the buffer pH in mobile phases composed of methanol and 1.0% TEAA in the ratio 20/80 on enantioresolution of CPPAs is shown in Fig. 9. The observed trends are rather similar for all the derivatives. The least effect of buffer pH on the *R*-values of 2,2-CPPA can be again attributed to the intramolecular interaction of

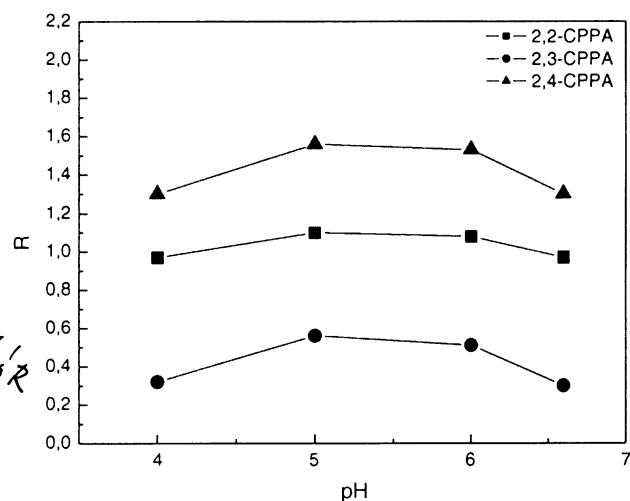


Fig. 9. Effect of the buffer pH on cLC enantioresolution of CPPAs using the teicoplanin-based CSP. Mobile phase composition: 20/80 (v/v) methanol/1.0% TEAA, pH 4.0–6.6.

both substituents on the aromatic ring. This is in accord with the lowest interaction (retention) of 2,2-CPPA in any mobile phase composition. Considering the *pK_a* value of CPPAs (*pK_a*(2,4-CPPA)=4.32 in DMSO/H₂O [39]) chiral recognition interactions are favored if the carboxyl group is dissociated. The same trend was observed for profen NSAIDs.

Through a series of TEAA concentration and buffer pH variations an optimized buffer composition of 1.0% TEAA, pH 5.0 was found for enantiomeric separation of CPPAs on the teicoplanin-based capillary column. This buffer should offer the basis for addition of teicoplanin if a separation system with an achiral capillary column, Nucleosil 100 C₁₈

Table 7

Effect of the concentration of teicoplanin in mobile phase on chromatographic data of CPPA herbicides using Nucleosil 100 C₁₈ HD

CPPA	Concentration of teicoplanin (mM)								
	0.00			0.05			0.10		
	<i>k</i> ₁	α	<i>R</i>	<i>k</i> ₁	α	<i>R</i>	<i>k</i> ₁	α	<i>R</i>
2,2-CPPA	4.06	1.00	0.00	4.85	1.19	1.38	7.85	1.36	1.58
2,3-CPPA	5.09	1.00	0.00	6.97	1.00	0.00	10.51	1.00	0.00
2,4-CPPA	5.12	1.00	0.00	6.03	1.00	0.00	11.89	1.32	1.40

The mobile phase composition: 30:70, methanol 0.1% TEAA; pH 5.0.

HD, is used for enantioseparation of CPPAs. The idea was to add the chiral selector to the mobile phase of the same composition, which yielded good enantioresolution with the teicoplanin-based CSP, it means 10/90 (v/v) methanol/1.0% TEAA, pH 5.0, for derivatives 2,2-CPPA and 2,3-CPPA and 30/70 (v/v) methanol/1.0% TEAA, pH 5.0, for 2,4-CPPA (Table 6). Using the achiral column retention of all derivatives of CPPA dramatically increased (to 150 min) even without addition of teicoplanin to the mobile phase composed of 10/90 (v/v), methanol/1.0% TEAA; pH 5.0. Moreover, solubility of teicoplanin in solutions with higher buffer concentration substantially decreased. For this reason, the mobile phase composed of methanol and 0.1% TEAA, pH 5.0, in the ratio 30/70, yielding retention of CPPAs about 30 min, was chosen as the basis. Table 7 shows the effect of concentration of teicoplanin in the mobile phase on the chromatographic data of CPPAs. The concentration of teicoplanin could not be increased above 0.1 mM because CPPAs did not then elute from the column. The teicoplanin concentration in the mobile phase is a very important factor to control the chiral recognition. The retention factors of all derivatives increased with increasing teicoplanin concentration. Enantiomeric separations of 2,2-CPPA and 2,4-CPPA were achieved already at 0.05 and 0.1 mM concentrations of teicoplanin, respectively. Enantiomers of 2,3-CPPA could not be separated under the studied conditions.

The results obtained in the comparable mobile phase (MeOH/0.1% TEAA, pH 5.0; 30/70, v/v) with teicoplanin bonded on CSP and free in the solution (Table 6 versus Table 7) show that better enantioseparation of 2,4-CPPA than of 2,2-CPPA was observed on the teicoplanin bonded CSP while the opposite result was obtained with teicoplanin added to the mobile phase. Significantly lower affinity of 2,3-CPPA to teicoplanin in both systems is obvious. Steric effects (both intra- and intermolecular) should be responsible for the different interaction possibilities of these position isomers with the chiral selector. Stabilization of the π -electron system (2,2- and 2,4-derivatives) may also play a role if π - π interactions are involved in the enantioresolution mechanism. Increased rigidity of these derivatives favors chiral recognition ability. Fig. 10 shows the separation of 2,4-CPPA enantiomers on teicoplanin-based CSP with two mobile phases differing in the TEAA concentration (Fig. 10A and B) and in the separation system composed of the achiral column and the mobile phase containing 0.1 mM teicoplanin (Fig. 10C). The best enantioresolution in a short analysis time was obtained with the teicoplanin CSP and the mobile phase containing 1.0% buffer (Fig. 10B), as was already discussed above. Comparison of the two separation systems, with the chiral and achiral column, both using the 0.1% buffer in the mobile phase, shows that baseline separation can be achieved only in the system composed of C-18 column and the chiral selector-contained mobile phase. However, such separation conditions have no practical use because the elution time is too long and bad peak shape makes the separation not suitable for quantitative analysis. The chromatogram evidenced that

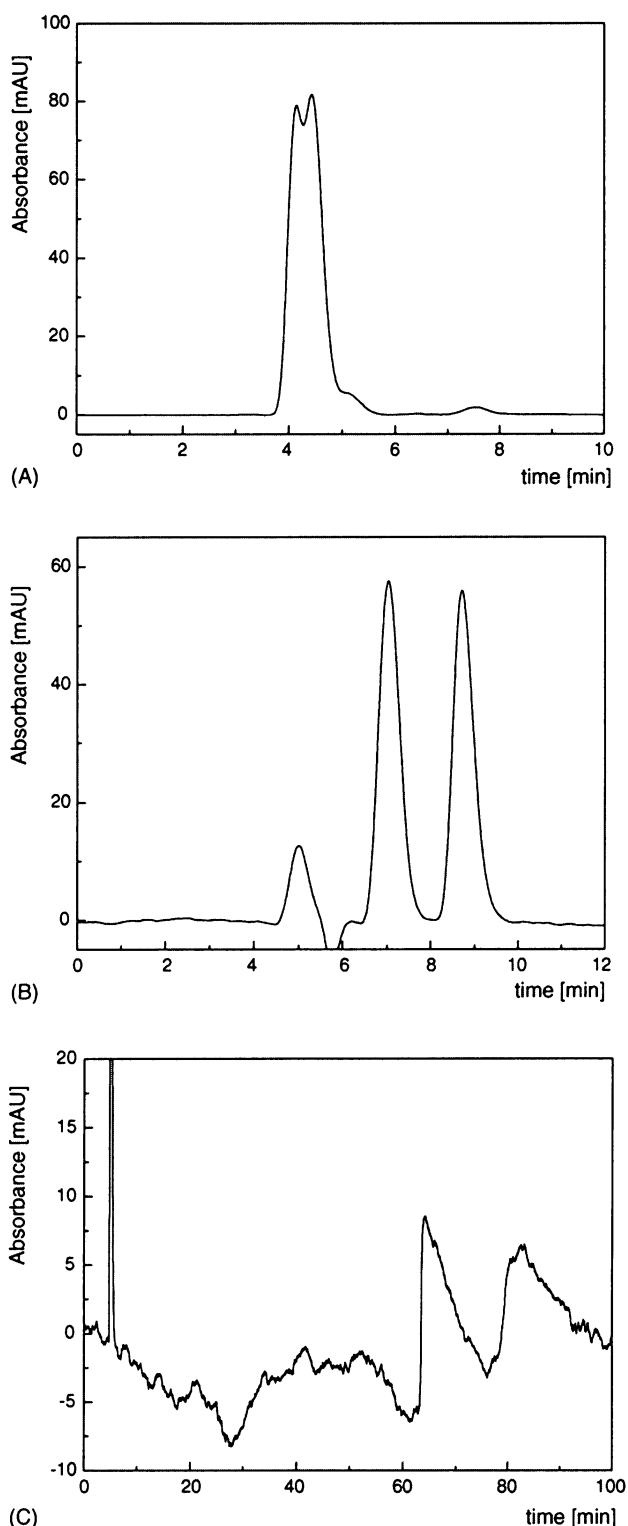


Fig. 10. Comparison of enantioseparation of 2,4-CPPA in mobile phase composition: (A) 30/70 (v/v) methanol/0.1% TEAA, pH 5.0; (B) 30/70 (v/v) methanol/1.0% TEAA, pH 5.0 on teicoplanin-based CSP; (C) 30/70 (v/v) methanol/0.1% TEAA, pH 5.0 with addition 0.1 mM teicoplanin on Nucleosil 100 C₁₈ HD.

a competing interaction of the analyte(s) with teicoplanin sorbed on the achiral stationary phase surface and that present in the mobile phase takes place in the separation system. This competition is responsible for the observed peak tailing.

4. Conclusion

Teicoplanin-based capillary column proved to be suitable for enantioseparation of some β -adrenergic antagonists, profen NSAIDs and chlorophenoxypropionic acid derivatives. Higher methanol content in the mobile phase was essential for enantioselective interactions of β -adrenergic antagonists with the CSP while profen NSAIDs and CPPAs were preferentially enantioresolved in mobile phases with low organic modifier content. In the reversed phase mode an increase of the TEAA buffer concentration (as the aqueous part of the mobile phase) resulted in a decrease of the retention of β -adrenergic antagonists but did not influence their chiral resolution so far. On the contrary, retention of profen NSAIDs and CPPAs was increased and their enantioresolution improved at higher buffer concentrations. Chiral separations of the various classes of enantiomers were found to be pH dependent. Regarding the structure of β -adrenergic antagonists significant influence of pH on their dissociation or protonization could not be expected. However, the effect of buffer pH on the CSP (the chiral selector, as well as, the silica gel support) resulted in better enantioresolution of β -adrenergic antagonists at higher pH value (5.0). Despite the different structures of profen NSAIDs and CPPAs (both possessing a carboxyl group next to the chiral carbon atom) compared to β -adrenergic antagonists similar effect of pH on their enantioresolution was found. It can be postulated that the effect of pH on ionization of the functional groups of teicoplanin plays an important role in the stereoselective interaction mechanism. Polar organic separation mode was advantageous for chiral separation of β -adrenergic antagonists. Teicoplanin added to the mobile phase in the separation system with an achiral capillary column yielded chiral separation of 2,2-CPPA and 2,4-CPPA. A very low teicoplanin concentration (0.1 mM) was sufficient for their baseline enantioresolution. Due to the combined interaction of the analytes with teicoplanin partly sorbed on the stationary phase and partly remained in the mobile phase the last separation system did not have sufficient separation efficiency.

