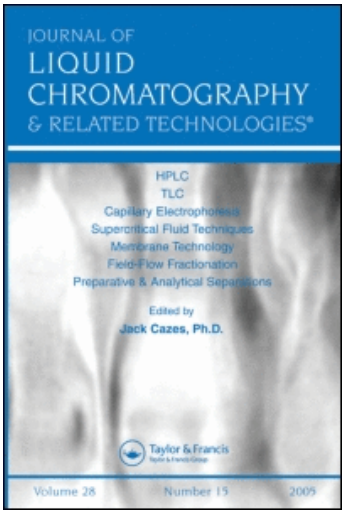

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HPLC STUDY ON ION-PAIRING ABILITY OF DEOXYCHOLIC ACID EPIMERS

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ABSTRACT

In line with the postulate that the physiological functions of bile acids, in particular their contribution to the regulation of the fat metabolism, may be related to their ion-pairing ability, this paper provides data on the chromatographic aspects related to their ion-pairing ability. The paper provides data about the chromatographic aspects of this subject. The ion-pairing (i.e. HPLC-retention-increasing) effects of the therapeutically applied deoxycholic acid epimers (7-deoxycholic acid, DOC; chenodeoxycholic acid, ChDOC; and ursodeoxycholic acid, UDOC) were studied in a reversed-phase HPLC system. As cationic partner substances, nine quaternary ammonium drug compounds were used as cationic partners The ion-pairing activities of the deoxycholic acids were found to be very strong, and much better than those of anionic ion-pairing agents such as pentanesulfonic acid and caproic acid. The natures of the binding of the DOC epimers by the C_{18} surface were also determined. While DOC and ChDOC formed double layers on the

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surface, UDOC was bound only in a monolayer. The differences in chromatographic behavior are interpreted on a stereochemical basis.

INTRODUCTION

One of the long-known physiological functions of bile acids is their role in the regulation of digestion. Their influence on the fat metabolism is mainly based on solubilization. Deoxy derivatives of cholic acid (3α,7α,12α-trihydroxy-5β-cholanic acid) as 3α,12α-dihydroxy-5β-cholanic acid (deoxycholic acid, DOC) and its epimers, the 3α,7α-(chenodeoxycholic acid, ChDOC) and 3α,7β-dihydroxy derivative (ursodeoxy-cholic acid, UDOC) have gained importance in therapy (1). The main field of indication is the inhibition of gallstone formation and the dissolution of gallstones (2–5). These drugs are also applied in the treatment of liver diseases (6) and biliary cirrhosis (7) in consequence of their hepatoprotective and anticholestatic effects (8,9). In the interpretation of the ability of UDOC and ChDOC to dissolve fat, emphasis is placed on intra- and intermolecular interactions (10) such as micelle or inclusion complex formation (11–15), other hydrophobic effects, or H-bond formation (16,17). Few data are to be found on the ion-pairing property of cholic acid or DOC (18–20), and even fewer papers deal with the relation between the ion-pairing effect and biological functions of bile acids, e.g. their influence on the biokinetics and membrane-penetrating ability of endogenous or exogenous (medicinal or alimentary) salts of biologically active cations (21,22).

The present work provides data on the ion-pairing activity of DOC and its epimers in a reversed-phase (RP) HPLC system: the RP HPLC behavior of various drug compounds often parallels their biological behavior (absorption, membrane penetration, and receptor activity) of certain drug compounds (23). Likewise, it is widely accepted that lipophilicity, a parameter frequently used in drug design (24,25) may be substituted by the chromatographic retention in an RPHPLC system, particularly on a C_{18}-based stationary phase. For our experiments, as model compounds, Quaternary ammonium (QA) drugs of different structural types were selected as model compounds for our experiments (Fig. 1); various QA drugs (e.g. anticholinergic spasmolytics) must reach the target organ (kidney, bile, etc.) following systemic application. The most probable mode of transport is via ion-pair absorption with lipophilic counter ions. Bile acids may serve as suitable partners in the ion-pairing process as they are present in high concentration in the GI tract. The ion-pairing activity of DOC and its epimers is quantified through the retention increase they cause in comparison with known anionic ion-pairing agents.