Acknowledgements

The authors acknowledge the financial support of this work from the Ministry of Education, Youth and Physical Training of the Czech Republic, grant no. 1893/2004 and from the Grant Agency of the Czech Republic, grant no. 203/03/0161. Special thanks must be expressed to Ing. J.

Planeta from the Institute of Analytical Chemistry, Czech Academy of Sciences, Brno, Czech Republic, for packing the teicoplanin-based capillary column.

References

- [1] T.J. Ward, A.B. Farris III, *J. Chromatogr. A* 906 (2001) 73.
- [2] T.J. Ward, *Anal. Chem.* 72 (2000) 4521.
- [3] T.J. Ward, T.M. Oswald, *J. Chromatogr. A* 792 (1997) 309.
- [4] C. Desiderio, S. Fanali, *J. Chromatogr. A* 807 (1998) 37.
- [5] M.P. Gasper, A. Berthod, U.B. Nair, D.W. Armstrong, *Anal. Chem.* 68 (1996) 2501.
- [6] S. Fanali, P. Catarcini, G. Blaschke, B. Chankvetadze, *Electrophoresis* 22 (2001) 3131.
- [7] F.H. Meyers, E. Jawetz, A. Goldheim, *Review of Medical Pharmacology*, seventh ed., Lange Medical Publications, Los Altos, 1980, p. 95.
- [8] B.N. Sigh, P. Denwania, K. Nadamane, A. Ward, E.M. Sorokin, *Drugs* 34 (1987) 115.
- [9] J. Ekelund, A. van Arkens, K.B. Hansen, K. Fich, L. Olsen, P.V. Petersen, *J. Chromatogr. A* 708 (1995) 253.
- [10] H.Y. Aboul-Enein, V. Serignese, *J. Liq. Chromatogr.* 16 (1993) 197.
- [11] H. Zhang, J.T. Stewart, M. Ujhelyi, *J. Chromatogr. B* 668 (1995) 309.
- [12] K.V. Penmetsa, Ch.D. Reddick, S.W. Fink, B.L. Kleintop, G.C. DiDonato, K.J. Volk, S.E. Klohr, *J. Liq. Chromatogr. Relat. Technol.* 23 (2000) 831.
- [13] X. Yang, T. Fukushima, T. Santa, H. Homma, K. Imai, *Analyst* 122 (1997) 1365.
- [14] J. Szymura-Oleksiak, M. Walczak, J. Bojarski, H.Y. Aboul-Enein, *Chirality* 11 (1999) 267.
- [15] B. Toussaint, B. Streel, A. Ceccato, Ph. Hubert, J. Crommen, *J. Chromatogr. A* 896 (2000) 201.
- [16] D.W. Armstrong, S. Chen, C. Chang, S. Chang, *J. Liq. Chromatogr.* 15 (1992) 545.
- [17] R. Bakhtiar, F.L.S. Tse, *Rapid Commun. Mass Spectrom.* 14 (2000) 1128.
- [18] B. Mistry, J.L. Leslie, N.D. Eddington, *J. Chromatogr. B* 758 (2001) 153.
- [19] H.Y. Aboul-Enein, V. Serignese, *Biomed. Chromatogr.* 13 (1999) 520.
- [20] A. Berthod, X. Chen, J.P. Kullman, D.W. Armstrong, *Anal. Chem.* 72 (2000) 1767.
- [21] M.E. Andersson, D. Aslan, A. Clarke, J. Roeraade, G. Hagman, *J. Chromatogr. A* 1005 (2003) 83.
- [22] A. Van Overbeke, W. Baeyens, H. Oda, H.Y. Aboul-Enein, *Chromatographia* 43 (1996) 599.
- [23] X.W. Teng, S.W.J. Wang, N.M. Davies, *J. Pharm. Biomed. Anal.* 33 (2003) 95.
- [24] E. Ameyibor, J.T. Stewart, *J. Liq. Chromatogr. Relat. Technol.* 20 (1997) 855.
- [25] F. Pehourcq, C. Jarry, B. Bannwarth, *Biomed. Chromatogr.* 15 (2001) 217.
- [26] Q. Sun, S.V. Olesik, *J. Chromatogr. B* 745 (2000) 159.
- [27] H.B. Lee, T.E. Peart, J.M. Carron, H. Tse, *J. Assoc. Off. Anal. Chem.* 74 (1991) 835.
- [28] H.R. Buser, M.D. Muller, *Chimia* 51 (1997) 694.
- [29] J.M. Schneiderheinze, D.W. Armstrong, A. Berthod, *Chirality* 11 (1999) 330.
- [30] Y. Okamoto, R. Aburatani, Y. Kaida, K. Hatada, *Chem. Lett.* (1998) 1125.
- [31] O. Azzolina, S. Collina, V. Ghislandi, *Il Farmaco* 48 (1993) 1401.
- [32] H. Riering, M. Sieber, *J. Chromatogr. A* 728 (1996) 171.

- [33] W.H. Pirkle, W. Lee, C.J. Welch, *Enantiomer* 2 (1997) 423.
- [34] G. Uray, N.M. Maier, *Enantiomer* 1 (1996) 211.
- [35] V. Vinkovic, D. Kontrec, V. Sunjic, L. Navarini, F. Zanetti, O. Az-zolina, *Chirality* 13 (2001) 581.
- [36] *Chirobiotic Handbook*, Advanced Separation Technology, Wippany, NY, USA, 2002.
- [37] A. Péter, E. Vékes, D.W. Armstrong, D. Tourwé, *Chromatographia* 56 (2002) 41.
- [38] A. Berthod, Y. Liu, C. Bagwill, D.W. Armstrong, *J. Chromatogr. A* 731 (1996) 123.
- [39] R.I. Nazareth, T.D. Sokoloski, D.T. Witiak, A.T. Hopper, *J. Pharm. Sci.* 63 (1974) 203.

Vancomycin as Chiral Selector for Enantioselective Separation of Selected Profen Nonsteroidal Anti-Inflammatory Drugs in Capillary Liquid Chromatography

BOŽENA KAFKOVÁ,¹ ZUZANA BOSÁKOVÁ,¹ EVA TESAŘOVÁ,^{2*} PAVEL COUFAL,¹
ANTONELLA MESSINA,³ AND MASSIMO SINIBALDI⁴

¹Department of Analytical Chemistry, Faculty of Science, Charles University, Czech Republic

²Department of Physical and Macromolecular Chemistry, Faculty of Science, Charles University, Prague, Czech Republic

³Department of Chemistry, University of Rome "La Sapienza", Rome, Italy

⁴CNR–Istituto di Metodologie Chimiche, Sezione Meccanismi di Reazione, c/o Department of Chemistry, University of Rome "La Sapienza", Rome, Italy

ABSTRACT The chiral selector vancomycin was used either as mobile phase additive or bound as a chiral stationary phase (CSP) for the stereoselective separation of seven racemic nonsteroidal anti-inflammatory drugs (NSAIDs), fenoprofen, carprofen, flurbiprofen, indoprofen, flobufen, ketoprofen, and suprofen, by capillary liquid chromatography. The effect of the type of stationary phase, the chiral column Chirobiotic V or the achiral stationary phases Nucleosil 100 C8 HD and Nucleosil 100 C18 HD, and the concentration of vancomycin in the mobile phase on separation of the drug enantiomers were evaluated. All the drugs, except flobufen, were successfully enantio-separated on Nucleosil 100 C8 HD with 4 mM vancomycin present in the mobile phase (composed of methanol and buffer) in the reversed phase mode. On the vancomycin-bonded chiral stationary phase, it was difficult to get enantio-separations of the profen NSAIDs. However, flobufen gave better enantio-separation on the vancomycin CSP. The better enantio-resolution of the majority of profen derivatives on the achiral columns with vancomycin added to the mobile phase can be attributed in particular to the higher separation efficiency of this capillary chromatographic system. In addition, vancomycin dimers, formed in the mobile phase, seem to offer a better steric arrangement for stereoselective interaction to these analytes than the vancomycin bonded on the CSP. These substantial differences in the CS structure significantly influence the chiral discrimination mechanism. *Chirality* 18: 531–538, 2006. © 2005 Wiley-Liss, Inc.

KEY WORDS: cLC; chiral separation; macrocyclic antibiotic; vancomycin-based chiral stationary phase; vancomycin chiral mobile phase additive

Chromatographic analysis of enantiomers is an important research field, now extended to include capillary liquid chromatography (cLC) studies.^{1,2} The cLC technique has certain advantages for enantiomeric separations, since it is easy to examine new types of chiral stationary phases (CSPs) that are normally rather expensive and usually available only in very small amounts. Furthermore, consumption of expensive chiral mobile-phase additives (CMPA) and other mobile-phase modifiers is lower, and the chromatographic efficiency and selectivity are often enhanced in contrast to classical high-performance liquid chromatography (HPLC).¹

Direct chiral separation can be achieved by the use of chiral stationary phases or chiral mobile-phase additives. Both arrangements have some advantages, but nowadays the system with chiral stationary phases is applied more frequently. The reason for this is the availability of a wide range of stable CSPs, which enable us to achieve highly

reproducible results.^{3–5} On the other hand, the use of achiral stationary phases with chiral mobile phase additives provides higher flexibility for selection of a suitable chiral selector (CS).^{6,7} Regarding the easier accessibility of the CS present in free solution for interaction with analytes, the chiral-discrimination mechanism can differ from that with the same chiral selector bonded on the stationary phase support. Moreover, the stoichiometry of the CS–analyte diastereoisomeric complex need not necessarily be 1:1.⁸ This possibility can bring an additional

*Correspondence to: Eva Tesařová, Department of Physical and Macromolecular Chemistry, Faculty of Science, Charles University, Albertov 2030, 128 43 Prague 2, Czech Republic. E-mail: tesarove@natur.cuni.cz
Received for publication 28 October 2005; Accepted 6 February 2006
DOI: 10.1002/chir.20284
Published online 24 April 2006 in Wiley InterScience (www.interscience.wiley.com).

improvement of chiral resolution. Therefore, the concentration of chiral selector in the mobile phase is, along with pH and organic-modifier content, the crucial factor influencing the enantioresolution.⁹ A combination of two different chiral additives can also substantially improve chiral recognition. Such an experimental setup is mostly employed in capillary electrophoresis.^{10–12} The choice of the separation arrangement, that is, chiral selector bonded or free, can make a substantial difference in the selectivity of enantioseparation. For example, the retention order of hexobarbital enantiomers was reversed when cyclodextrin CS was added to the mobile phase instead of using the cyclodextrin-bonded CSP.¹³ In some cases, an improvement of chiral separation can be achieved by combining a chiral stationary phase with a chiral mobile phase. Synergistic effects have been observed with the use of a structurally different chiral mobile-phase additive and bonded chiral selector, for example, D-(+)-camphorsulphonic acid as CMPA with β -cyclodextrin CSP in HPLC.¹⁴ Structurally related chiral selectors, for example, vancomycin as CMPA with immobilized ristocetin A as CSP, also provided for improved enantioresolution.¹⁵ Good results have been obtained with CSP and CMPA based on homologous chiral selectors in opposite configurations.¹⁶ In addition, the simultaneous use of vancomycin¹⁷ or norvancomycin¹⁸ both as stationary-phase and mobile-phase additives has been investigated. The explanation of the retention and enantiodiscrimination mechanism in these combined chromatographic separation systems is often rather curious.

Macrocyclic antibiotics (MAs), among them glycopeptides (namely, vancomycin, teicoplanin and ristocetin A), have been frequently employed in HPLC, capillary electrophoresis, and capillary electrochromatography.^{19–21} These compounds have a great variety of functional groups, cavities, and numerous stereogenic centers, which offer many interaction possibilities, including hydrogen bonding, electrostatic, and π - π interactions, inclusion, steric interactions, dipole stacking, or combinations thereof.

Profen nonsteroidal anti-inflammatory drugs (NSAIDs) represent an important group of pharmaceutical compounds that exhibit optical activity. It is known that individual enantiomers of some profen NSAIDs have different therapeutic activities.²² Therefore, various enantioresolution methods have been proposed for their separation. In HPLC the most frequently used CSPs for enantioseparation of individual profen NSAIDs are based on protein,^{23,24} polysaccharide,^{25,26} cyclodextrin,^{27,28} and macrocyclic antibiotic^{29–31} chiral selectors. A set of profens (fenoprofen, flurbiprofen, ibuprofen, indoprofen, ketoprofen, and suprofen) was baseline enantioresolved on avoparcin-based CSP in normal separation mode²⁹ and on teicoplanin-based CSP in reversed separation mode.³⁰ Vancomycin-based CSP (Chirobiotic V) was shown to be suitable for chiral separation of flurbiprofen and ketoprofen in buffer-organic mobile phase with a low amount of the organic modifier.³¹ Four stereoisomers of loxoprofen were baseline resolved on a cellulose derivative CSP Chiralcel OJ.³² Only a few studies employed chiral mobile-phase additives (mostly cyclodextrin chiral selectors) for

Chirality DOI 10.1002/chir

enantioseparation of profen NSAIDs in HPLC.^{33,34} Successful enantioseparations of ketoprofen and fenoprofen were obtained only with hydroxypropyl β -cyclodextrin.³³ Improved chiral selectivity was reported for flurbiprofen on the Chirobiotic V column after addition of vancomycin to the mobile phase.¹⁷

The capillary liquid chromatography technique has not yet been employed for chiral separation of profens. Our previous work utilizing teicoplanin as chiral selector in cLC for the enantioseparation of profens did not give satisfactory results for these compounds.³⁵

The aim of this study was to evaluate the chromatographic behavior of two direct cLC approaches for the enantioseparation of chiral drugs, fenoprofen, carprofen, flurbiprofen, indoprofen, flobufen, ketoprofen, and suprofen. As the role of the achiral stationary phase in the enantioselective separation systems is not always clearly interpreted,^{36–38} two capillary columns, Nucleosil 100 C8 HD and Nucleosil 100 C18 HD, were investigated with vancomycin added to the mobile phase. The second way of direct separation was vancomycin CSP Chirobiotic V and achiral mobile phase. Both the separation options were compared in order to elucidate the differences in the separation mechanism in the systems with free and bonded vancomycin CS.

MATERIALS AND METHODS

Materials

Mobile phases were prepared from the following compounds and solvents: methanol (MeOH), LiChrosolv, purity >99.8% (Merck, Darmstadt, Germany); triethylamine (TEA), purity >99.5% (Fluka, Buchs, Switzerland), and acetic acid, p.a. 99% (Lachema, Brno, Czech Republic). Free vancomycin chiral selector, as the mobile phase additive, was provided by Astec (Whippany, NJ, USA). Water used for preparation of all the mobile phases was purified with a Milli-Q water purification system (Millipore, USA).

Triethylamine acetate (TEAA) buffers, 0.1%, pH 5.0, containing various percentages of MeOH were used as mobile phases with vancomycin-based CSP.

The mobile phases 50/50 (v/v) methanol/0.1% TEAA, pH 5.0, with various concentrations (1.0–4.0 mM) of vancomycin were used if achiral stationary phases were employed. Vancomycin was dissolved in pure buffer and then appropriate amount of MeOH was added. The eluents were sonicated for at least 10 min just before use.

Racemic profen NSAIDs, fenoprofen, carprofen, flurbiprofen, indoprofen, flobufen, ketoprofen, and suprofen; all p.a. purity, were obtained from Sigma-Aldrich (St. Louis, MO, USA). All the compounds studied were injected as 1.0 mg mL⁻¹ methanolic solutions. The chemical structures of the analytes are shown in Figure 1.

Capillary Liquid-Chromatography Method

An ISCO syringe pump model 100 DM (Lincoln, NE, USA), a Valco injection valve with a 60 nl internal loop (Schenkon, Switzerland), and a Linear UV-VIS 205 detec-

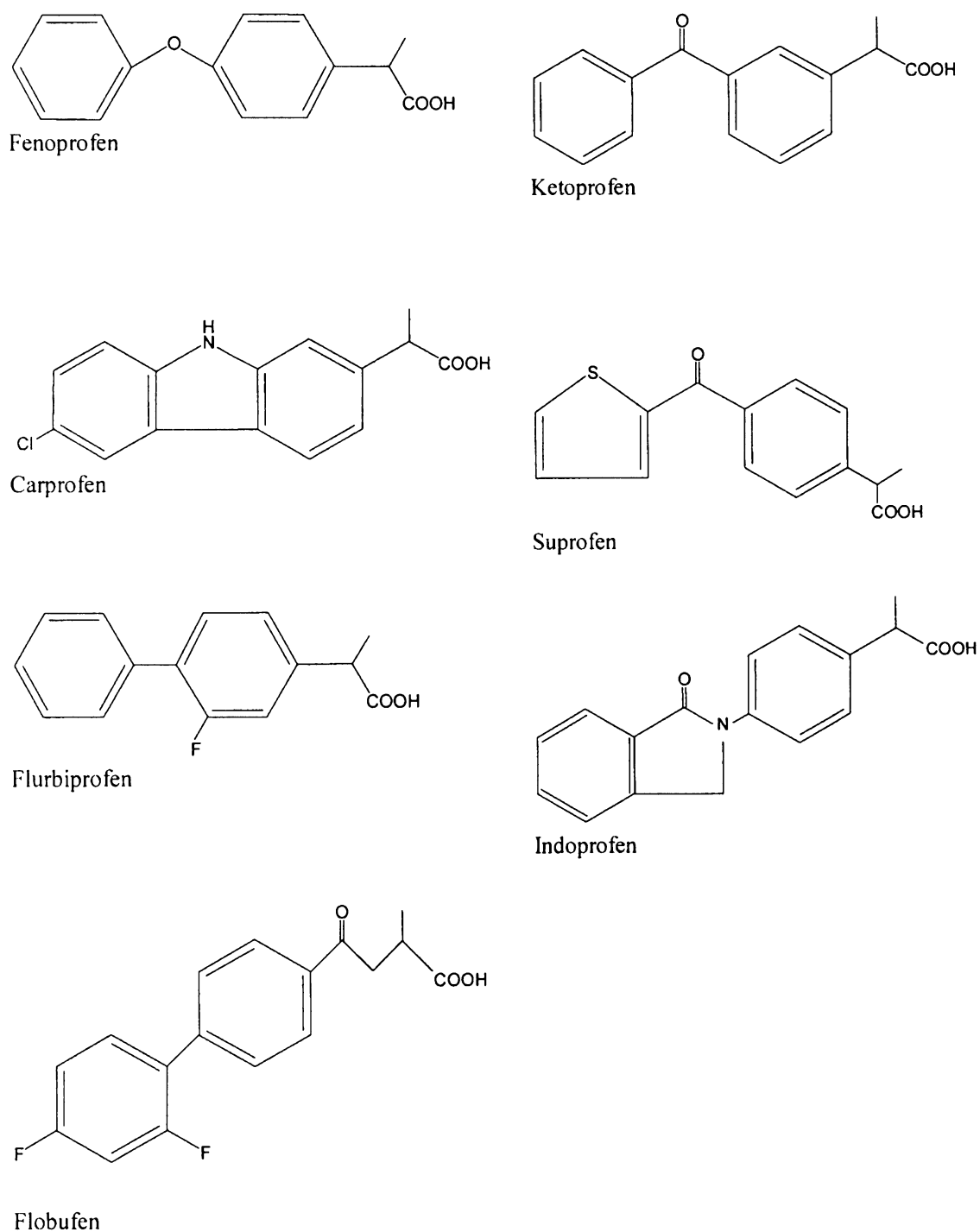


Fig. 1. Chemical structures of the studied profen NSAIDs.

tor equipped with a CE on-column flow cell (San Jose, CA, USA) were applied for the cLC experiments.

Fused-silica capillary columns of 25 cm × 300 μm I.D. packed with 5 μm Nucleosil 100 C8 HD or 5 μm Nucleosil 100 C18 HD were purchased from GROM (Herrenberg-Kayh, Germany).

A fused-silica capillary column of 25 cm × 320 μm I.D. packed with vancomycin bonded to silica gel (Chirobiotic

V), particle size 5 μm (Astec, Whippany, NJ, USA) was prepared at the Institute of Analytical Chemistry, Czech Academy of Sciences, Brno, Czech Republic. The frits were prepared as described by Cortes et al.³⁹ and slurry procedure packing was performed according to Planeta et al.⁴⁰

The column inlet was installed in the injection valve using a 5 cm polyether ether ketone (PEEK) sleeve (500 μm

Chirality DOI 10.1002/chir

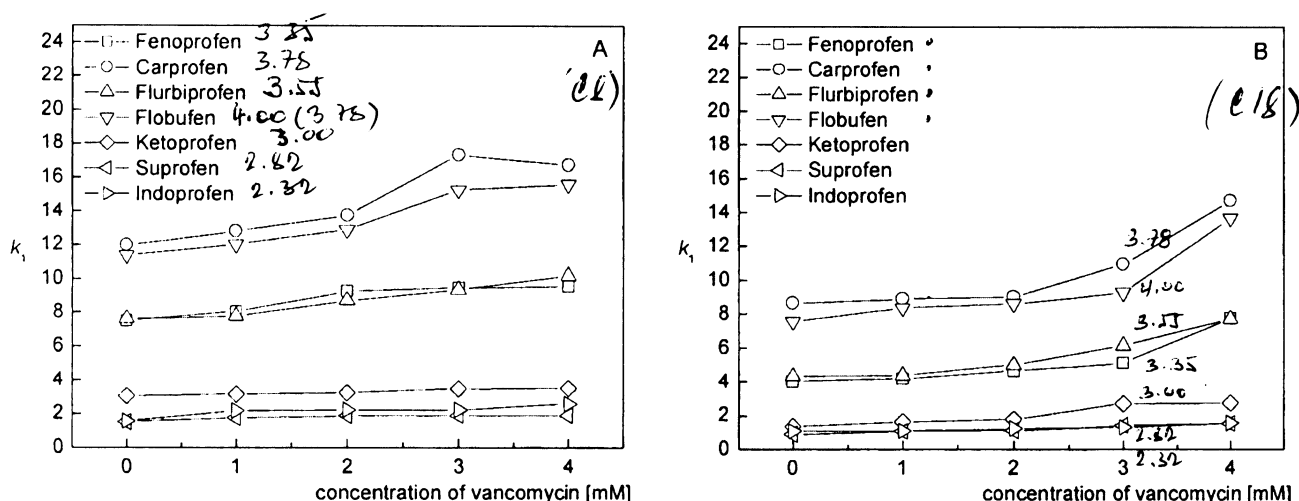


Fig. 2. Effect of the vancomycin concentration in the mobile phase on retention of profen NSAIDs on (a) Nucleosil 100 C8 HD and (b) Nucleosil 100 C18 HD. Mobile-phase composition: 50:50 (v/v) MeOH-0.1% TEAA, pH 5.0 with addition of vancomycin (k_1 is the retention factor of the first eluting enantiomer).