Excellent reviews of chromatographic bile acid separation were recently published by Roda et al. (26) and Scalia et al. (27) Roda et al. (28) used an HPLC/MS system for the determination of bile acids. The majority of HPLC

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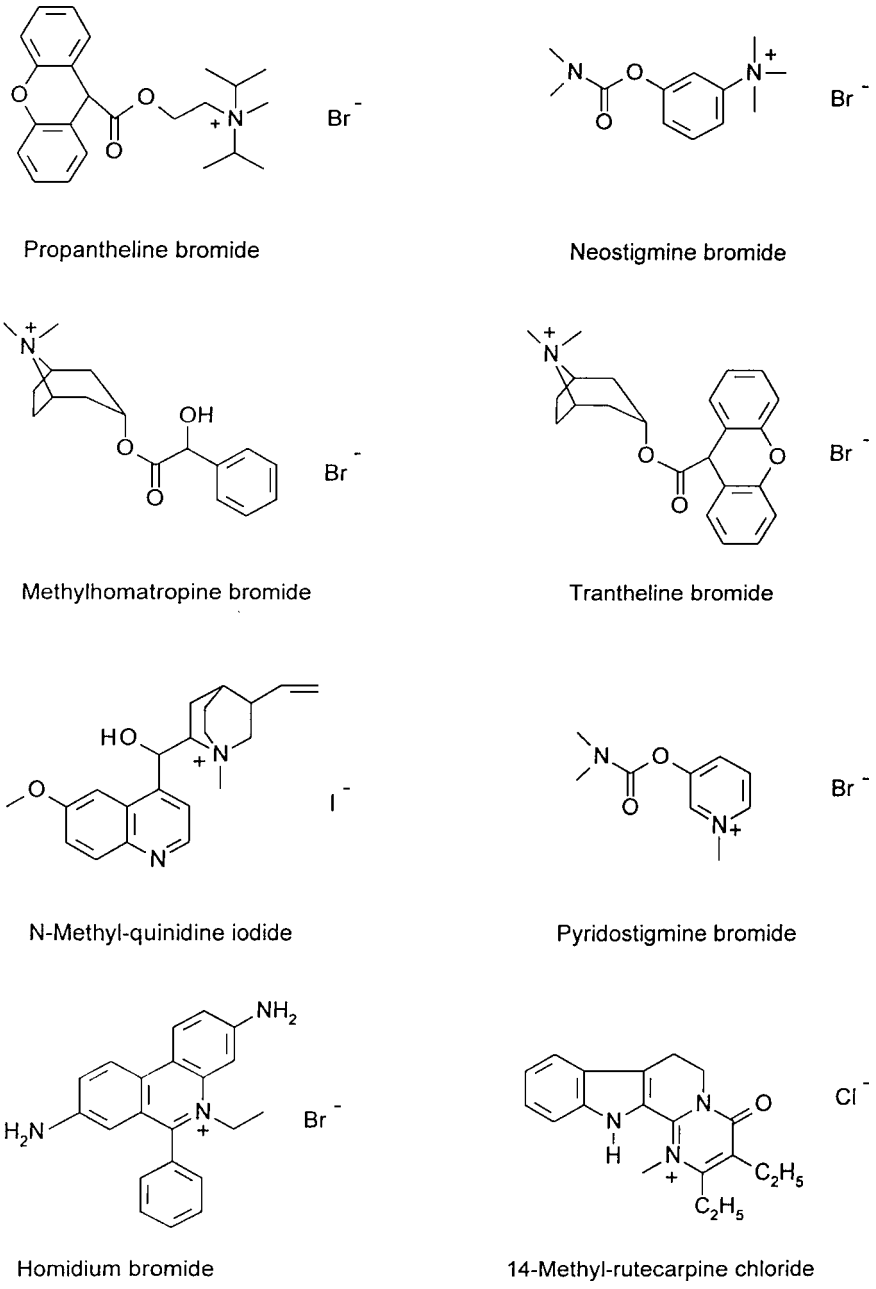

Figure 1. Formulae of the quaternary ammonium model compounds.

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methods used for DOC analysis have involved RP systems, with acetonitrile as the most commonly used organic modifier. Such conditions have been applied to study the UDOC contents of drugs and their purity profile (29,30). Scalia et al. (31,32) measured the dissolution of UDOC from drug preparations. The HPLC separation of UDOC and ChDOC was utilized in the determination of the latter as an impurity in UDOC preparations (33), on the basis of a review of the purity profile of ChDOC (34). NMR spectroscopy has proved to be a suitable method for the selective determination of DOC epimers after HPLC separation (35,36).

Since the present work involves a study of the ion-pairing activity of DOC and its epimers, the binding of the tested acids by the C_{18}-surface was first determined by the break through method (37–39). Interestingly, the chromatographic (binding) behavior on the C_{18}-surface, even relatively weak, shows differences in the epimeric structures.

EXPERIMENTAL

Chemicals

Methylhomatropine bromide (Gyógyért, Budapest); pyridostigmine bromide, neostigmine bromide, and homidium bromide samples (Sigma); propantheline bromide (Chinoin, Budapest); trantheline bromide (EGIS, Budapest); quinidine methoiodide and 14-methyl-rutecarpine, were synthesized in our laboratory and tested for identity and purity by elemental analysis and by HPLC.

Reagents

Methanol for HPLC (Chemolab, Budapest) and buffer solutions were prepared by mixing appropriate volumes of 0.025 M aqueous solutions of potassium dihydrogenphosphate and disodium hydrogenphosphate (KH_2PO_4, $Na_2HPO_4 \cdot 2H_2O$) of analytical grade (Reanal, Budapest). The pH of the solutions was tested by potentiometry with an accuracy of ± 0.02 pH unit. The water used was deionized and double distilled. Triethylamine (Sigma); Pentanesulfonic acid Na; PSA (Aldrich), Caproic acid Na (Sigma).

Chromatography

Stock solutions of the model QA compounds were prepared at a concentration of 0.001 M in methanol. 10 μL was injected onto an ISCO HPLC system consisting of a Model 2350 delivery pump and injector unit. A V^4 absorbance detector and a Hewlett Packard integrator HP 3396 Series II. were used. The

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chromatographic effluent was monitored at 220 nm. The C_{18}-column (Hypersil ODS, particle size 5 μm, 250 $\times$ 4.6 mm I.D.) was purchased from BST Ltd., Budapest. The mobile phase composition was methanol-phosphate buffer (pH 7.4 or 4.0) (60:40, v/v); flow rate 1.0 mL/min. All separations were carried out at room temperature (25 $\pm$ 2°C). Retention factors (k') were calculated via the equation $k' = t_r - t_o/t_o$, where $t_o = 2.8$ ($\pm$0.03) min. The void volume was determined from the retention time of methanol. All retention times used in the calculations are the mean of results of three individual measments. The column was brought to the initial state by washing with a methanol–water mixture (40:60, v/v) and then with methanol, each for 60 min; for eluents containing deoxycholic acids, the above washing mixture was acidified to pH 3 with phosphoric acid. The pH measurements were made with a Radiometer PHM 93 reference pH-meter, calibrated with Titrisol for buffer solutions (Merck).

Log P Determination

The apparent partition coefficients (log P') of ion-pairs were determined by a shake-flask technique in an octanol/pH 7.4 Sörensen buffer system. The experimental details have been described in a report (41) on an extensive study of the ion-pair partition of QAs with different counter ions. In that report, the effects of counter ions on the lipophilicity of QAs were investigated by using increasing concentrations of the counter ions (the molar ratio QA:Y was varied from 1:1 to 1:50). In the present paper, we present only some log P' data (Tab. 3), such as the intrinsic lipophilicity of QA drugs (in bromide salt form, molar ratio 1:1) and the log P' data on QA ion-pairs with CA and DOC at molar ratio 1:50, in order to make a comparison with their chromatographic behavior.

Calculations

The geometry for DOC and its epimers was optimized with the molecular mechanical MM+ method, followed by the semi-empirical PM3 method, using the HYPERCHEM (version 4.1) molecular modelling package. The binding data were calculated by relating the amount of ion-pairing agent bound by a specific surface area of the C_{18}-sorbent of 180 m^2/g and total filling capacity of the column, with 2.50 g.