I.D.) and a PEEK finger-tight fitting, and the column outlet was connected by PTFE tubing to a 100 μm I.D. fused-silica capillary with the detection window 7 cm from the column outlet.

The flow rate of all the mobile phases used was 3 $\mu\text{L min}^{-1}$. The detection wavelength for the profen NSAIDs was 270 nm. Chromatograms were recorded and evaluated employing CSW computer software provided by DataApex (Prague, Czech Republic).

RESULTS AND DISCUSSION

Enantioselective Separations of Profen NSAIDs Using Vancomycin as the Mobile-Phase Additive and Achiral Columns

Two achiral capillary columns with different lengths of chemically bonded hydrocarbon chains, Nucleosil 100 C8 HD and Nucleosil 100 C18 HD, were examined for enantioseparation of selected profen NSAIDs with vancomycin as the mobile-phase additive. The role of the achiral stationary phases has been investigated with cyclodextrins as chiral mobile-phase additives, but some contradictory results have been described in the literature.^{36–38} Therefore, we wanted to examine also the effect of the stationary-phase structure on retention and enantioseparation of the profen NSAIDs in this work. Based on the preliminary results, MeOH-0.1% TEAA buffer, pH 5.0 in the volume ratio of 50:50 was selected as suitable mobile phase, to which vancomycin was added.

The effect of vancomycin concentration in the mobile phase on retention of the profen NSAIDs on the both columns is compared in Figure 2. Generally, greater retention of all the derivatives was found on Nucleosil 100 C8 HD. These results indicate that, although in the reversed-phase to separation mode hydrophobic interactions predominate, polar/ionic interactions are also involved in the retention mechanism. In general, the latter interaction forces have more pronounced effect on the stationary phase with shorter hydrocarbon chains (owing to the

Chirality DOI 10.1002/chir

more easily accessible silica-gel surface there).^{41,42} Increasing the vancomycin concentration resulted in increased retention. This trend could be observed more clearly on the C-18 stationary phase at higher chiral-selector concentration. Similar retention behavior, with almost no change of retention parameters with vancomycin concentration, was observed for structurally related derivatives, (suprofen, indoprofen and ketoprofen). Two pairs of derivatives, flurbiprofen with fenoprofen and flobufen with carprofen, also exhibited similar trends of retention factors, on both columns. A higher retention noticed at the higher vancomycin concentration can be attributed to a stronger interaction of the analyte-vancomycin complex with the stationary surface. The complex can be created in the mobile phase first and then the vancomycin-analyte complex can interact with the stationary phase. Vancomycin from the mobile phase can be also adsorbed on the achiral stationary phase before the vancomycin-analyte interaction is formed. Most probably a combination of both possibilities is involved in the retention mechanism. The latter possibility seems to take place predominantly on the C-8 column (compare Figs. 2a and 2b). The portion of vancomycin chiral selector adsorbed on the surface is affected by both the stationary-phase structure and mobile-phase composition, so it need not be necessarily the same on the both columns.¹⁷ An interesting feature influencing the interaction mechanism of analytes with vancomycin as CMPA is the formation of vancomycin dimers in solution,^{43,44} as described by many authors.^{43–48} Experimental approaches as well as computer simulation have been used to characterize the dimer formation. Acetate, which is present also in our mobile phase, has been reported to enhance dimerization.^{46,47} The dimerization of vancomycin seems to promote its affinity to carboxyl-containing analytes.⁴⁸ Higher stability of dimers probably creates more suitable conformation for chiral recognition of these analytes and can be also responsible for the increased retention observed in our experiments at higher vancomycin concentration.

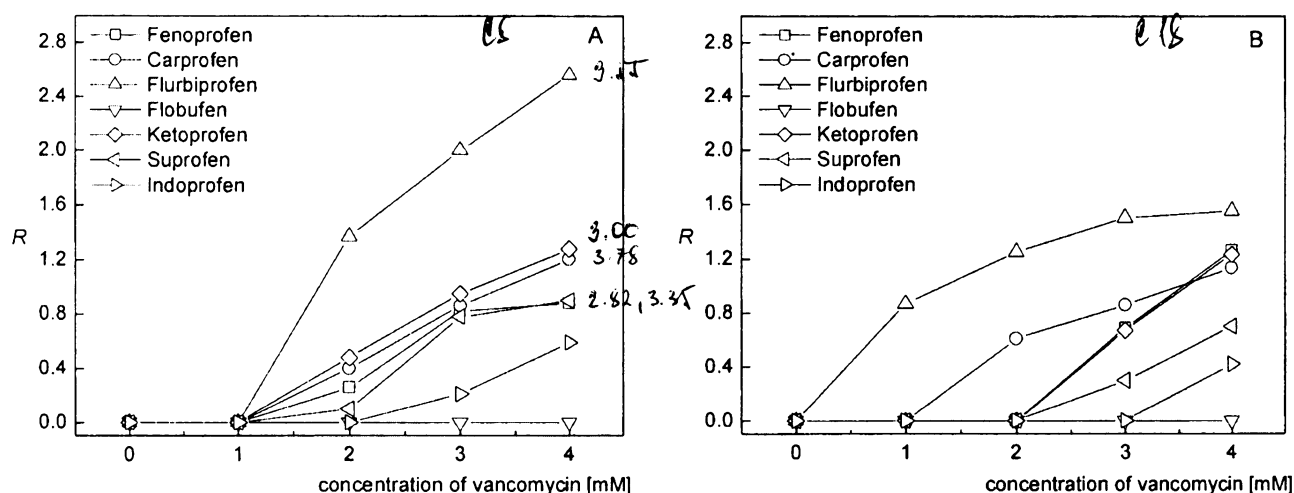


Fig. 3. Effect of vancomycin concentrations in the mobile phase on resolution of profen NSAIDs on (a) Nucleosil 100 C8 HD and (b) Nucleosil 100 C18 HD. Mobile-phase composition: 50:50 (v/v) MeOH-0.1% TEAA, pH 5.0 with addition of vancomycin (R is the resolution).

Resolution values on both columns were plotted versus vancomycin concentration, as shown in Figure 3. It is obvious that with increasing concentration of vancomycin, resolution of enantiomers of profen NSAIDs increased (with the exception of flobufen). The analytes mostly gave better enantioresolution on the Nucleosil 100 C8 HD capillary column than on the Nucleosil C18 HD with the mobile phase 50:50 (v/v) MeOH-0.1% TEAA, pH 5.0 with 4 mM vancomycin added (see also the chromatograms in Fig. 4). Table 1 summarizes the efficiency values obtained on both achiral columns at various concentrations of vancomycin in the mobile phase. It is obvious that the efficiencies are slightly higher on the Nucleosil 100 C8 HD stationary phase at any compared vancomycin concentration. Also, with increasing concentration of vancomycin in the mobile phase starting from the 2 mM concentration, the efficiency on the both columns slightly increases. For example, for flurbiprofen the respective values are 15,700 and 22,500 theoretical plates per meter (tp/m) in the mobile phase without and with 4 mM vancomycin on Nucleosil C18 stationary phase, and the corresponding values on Nucleosil C8 are 18,900 and 25,800 tp/m. The increased resolution values on Nucleosil 100 C8 HD (in comparison with Nucleosil 100 C18 HD) or generally, at higher vancomycin concentrations can be influenced by the corresponding higher efficiencies. The initial decrease of efficiencies with the mobile phase containing 1 mM vancomycin is difficult to explain. It is also difficult to judge if there is any relationship between the efficiency values and adsorption of vancomycin on the stationary surface. In general, better peak symmetry was observed on the stationary phase with shorter hydrocarbon chains (the asymmetry values' range was 1.1–2.3, versus 1.4–3.6 on the column with octadecyl chains). Selectivities were similar, ranging from 1.05 to 1.18, on both stationary phases and increased slightly with vancomycin concentration. The highest enantioresolution values for all the studied derivatives were obtained at the highest vancomycin concentration used (Fig. 3),

while the initial vancomycin amount necessary for at least partial enantioseparation was rather different for the individual pairs of enantiomers. Any general trend for the whole set of profen NSAIDs is rather difficult to discern for any of the columns. The baseline separation of flurbiprofen enantiomers was achieved with 2 mM vancomycin in the mobile phase on the both columns. On the contrary, the first indication of the enantioseparation of indoprofen was observed at 3 mM vancomycin on Nucleosil C8, while on Nucleosil C18 it was with 4 mM vancomycin (see Fig. 3). Flobufen was the only derivative that was not enantioresolved either on Nucleosil C8 or on Nucleosil C18 stationary phases at any chiral selector concentration.

The retention behavior of the profen NSAIDs studied was similar on both columns but higher retention and

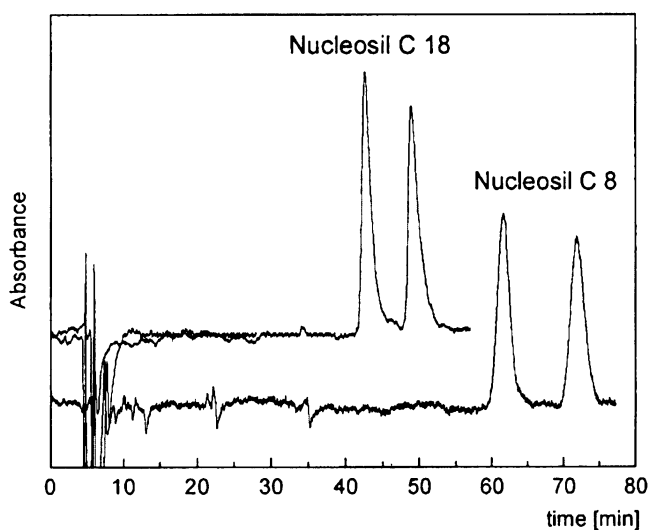


Fig. 4. Chromatograms of flurbiprofen on two different achiral stationary phases with vancomycin as the mobile-phase additive. Stationary phases: Nucleosil 100 C18 HD, Nucleosil 100 C8 HD; mobile-phase composition: 50:50 (v/v) MeOH-0.1% TEAA, pH 5.0, with 4mM vancomycin.