RESULTS AND DISCUSSION

The ion-pairing effects of the phosphate ions in the eluent on the cations was first tested. The results are given in Table 1. These experiments were performed at

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Table 1. The Influence of Phosphate Ions on the Retention (log k') of QA Cations[a]

	Phosphate Concn., mM (pH = 4.0)			Phosphate Concn., mM (pH = 7.4)		
Compound[b]	25	50	100	25	50	100
Methylhomatropine	−0.721	−0.854	−0.873	−0.268	−0.387	−0.509
Pyridostigmine	−0.553	−0.770	−0.824	−0.027	−0.215	−0.367
Neostigmine	−0.602	−0.745	−0.770	−0.122	−0.252	−0.382
14-Methyl-rutecarpine	−0.305	−0.410	−0.450	0.148	0.039	−0.066
	[c]0.566	0.560	0.582	[c]0.553	0.566	0.553
N-Methyl-quinidine	−0.034	−0.201	−0.620	0.396	0.322	0.196
				[c]0.945	0.921	0.814
Homidium	0.077	−0.032	−0.051	0.371	0.306	0.212
Propantheline	0.548	0.518	0.526	1.056	0.923	0.836
Trantheline	0.563	0.494	0.486	0.992	0.856	0.779

[a]Eluent:methanol–phosphate buffer, 60:40 v/v %.
[b]Anions as in the experimental part.
[c]Second-peak formation (presumably related to the dibasic character of the model compound) is observed (see also Tabs. 2 and 5).

the physiological pH of 7.4, where the phosphate content exists mainly (≅68%) in the form of secondary phosphate, accompanied by a significant amount (≅31%) of primary phosphate anions, and also at pH 4, when $H_2PO_4^-$ is practically the only phosphate species present. It is clear, that with an increase of the phosphate concentration, there is a successive decrease in retention of the QAs. This finding suggests that the ion-pairing effect of phosphate observed earlier (40) is suppressed by the desorbing effect (change of the ionic strength, etc.) of the mobile phase. It may also be seen, that the retention decrease at pH 4 slows down at higher phosphate concentration indicating an increasing contribution of ion-pairing to the retention. Figure 2 demonstrates that phosphate ions completely replace the bromide or iodide anions of the parent compounds.

Table 2 reports the retention data on the model QAs in chromatographic systems containing DOC or one of the better-known anionic ion-pairing agents, as (PSA or CA). The retention values of the eight QAs fall into two groups: homidium and tranthelinium are bound much more firmly than the other compounds by the C_{18} surface. The increases in the retention values in the presence of the anionic ion-pairing agents are indicative of ion-pair formation. The retention-increasing effects of DOC is significantly stronger than those of the other two acids. This difference may be explained by the differences in lipophilicity of the ion-pairing agents and also by selective structural and adsorptive properties. These results demonstrate the extremely strong ion-pairing ability of DOC. It may be noted that HPLC retention parameters such as the octanol/water partition coefficients (log P') of ion-pairs measured by a shake-flask technique (41). Table 3 also indicate a