Chirality DOI 10.1002/chir

TABLE 1. Comparison of the efficiencies (in tp/m) of the separation systems composed of achiral stationary phases Nucleosil C8 or Nucleosil C18 and mobile phases (MeOH-0.1% TEAA, pH 5.0) with various vancomycin concentrations

Mobile phase	50:50 (v/v) MeOH-0.1% TEAA pH 5.0									
	Column	C8					C18			
Vancomycin	0 mM	1 mM	2 mM	3 mM	4 mM	0 mM	1 mM	2 mM	3 mM	4 mM
Fenoprofen	28,930	17,350	31,330	35,300	39,340	15,600	9,140	14,000	24,400	30,420
Carprofen	24,090	13,260	28,390	32,850	34,150	16,260	10,650	22,400	31,500	32,880
Flurbiprofen	18,900	12,350	22,610	24,970	25,800	15,700	14,400	19,200	20,030	22,500
Flobufen	21,310	17,350	23,400	30,600	32,640	9,100	8,400	13,100	21,600	29,900
Ketoprofen	14,000	8,200	16,120	19,200	25,850	5,050	3,500	5,900	23,220	24,510
Suprofen	6,650	6,600	9,820	11,420	14,510	4,030	3,000	4,850	12,100	13,600
Indoprofen	11,600	8,700	12,510	14,720	17,550	2,950	2,600	4,140	10,750	12,940

better enantioresolution were observed using the stationary phase with shorter hydrocarbon chains. These results are in accord with those obtained with similar columns using cyclodextrin as chiral selector.³⁶ As to the enantioresolution, the steric fit of the majority of profen derivatives into the structure of the dimerized vancomycin seems to be stereoselective and helps substantially in chiral discrimination. This explanation corresponds with the literature.⁴⁸

Enantioseparations of Profen NSAIDs Using Vancomycin-Bonded CSP

In the next part of our work, vancomycin CSP was used with an achiral mobile phase employed earlier that is, 50:50 (v/v) MeOH-0.1% TEAA, pH 5.0, but without vancomycin. This resulted in very short retention times and no enantioseparation. For this reason, the MeOH content of the mobile phase was reduced to lower values (in the range of 20–30%). The retention factors and enantioresolution values are summarized in Table 2. In general, the retention of profen NSAIDs increased with decreasing content of methanol in the mobile phase. The resolution, being affected by retention, selectivity, and efficiency, was not so straightforward. The separation selectivities were around 1.1, but undesirable peak broadening in some cases resulted in lowering of the resolution values at reduced methanol contents in the mobile phase. These results are in accord with our previous

experience with a commercial Chirobiotic V column in HPLC.⁴⁹

Five derivatives of the profen NSAIDs were partially enantioresolved on the vancomycin-based CSP, but with lower resolution values than on Nucleosil 100 C8 HD and 4 mM vancomycin in the mobile phase. The best enantioresolution was obtained again for flurbiprofen and also for suprofen. Fenoprofen and indoprofen enantiomers remained unresolved. It is obvious from the literature that substantial variation of separation conditions is necessary to give chiral separation of diverse profen derivatives, as has also been reported on macrocyclic antibiotic-based CSPs.^{29–31} Spatial arrangement of the bonded vancomycin is probably less suited for the chiral interactions with these analytes. Moreover, dimer formation, which was reported to improve stereoselective interaction, cannot be expected here.^{43–48} Surprisingly, relatively good results were obtained for flobufen, as shown in Figure 5. The best enantioresolution of flobufen was achieved with 25% MeOH in the mobile phase for the vancomycin CSP. The efficiency of the first eluted peak of flobufen was 17,000 theoretical plates per meter. Retention of flobufen at the lowest content of MeOH (20%) was higher, but severe broadening of both peaks decreased the enantioresolution. Successful enantioselective separation of flobufen (compared to enantioresolution on the achiral columns with addition of vancomycin to the mobile phase) can be explained by its structure. The aromatic ring is not

TABLE 2. Effect of methanol contents in the mobile phase (MeOH-0.1% TEAA, pH 5.0) on chromatographic data of profen NSAIDs using the vancomycin-based CSP

Profen NSAIDs	MeOH					
	30%		25%		20%	
	k_1	R	k_1	R	k_1	R
Fenoprofen	1.24	0.00	1.79	0.00	2.03	0.00
Carprofen	2.91	0.00	4.93	0.00	6.07	0.26
Flurbiprofen	1.53	0.70	2.13	0.60	2.43	0.63
Flobufen	1.42	0.38	3.13	0.76	3.87	0.70
Ketoprofen	0.37	0.00	1.89	0.44	2.25	0.44
Suprofen	1.43	0.71	2.10	0.42	2.42	0.40
Indoprofen	2.14	0.00	3.14	0.00	4.02	0.00

k_1 is the retention factor of the first eluting enantiomer; R is the resolution.

Chirality DOI 10.1002/chir

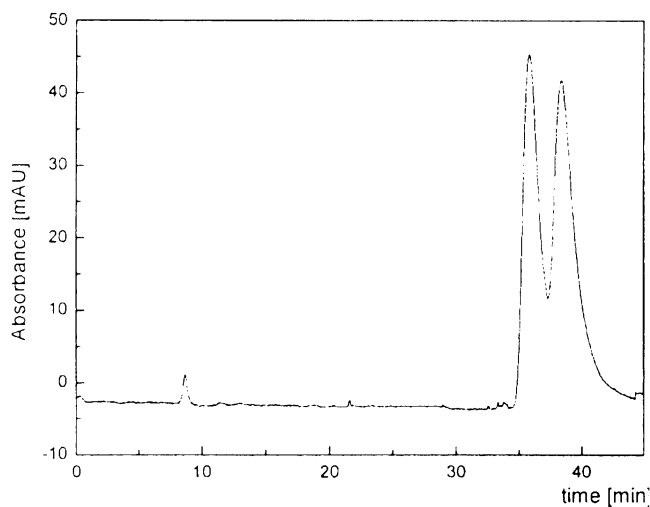


Fig. 5. Chromatogram of enantioseparation of flobufen on vancomycin CSP. Mobile-phase composition: 25:75 (v/v) MeOH-0.1% TEAA, pH 5.0.

attached directly to the asymmetric center, but the linking keto group contributes to chiral discrimination. This result corresponds with our observations from the comparison of vancomycin bonded CSPs with various densities of the chiral selector coverage.¹⁹ The enantiomers of flobufen were baseline resolved on the CSP with lower coverage of vancomycin, while only partial separation was achieved on the higher-density stationary phase. These results obtained with flobufen were opposite from those of all the other profens studied. Flobufen seems to favor greater distance of the CS molecules from each other. From this point of view, the dimerized vancomycin in the mobile phase seems to be less suitable for the stereoselective interaction. A change of conformation of vancomycin caused by its binding to the carrier is another possible explanation of this result.

CONCLUDING REMARKS

The chiral selector vancomycin yields better enantioseparations of the profen derivatives when used as a mobile phase additive than as a stationary phase. The free vancomycin molecules (in solution), at which none of the interaction sites are blocked by binding of the chiral selector to the support, offer additional possibilities for enantioselective interactions compared to vancomycin molecules bonded on silica gel. The spatial arrangement of the dissolved CS seems to be better suited for interaction with the analytes. Moreover, vancomycin in the mobile phase can dimerize and the interactions of the acidic analytes with the dimerized CS were reported to be advantageous for chiral discrimination.¹⁸ In accordance with the results of a study¹⁷ in which HPLC system of vancomycin bonded CSP and vancomycin CMPA were used simultaneously, we have found it difficult to achieve enantioseparations of profen NSAIDs using vancomycin CSP. On the other hand, we have shown that the use of the vancomycin CSP is not, in principle, important if the stationary

phase shows sufficiently high efficiency and is compatible with the mobile phase and the chiral selector.

The behavior of flobufen, which was enantioresolved on vancomycin CSP with better result than on achiral columns with addition of vancomycin to the mobile phase, differed from the other NSAIDs studied.

ACKNOWLEDGMENTS

The authors acknowledge the financial support of this work from the Grant Agency of the Czech Republic under grant no. 203/03/0161. Special thanks must be expressed to Ing. J. Planeta Ph.D. from the Institute of Analytical Chemistry, Czech Academy of Sciences, Brno, Czech Republic, for packing the vancomycin-based capillary column.

References

1. Vissers JPC. Recent developments in microcolumn liquid chromatography. *J Chromatogr A* 1999;856:117-143.
2. Liu Z, Otsuka K, Terabe S, Motokawa M, Tanaka N. Physically adsorbed chiral stationary phase of avidin on monolithic silica column for capillary electrochromatography and capillary liquid chromatography. *Electrophoresis* 2002;23:2973-2981.
3. Haginaka J. Pharmaceutical and biomedical applications of enantioseparations using liquid chromatographic techniques. *J Pharm Biomed Anal* 2002;27:357-372.
4. Thompson R. A practical guide to HPLC enantioseparations for pharmaceutical compounds. *J Liq Chromatogr* 2005;28:1215-1231.
5. Beesly TE, Scott RPW. Chiral chromatography. Separation science series. Chichester, UK: John Wiley & Sons; 1998.
6. Ahuja S. Chiral separations: Overview. In: Ahuja S, editor. Chiral separations by liquid chromatography. Washington, DC: American Chemical Society; 1991.1-26 p.
7. Sharp VS, Risley DS. Evaluation of the macrocyclic antibiotic LY333328 as a chiral selector when used as a mobile phase additive in narrow bore HPLC. *Chirality* 1999;11:75-81.
8. Hilton ML, Armstrong DW. Contribution of cyclodextrin and derivatives to liquid chromatography. In: Duchene D, editor. New trends in cyclodextrins and derivatives. Paris, France: Editions de Santé; 1991. p 515-549.
9. Tesařová E, Armstrong DW. Enantioselective separation. In: Deyl Z, editor. Advanced chromatographic and electromigration methods in bioscience. Amsterdam, The Netherlands: Elsevier; 1998.197-256 p.
10. Ward TJ, Farris AB, Woodling K. Synergistic chiral separations using the glycopeptides ristocetin A and vancomycin. *J Biochem Biophys Methods*. 2001;48:163-174.
11. Rizvi SAA, Akbay C, Shamsi SA. Polymeric alkenoxy amino acid surfactants: II. Chiral separations of β -blockers with multiple stereogenic center. *Electrophoresis* 2004;25:853-860.
12. Kuhn R. Enantiomeric separation by capillary electrophoresis using a crown ether as chiral selector. *Electrophoresis* 1999;20:2605-2613.
13. Krstulovic AM. Chiral separation by HPLC. New York: Ellis Horwood Limited; 1989.
14. Kuijpers PK, Gerding TK, Gerhardus JJ. Influence of the combination of a β -cyclodextrin stationary phase and D(+)-camphorsulphonic acid as a mobile phase additive on enantioselectivity. *HPLC '92*, Baltimore, MD, 1992.
15. Slama I, Jourdan E, Grosset C, Ravel A, Villet A, Peyrin E. Role of the vancomycin-ristocetin heterodimerization on the enantioselectivity of D,L-tryptophan and D,L-dansyl tryptophan. *J Chromatogr B* 2003;795:115-121.
16. Duff KJ, Gray HL, Gray RJ, Bahler CC. Chiral stationary phases in concert with homologous chiral mobile-phase additives-push-pull model. *Chirality* 1993;5:201-206.

17. Sun Q, Olesik SV. Chiral separation by simultaneous use of vancomycin as stationary phase chiral selector and chiral mobile phase additive. *J Chromatogr B* 2000;745:159–166.
18. Ding GS, Huang XJ, Liu Y, Wang JD. Chiral separation of racemates of drugs and amino acid derivatives by high-performance liquid chromatography on a norvancomycin-bonded chiral stationary phase. *Chromatographia* 2004;59:443–449.
19. Ward TJ, Farris AB. Chiral separations using the macrocyclic antibiotics: A review. *J Chromatogr A* 2001;906:73–89.
20. Aboul-Enein HY, Ali I. Macrocyclic antibiotics as effective chiral selectors for enantiomeric resolution by liquid chromatography and capillary electrophoresis. *Chromatographia* 2000;52:679–691.
21. Desiderio C, Fanali S. Chiral analysis by capillary electrophoresis using antibiotics as chiral selector. *J Chromatogr A* 1998;807:37–56.
22. Sheldon RA. *Chirotechnology*. New York: Marcel Dekker; 1993.56 p.
23. Hermansson J, Hermansson I. Dynamic modification of the chiral bonding properties of a Chiral-AGP column by organic and inorganic additives—separation of enantiomers of antiinflammatory drugs. *J Chromatogr A* 1994;666:181–191.
24. Hofstetter H, Hofstetter O, Schurig V. Rapid separation of enantiomers in perfusion chromatography using a protein chiral stationary phase. *J Chromatogr A* 1997;764:35–41.
25. Teng XW, Wang SWJ, Davies NM. Stereospecific high-performance liquid chromatographic analysis of flurbiprofen: Application to pharmacokinetic studies. *J Pharm Biomed Anal* 2003;33:95–100.
26. VanOverbeke A, Baeyens W, Oda H, Aboul-Enein HY. Direct enantiomeric HPLC separation of several 2-arylpropionic acids, barbituric acids and benzodiazepines on Chiracel OJ-R chiral stationary phase. *Chromatographia* 1996;43:599–606.
27. Gilar M, Tesarova E, Deyl Z. Influence of mobile phase composition on retention and enantioseparation of profens in HPLC on the β -cyclodextrin stationary phase. *Chem Listy* 1996;90:461–466.
28. Besson MD, Vigh J. Examination of the retention behavior of undervatized profen enantiomers on cyclodextrin silica stationary phases. *J Chromatogr A* 1993;634:197–204.
29. Ekborg-Ott KH, Kullman JP, Wang XD, Gahm K, He LF, Armstrong DW. Evaluation of the macrocyclic antibiotic avoparcin as a new chiral selector for HPLC. *Chirality* 1998;10:627–660.
30. Armstrong DW, Liu YB, Ekborg-Ott KH. Covalently bonded teicoplanin chiral stationary phase for HPLC enantioseparations. *Chirality* 1995;7:474–497.
31. Pehourcq F, Jarry C, Bannwarth B. Chiral resolution of flurbiprofen and ketoprofen enantiomers by HPLC on a glycopeptide-type column chiral stationary phase. *Biomed Chromatogr* 2001;15:217–222.
32. Kanazawa H, Tsubayashi A, Nagata Y, Matsushima Y, Mori C, Kizu J, Higaki M. Stereospecific analysis of loxoprofen in plasma by chiral column liquid chromatography with a circular dichroism-based detector. *J Chromatogr A* 2002;948:303–308.
33. Ameyibor E, Stewart JT. Enantiomeric HPLC separation of selected chiral drugs using native and derivatized β -cyclodextrins as chiral mobile phase additives. *J Liq Chromatogr & Rel Technol* 1997;20:855–869.
34. Patel BK, Valentova J, Hutt AJ. Chromatographic separation and enantiomeric resolution of flurbiprofen and its major metabolites. *Chromatographia* 2002;55:135–142.
35. Kafkova B, Bosakova Z, Tesarova E, Coufal P. Chiral separation of β -adrenergic antagonists profen non-steroidal anti-inflammatory drugs and chlorophenoxypropionic acid herbicides using teicoplanin as the chiral selector in capillary liquid chromatography. *J Chromatogr A* 2005;1088:82–93.
36. Takeuchi T, Nagae N. Stationary phase effect on enantioselectivity of dansyl phenylalanine in microcolumn liquid-chromatography with γ -cyclodextrin as mobile phase additive. *J Chromatogr* 1992;595:121–124.
37. Walhagen A, Edholm LE. Chiral separation on achiral stationary phases with different functionalities using β -cyclodextrin in the mobile phase and application to bioanalysis and coupled columns. *Chromatographia* 1991;32:215–223.
38. Peyrin E, Guillaume YC. Peculiarities of the mechanism of retention of imidazole derivatives when using hydroxypropyl- β -cyclodextrin as mobile phase additive. *Chromatographia* 1999;49:691–698.
39. Cortes HJ, Pfeiffer CD, Richter BE, Stevens TS. Porous ceramic bed supports for fused silica packed capillary columns used in liquid chromatography. *J High Resolut Chromatogr* 1987;10:446–448.
40. Planeta J, Karasek P, Vejrosta J. Development of packed capillary columns using carbon dioxide slurries. *J Sep Sci* 2003;26:525–530.
41. Sandi A, Nagy M, Szepeszy L. Characterization of reversed-phase columns using the linear free energy relationship. III. Effect of the organic modifier and the mobile phase composition. *J Chromatogr A* 2000;893:215–234.
42. Unger KK. Adsorbents in column liquid chromatography. In: Unger KK, editor. *Packings and phases in chromatographic techniques*. *Chromatogr Sci Series Vol 47*; New York: Marcel Dekker; 1990.331–470 p.
43. Slama I, Dufresne C, Jourdan E, Fahrat F, Villet A, Ravel A, Grosset C, Peyrin E. Vancomycin dimerization and chiral recognition studied by high-performance liquid chromatography. *Anal Chem* 2002;74:5205–5211.
44. Jourdan E, Haroun M, Slama I, Ravel A, Grosset C, Villet A, Peyrin E. Use of an amino stationary phase to study the vancomycin dimerization dependence on solute enantioselectivity. *J Liq Chromatogr* 2003;26:1027–1039.
45. Gerhard U, Mackay JP, Maplestone RA, Williams DH. The role of the sugar and chlorine substituents in the dimerization of vancomycin antibiotics. *J Am Chem Soc* 1993;115:232–237.
46. McPhail D, Cooper A. Thermodynamics and kinetics of dissociation of ligand-induced dimers of vancomycin antibiotics. *J Chem Soc Faraday Trans* 1997;93:2283–2289.
47. Shiozawa H, Zerella R, Bardsley B, Tuck KL, Williams DH. Noncovalent bond lengths and their cooperative shortening: Dimers of vancomycin group antibiotics in crystal and in solution. *Helv Chim Acta* 2003;86:1359–1370.
48. Kang JW, Bischoff D, Jiang ZJ, Bister B, Sussmuth RD, Schurig V. A mechanistic study of enantiomeric separation with vancomycin and balhimycin as chiral selectors by capillary electrophoresis. Dimerization and enantioselectivity. *Anal Chem* 2004;76:2387–2392.
49. Bosakova Z, Curinova E, Tesarova E. Comparison of vancomycin based stationary phases with different chiral selector coverage for enantioselective separation of selected drugs in HPLC. *J Chromatogr A* 2005;1088:94–103.

PHYSICOCHEMICAL PROPERTIES OF 9-(ALKYLSULFANYL)- AND 9-(ARYLSULFANYL)ACRIDINE DERIVATIVES AND THEIR INTERACTION WITH (2-HYDROXYPROPYL)CYCLODEXTRINSIrena NĚMCOVÁ^{a1,*}, Karel NESMĚRÁK^{a2}, Božena KAFKOVÁ^a and † Jan SEJBAL^b^a Department of Analytical Chemistry, Faculty of Science, Charles University, Albertov 6, CZ-128 43 Prague 2, Czech Republic; e-mail: ¹ inemcova@natur.cuni.cz, ² nesmerak@natur.cuni.cz^b Department of Organic and Nuclear Chemistry, Faculty of Science, Charles University, Albertov 6, CZ-128 43 Prague 2, Czech Republic

Received September 2, 2005

Accepted December 12, 2005

Dedicated to Professor Jaroslav Podlaha on the occasion of his 70th birthday.

Acid-base properties and aggregations of 9-(alkylsulfanyl)- and 9-(arylsulfanyl)acridine derivatives were studied as a part of systematic study of physicochemical properties of these compounds synthesized as potential drugs. The effect of the (2-hydroxypropyl)cyclodextrins on these properties and their association with the acridines was also followed.

Keywords: Cyclodextrins; Sulfanylacridines; Dissociation constants; Aggregation; Inclusion complexes; Association constants; Dissolution; NMR spectroscopy.

In the frame of the study of basic properties of newly synthesized 9-(alkylsulfanyl)- and 9-(arylsulfanyl)acridines, we have published the effect of substituents on the electron-donor properties¹ of these compounds. We also reported electrochemical oxidation of these compounds as the model of their possible biotransformation². Chromatographic methods for determination of these compounds were also proposed^{3,4}.

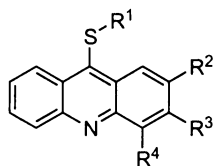
Sulfanylacridine derivatives⁵ are pharmacologically active mainly against microbial^{6,7} and protozoal infections^{8,9}. Recently, these compounds were also tested in treatment of Alzheimer¹⁰ and Creutzfeld–Jacob¹¹ diseases. Based on the analogy to other polycyclic aromatic compounds with heterocyclic nitrogen, they could also be active in the reversal of multidrug resistance¹² (by inhibition of transmembrane transport¹³).

Cyclodextrins¹⁴ are cyclic oligosaccharides consisting of six (α -cyclodextrin), seven (β -cyclodextrin) or eight (γ -cyclodextrin) glucose units connected by α -1,4-glycoside bonds. The cavity of cyclodextrins is weakly hydrophobic

relative to the hydrophilic exterior with primary and secondary hydroxy groups. Cyclodextrins form inclusion complexes based on interactions of noncovalent nature (electrostatic interactions, van der Waals forces, π - π interactions) and steric effects¹⁵. These complexes are often used in analytical chemistry¹⁶. In pharmaceutical preparations they improve the solubility and stability of pharmaceuticals¹⁷, and are used as carriers of active substances in biological systems and to retard the release of active substances from the pharmaceutical matrix¹⁸.

We have studied acid-base properties, aggregation, and solubility of newly synthesized 9-sulfanylacridine derivatives (Table I) and the effect of cyclodextrins on these properties. The association constants of the formed inclusion complexes were also determined. The (2-hydroxypropyl)cyclodextrins were used as they are recommended as more soluble and less toxic than the unsubstituted ones¹⁹ and they are widely used in pharmaceutical research^{20,21}.

TABLE I
Structures of studied 9-sulfanylacridines



Compound	R ¹	R ²	R ³	R ⁴
BG 51	CH ₃	H	H	H
BG 138	CH ₃	OCH ₃	H	H
BG 55	C ₂ H ₅	H	H	H
BG 180	C ₂ H ₅	H	H	OCH ₃
BG 980	<i>n</i> -C ₃ H ₇	OCH ₃	H	H
BG 375	<i>n</i> -C ₃ H ₇	H	H	OCH ₃
BG 376	<i>n</i> -C ₆ H ₁₃	H	H	OCH ₃
BG 238	CH ₂ CH ₂ N(CH ₃) ₂	H	NH ₂	H
BG 186	CH ₂ CH ₂ N(CH ₂ CH ₃) ₂	H	H	OCH ₃
BG 979		OCH ₃	H	H
BG 463		H	NH ₂	H

EXPERIMENTAL

Instruments and Operating Conditions

Determination of dissociation constants was performed by potentiometric titration using an automatic microburette ABU 80 (Radiometer) and pH-meter pHM 64 (Jenway). The compounds ($c = 1 \times 10^{-4} \text{ mol l}^{-1}$ in 9% ethanol) were titrated with 0.1 M HCl; the constant ionic strength of 0.1 was maintained with KCl. The pK_a values were evaluated from the titration curves obtained. Aggregation and determination of the association constants were studied by UV spectrophotometry using a HP-8455 diode-array spectrophotometer (Hewlett-Packard) with quartz cuvettes with 1-cm absorption layer. As it is known that presence of inorganic salts supports the aggregation, it was not possible to adjust the pH of solution by buffers. Therefore, the pH was maintained by addition of appropriate volume of 0.1 M HCl or 0.1 M NaOH. The ionic strength of solutions was not set. ^1H NMR spectra were measured by Varian^{UNITY} Inova 400 at 399.95 MHz in deuterium oxide at 25 and 50 °C. 2-Methylpropan-2-ol (δ 1.25 ppm) was used as a standard. The COSY spectra were measured using standard double pulsed sequence in the absolute mode. The NOESY and ROESY spectra were measured in the standard double pulsed sequence as phase-sensitive experiments (mixing time 0.3 s for NOESY and 0.1 s for ROESY). All 2D experiments were made in the spectral window 5000 Hz for proton signals. The solubility measurements were performed using an Erweka Dissolution Tester DT 6R and a Shimadzu UV-160 HIM 226 spectrophotometer. The inclusion complexes were prepared²² by precipitation and by mixing (which was found to be more effective) with the ratio of compound:cyclodextrin 1:3.

Chemicals and Solutions

The studied compounds were synthesized by the previously described method²³, their identity was checked by elemental analysis and NMR and their purity by HPLC and GC-MS. Stock solutions of the studied derivatives were prepared with a concentration of $1 \times 10^{-3} \text{ mol l}^{-1}$ in 90% ethanol. The (2-hydroxypropyl)cyclodextrins (Sigma Aldrich) were used as supplied. Their stock solutions with a concentration of 0.25 mol l^{-1} were prepared in distilled water. All other used chemicals were of analytical grade.

RESULTS AND DISCUSSION

Acid-Base Properties of 9-Sulfanylacridines

Acid-base properties of biological active compounds are very important characteristics both from the theoretical and practical viewpoints. The effect of molecular structure on acid-base equilibria is an important topic in modern organic chemistry. Moreover, the knowledge of dissociation constants is necessary in the prediction of biological activity using the QSAR method. From the pharmaceutical viewpoint, acid-base properties are very important for drug interactions with biological systems.

The determined pK_a values of studied 9-sulfanylacridines are summarized in Table II. We have also calculated the pK_a values using the Pallas 3.0 program (CompuDrug Chemistry); their values are also given in Table II. In the case of compounds with more nitrogen atoms, the calculated lower pK_a values refer to heterocyclic nitrogens. Only one pK_a value is obtained (both experimentally and by calculation) in the acid range for compounds with NH_2 group near to heterocyclic nitrogen atom because of the strong N–N interaction. It was impossible to obtain the pK_a value for the strongly basic group $-CH_2CH_2N(CH_2CH_3)_2$. The determined and calculated pK_a values correlate satisfactorily (Fig. 1); therefore, the program Pallas can be used for the prediction of pK_a values of compounds in this structure group. In the presence of (2-hydroxypropyl)cyclodextrins (up to concentrations of 0.01 mol l^{-1}) the values of dissociation constants decrease slightly (in tenths of unit). The potential presence of (2-hydroxypropyl)cyclodextrins as drug carriers would not thus affect the acid-base equilibria of studied compounds.

Aggregation of 9-Sulfanylacridines

As it follows from the previous part that the pK_a values of all the studied compounds are in the range of 5–7, all the following studies were performed at pH 3 (protonized forms of the compounds) and pH 8 (free bases),

TABLE II
Experimental and calculated dissociation constants of studied 9-sulfanylacridines

Compound	pK_a	
	experimental ^a	calculated
BG 51	5.34	5.61
BG 138	6.40	6.51
BG 55	5.68	5.45
BG 980	6.23	6.56
BG 238	7.10	7.20; 8.45
BG 186	6.13	6.88; 9.15
BG 979	6.13	6.73
BG 463	5.51	6.23

^a Relative standard deviations are lower than 2.5%.

which is near the physiological pH. We have found that the free bases of some 9-sulfanylacridines aggregate at very low concentrations in aqueous solution, in analogy to the aggregation of other acridine derivatives²⁴. New absorption bands with absorption maxima at longer wavelengths are formed in the absorption spectra of the studied compounds. Generally²⁵,

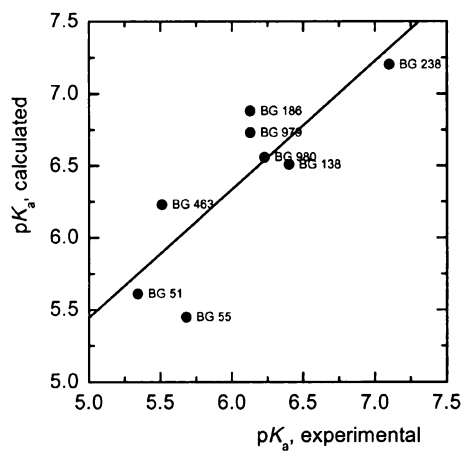


FIG. 1
The plot of calculated vs measured values of dissociation constant for studied 9-sulfanylacridines ($r = 0.8307$)

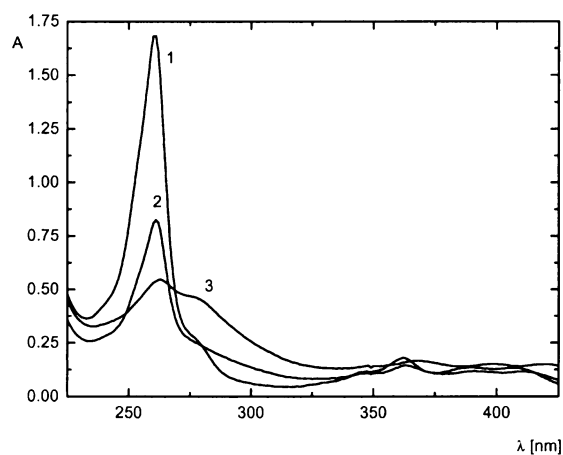


FIG. 2
The absorption spectra of compounds BG 138 (1), BG 980 (2), and BG 979 (3) ($c = 2 \times 10^{-5} \text{ mol l}^{-1}$, pH 8)

the absorption maxima of "head-to-tail" aggregates are shifted bathochromically relative to the monomer (*J*-bands are formed); the absorption maxima of "head-to-head" aggregates are shifted hypsochromically (*H*-bands). According to our results, the degree of aggregation of the studied compounds depends on the type of substituent on the sulfur atom. As an example, the absorption spectra of compounds with increasing alkyl length (BG 138 and BG 980) and with the phenyl group in the side chain (BG 979) are given in Fig. 2. The absorbance of the absorption maxima of monomers ($\lambda \cong 260$ nm) decrease and the new absorption maxima of aggregates ($\lambda \cong 280$ nm) are formed. We have found that the presence of ethanol affects the aggregation, too. However, we have determined that this effect begins at the concentration of ethanol higher than 10%; the concentration of ethanol in the measured solutions was lower than 1%. The protonized forms of derivatives do not aggregate because of the repulsion of their positive charges.

In the presence of (2-hydroxypropyl)cyclodextrins (Fig. 3), the absorbance of absorption maximum of aggregate ($\lambda \cong 280$ nm for all aggregates) decreases and the absorbance of absorption maximum of monomer of base ($\lambda \cong 260$ nm) increases. Thus, (2-hydroxypropyl)cyclodextrins suppress the aggregation, as only monomers can be included in the cavity and can form the inclusion complexes. Mainly monomeric forms are pharmaceutically

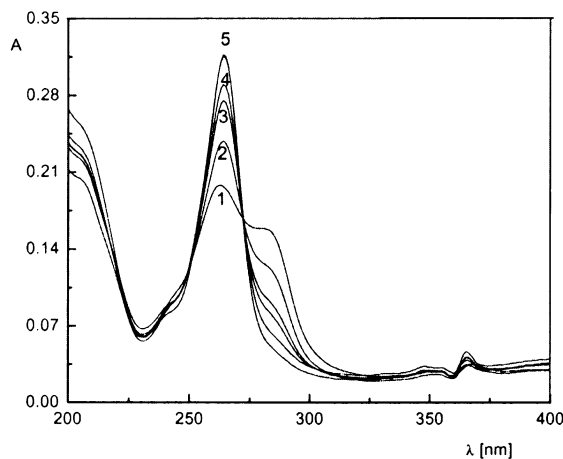


FIG. 3

The absorption spectra of compound BG 375 ($c = 1 \times 10^{-5}$ mol l⁻¹, pH 8) in presence of growing concentration of (2-hydroxypropyl)- α -cyclodextrin (in mol l⁻¹): 0 (1), 2×10^{-3} (2), 4×10^{-3} (3), 6×10^{-3} (4), 8×10^{-3} (5)

active and, therefore, (2-hydroxypropyl)cyclodextrins could have a positive effect in medical formulations.

Association Constants of Inclusion Complexes

The formation of the inclusion complexes in the presence of increasing concentrations of (2-hydroxypropyl)cyclodextrins results in increase in the absorbance of the main absorption band of the base ($\lambda_{\text{max}} \cong 260$ nm for all the compounds). The protonized forms of the compounds do not interact with (2-hydroxypropyl)cyclodextrins. The stoichiometry and association constants K_{as} of inclusion complexes of the selected compounds were evaluated from their absorption spectra using the Benesi–Hildebrand method²⁶, i.e. the linear dependence of $1/\Delta A$ vs $1/c$ for the complexes with the stoichiometry 1:1. As an example, the absorption spectra of the negligibly aggregating compound BG 138 in the presence of increasing concentration of (2-hydroxypropyl)- α -cyclodextrin are given in Fig. 4.

The binding isotherms of BG 138 and of the aggregating compounds BG 980 are given in Fig. 5. It follows from this figure that the degree of interaction depends on the compound structure and on the cavity size of (2-hydroxypropyl)cyclodextrins. The constant absorbance value is attained for the interaction with (2-hydroxypropyl)- β -cyclodextrin in both cases, in-

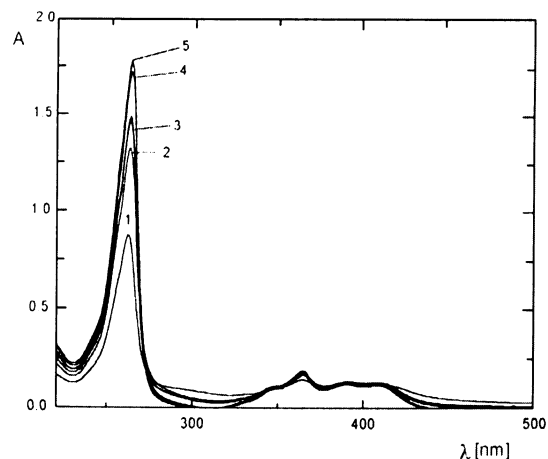


FIG. 4

The absorption spectra of compound BG 138 ($c = 1 \times 10^{-5}$ mol l^{-1} , pH 8) in presence of growing concentration of (2-hydroxypropyl)- α -cyclodextrin (in mol l^{-1}): 0 (1), 6×10^{-3} (2), 8×10^{-3} (3), 1×10^{-2} (4), 2.5×10^{-2} (5)

dicating that the complexes are formed at the used (2-hydroxypropyl)- β -cyclodextrin concentrations. The continually increasing absorbance in the interaction with (2-hydroxypropyl)- α -cyclodextrin and (2-hydroxypropyl)- γ -cyclodextrin indicates that the complexes are weaker and a higher excess of cyclodextrins is necessary for their formation. However, this could not be realized because of the insufficient solubility of (2-hydroxypropyl)-cyclodextrins.

The stoichiometry sulfanylacridine:cyclodextrin 1:1 was found in all cases. The obtained values of K_{as} are given in Table III. Thus, the K_{as} values decrease with increasing alkyl length in the interaction with (2-hydroxypropyl)- α -cyclodextrin. The highest values of K_{as} of the inclusion complexes of the compound with alkylsulfanyl group were obtained for the interaction with (2-hydroxypropyl)- β -cyclodextrin, the derivative with phenylsulfanyl group forms the most stable complex with (2-hydroxypropyl)- γ -cyclodextrin.

NMR Study of Aggregation and Association with (2-Hydroxypropyl)cyclodextrins

The aggregation of 9-sulfanylacridines and their association with (2-hydroxypropyl)cyclodextrins were studied by ^1H NMR, 2D-NOESY, and ROESY in detail²⁷. The concentration and temperature dependences of ^1H NMR spectra confirmed aggregation of the studied compounds; the aggregation is suppressed in the presence of (2-hydroxypropyl)cyclodextrins. In the

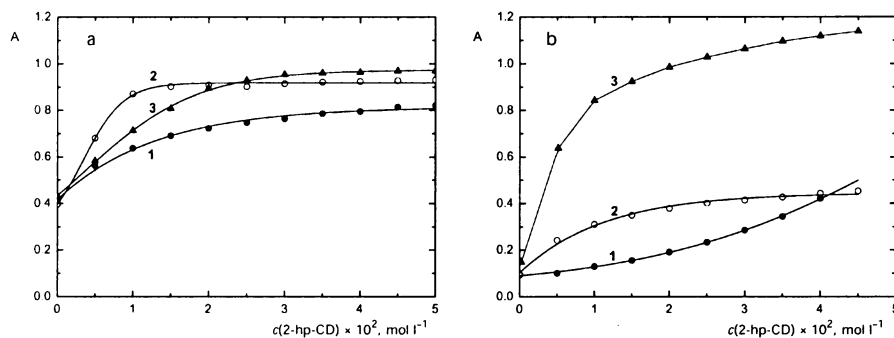


FIG. 5

The binding isotherms of compounds BG 138 (a) and BG 980 (b) in the presence of (2-hydroxypropyl)- α -cyclodextrin (1), (2-hydroxypropyl)- β -cyclodextrin (2), and (2-hydroxypropyl)- γ -cyclodextrin (3). Measurements were performed against blank solutions with the same concentrations of the cyclodextrins; $c(\text{compound}) = 2.5 \times 10^{-6} \text{ mol l}^{-1}$, pH 8)

^1H NMR spectra of BG 180, the considerable proton shifts in the position of 3 and 5 of glucose units of cyclodextrins give evidence on the occupation of cyclodextrin cavity. The protons in the position 6 and 6' of glucose units are affected, too. The space contacts of cyclodextrin protons in the position 6 with the acridine protons in the positions 1, 2, 7, and 8 are significant in 2D NOESY. The ethyl group on the sulfur is in contacts with cyclodextrin protons in the position 3 and 5 in such a way that the $-\text{CH}_3$ is nearer to H-3, and $-\text{CH}_2-$ is nearer to H-5. It can be concluded that this ethyl group is inserted into narrow rim of cyclodextrin cavity. Similarly, compound BG 979 interacts with (2-hydroxypropyl)- β -cyclodextrin and (2-hydroxypropyl)- γ -cyclodextrin by insertion its phenyl ring into cyclodextrin cavity. No signs of the insertion of acridine rings were found in any of the cases. The interaction with the protonized forms is very weak; the compounds are near to the narrow rim of cyclodextrin cavity.

The Solubility of 9-Sulfanylacridines

The effect of cyclodextrins on the solubility of drugs is one of the often studied effects²⁸. Generally, the inclusion complexes with cyclodextrins are more water soluble than the original drugs²⁹. We have studied the effect of (2-hydroxypropyl)cyclodextrins on the solubility of compounds BG 375

TABLE III
The association constants of studied 9-sulfanylacridines with (2-hydroxypropyl)cyclodextrins

Compound	$K_{\text{as}}, \text{mol}^{-1}\text{l}^{\text{a}}$		
	α -CD	β -CD	γ -CD
BG 138	946	1720	587
BG 180	462	149	42
BG 980	52	459	373
BG 375	73	666	145
BG 376	36	220	74
BG 979	$-^b$	288	496
BG 463	$-^b$	312	262

^a Relative standard deviations are lower than 15%. ^b Negligible interaction.

and BG 980. Their solubility increases about six times (Fig. 6). Thus, the use of cyclodextrins as carriers of 9-sulfanylacridines would increase their biological availability.

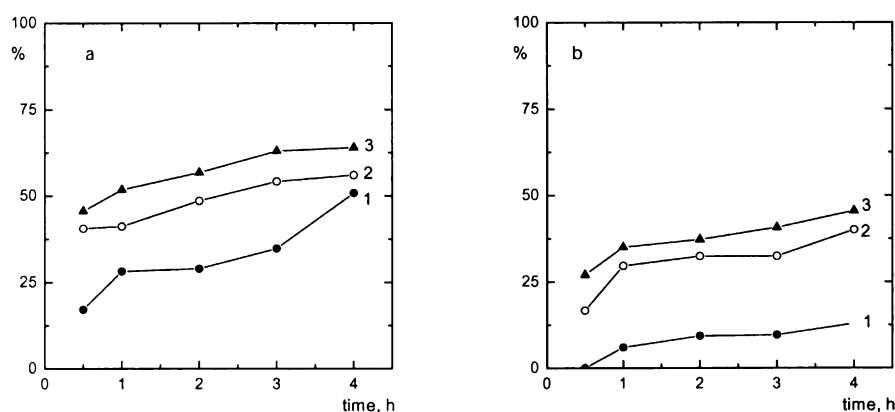


FIG. 6

The solubility of compounds BG 375 (a) and BG 980 (b) in the presence of (2-hydroxypropyl)- α -cyclodextrin (1), (2-hydroxypropyl)- β -cyclodextrin (2), and (2-hydroxypropyl)- γ -cyclodextrin (3). The solubility is related to the batch amount of compound

CONCLUSION

The dissociation constants of the studied 9-sulfanylacridines, differing in the length of alkylsulfanyl groups and with phenylsulfanyl groups, substituted in the acridine ring by methoxy or amino groups, range between 5 and 7. The effect of (2-hydroxypropyl)cyclodextrins on these values is very small (decrease in pK_a values of tenths of units). The free bases of 9-sulfanylacridines aggregate in aqueous solutions. (2-Hydroxypropyl)cyclodextrins suppress the aggregation by the formation of inclusion complexes with monomeric form of 9-sulfanylacridines. The association constants of these complexes were determined; their values depend on both the structure of the compound and on the cyclodextrin cavity size. The formation of inclusion complexes improves the solubility of studied compounds.

This work was supported by COST B 16 Action. The authors are grateful to Prof. J. Barbe, Faculty of Pharmacy, Université de la Méditerranée for the supply of studied compounds.

REFERENCES

1. Němcová I., Nesměrák K., Jelínek I., Němec I., Barbe J.: *Anal. Lett.* **2001**, *34*, 1223.
2. Nesměrák K., Němec I., Štícha M., Němcová I., Horká V.: *Anal. Lett.* **2002**, *35*, 1617.
3. Kafková B., Tesařová E., Suchánková J., Bosáková Z., Coufal P.: *J. Sep. Sci.* **2003**, *26*, 686.
4. Kutnerová B., Jelínek I., Štícha M., Němcová I.: *Anal. Lett.* **2004**, *37*, 263.
5. Barbe J., Galy A. M., Galy J. P.: *Trends Heterocycl. Chem.* **1993**, *3*, 1.
6. Cremieux A., Chevalier J., Sharples D., Berny H., Galy A. M., Brouant P., Galy J. P., Barbe J.: *Res. Microbiol.* **1995**, *146*, 73.
7. Nesměrák K., Pospíšek M., Zikánová B., Němec I., Barbe J., Gabriel J.: *Folia Microbiol. (Prague)* **2002**, *47*, 118.
8. Berny H., Bsiri N., Charbit J. J., Galy A. M., Soyfer J. C., Galy J. P., Barbe J., Sharples D., Mesa-Valle C., Mascaro C., Osuna A.: *Arzneimittel-Forsch./Drug Res.* **1992**, *42*, 674.
9. Obexer W., Schmid C., Barbe J., Galy P. J., Brun R.: *Trop. Med. Parasitol.* **1995**, *46*, 49.
10. Demeunynck M., Charmantray F., Martelli A.: *Curr. Pharm. Des.* **2001**, *7*, 1703.
11. Vogtherr M., Crimme S., Elshorst B., Jacobs D. M., Fiebig K., Griesinger C., Zahn R.: *J. Med. Chem.* **2003**, *46*, 3563.
12. Hever A., Santelli-Rouvier C., Brouant P., El Khyari S., Molnar J., Barra Y., Barbe J.: *Anticancer Res.* **1998**, *18*, 3053.
13. Castaing M., Brouant P., Loiseau A., Santelli-Rouvier C., Santelli M., Alibert-Franco S., Mahamoud A., Barbe J.: *J. Pharm. Pharmacol.* **2000**, *52*, 289.
14. Szejtli J.: *Chem. Rev.* **1998**, *98*, 1743.
15. Connors K. A.: *Chem. Rev.* **1997**, *97*, 1325.
16. Mosinger J., Tománková V., Němcová I., Zýka J.: *Anal. Lett.* **2001**, *34*, 1979.
17. Loftsson T., Brewster M. E.: *J. Pharm. Sci.* **1996**, *85*, 1017.
18. Uekama K., Hirayama F., Irie T.: *Chem. Rev.* **1998**, *98*, 2045.
19. Gould S., Scott R. C.: *Food Chem. Toxicol.* **2005**, *43*, 1451.
20. Monnaert V., Betbeder D., Fenart L., Bricout H., Lenfant A. M., Landry C., Cecchelli R., Monflier E., Tilloy S.: *J. Pharmacol. Exp. Ther.* **2004**, *311*, 1115.
21. Trapani G., Lopodota A., Boghetich G., Latrofa A., Franco M., Sanna E., Liso G.: *Int. J. Pharm.* **2003**, *268*, 47.
22. Kreaz R. M. A., Dombi G., Kata M.: *J. Inclusion Phenom. Macrocycl. Chem.* **1998**, *31*, 189.
23. Bsiri N., Johnson C., Kayirere M., Galy A. M., Galy J. P., Barbe J., Osuna A., Mesa-Valle C., Castilla-Calvente J. J., Rodrigues-Cabezas M. N.: *Ann. Pharm. Fr.* **1996**, *54*, 27.
24. Neumann M. G., Gehlen M. H.: *J. Colloid Interface Sci.* **1990**, *135*, 209.
25. Bohn P. W.: *Annu. Rev. Phys. Chem.* **1993**, *44*, 37.
26. Connors K. A.: *Binding Constants. The Measurement of Molecular Complex Stability.* John Wiley, New York 1987.
27. Hahnová A.: *M.S. Thesis.* Charles University, Prague 2005.
28. Brewster M. E., Loftsson T.: *Pharmazie* **2002**, *57*, 94.
29. Albers E., Muller B. W.: *Crit. Rev. Ther. Drug Carrier Syst.* **1995**, *12*, 311.