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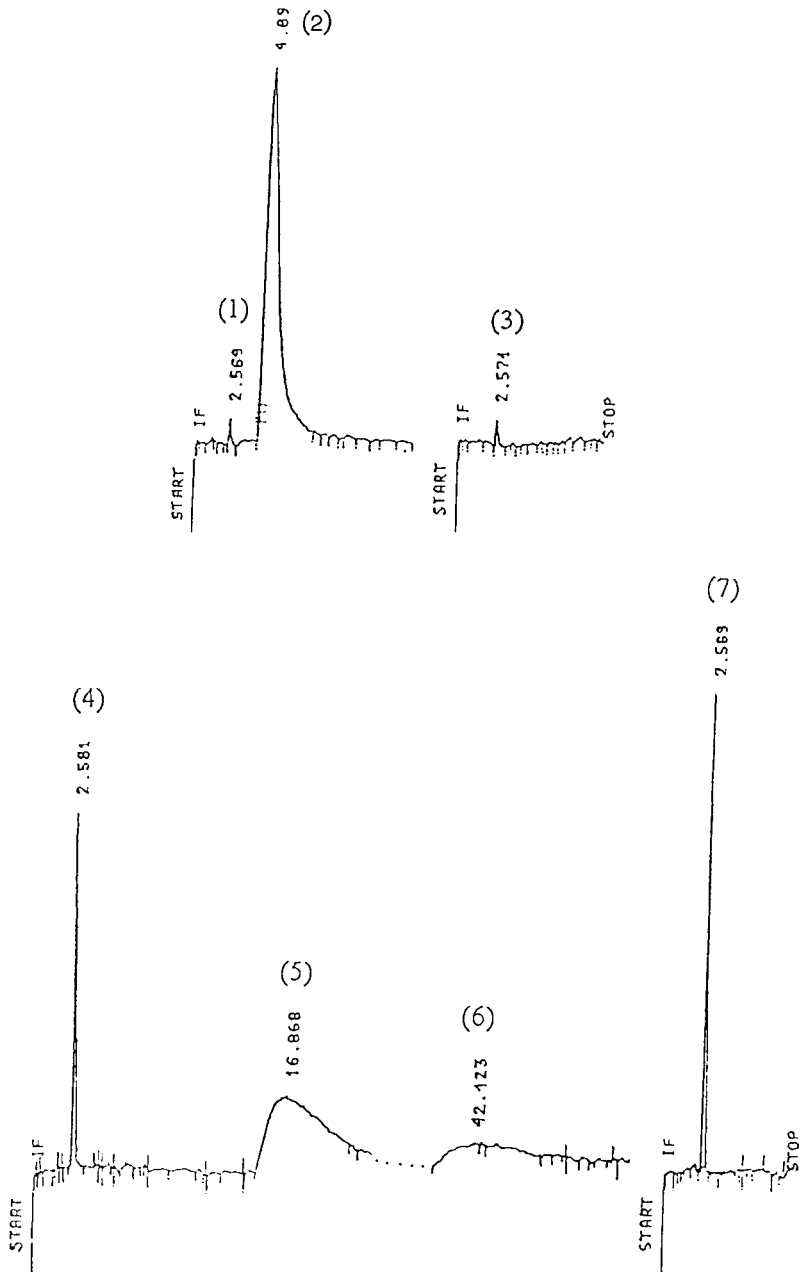

Figure 2. The release of bromide and iodide ions in response to phosphate ions. Eluent: methanol-phosphate buffer (pH = 7.4), 60:40 v/v. (1,2) Injected: Methylhomatropine bromide, 0.004 M. (3) Potassium bromide, 0.004 M. (4,5,6) Quinidine methoiodide, 0.0001 M. (7) Potassium iodide 0.0001 M. As solvent, the eluent itself was used.

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Table 2. Ion-Pairing Effects of DOC, PSA and CA[a]

	Retention, log k'			
	Ion-Pairing Agent in Eluent ($c = 0.002$ M)			
Compound[b]		DOC	PSA	CA
Methylhomatropine	−0.268	0.241	−0.268	−0.252
Pyridostigmine	−0.027	0.303	−0.066	0.009
Neostigmine	−0.122	0.303	−0.092	−0.027
14-Methylrutecarpine	0.148	0.520	0.207	0.260
	0.553	0.615	0.551	0.550
N-Methylquinidine	0.396	0.820	0.509	0.564
	0.945	1.226	0.943	1.033
Homidium	0.371	0.828	0.508	0.555
Propantheline	1.056	1.463	1.165	1.180
Trantheline	0.992	1.359	1.046	1.072

[a]Eluent:methanol–phosphate buffer (pH = 7.4), 60:40 v/v % + anionic ion-pairing agent.
[b]Anions as in the experimental part.

marked ion-pairing and, hence, a lipophilicity-increasing effect of the deoxycholate ion.

The binding data on the three deoxycholic acid epimers, PSA and CA are listed in Table 4. Unlike UDOC, ChDOC and DOC form a bilayer on the C_{18} surface, as revealed by the two-wave character of their break-through curves (Fig. 3).

Table 3. Comparative Data on Octanol/Water Partition of Quaternary Ammonium Drug Ion-Pairs (41)

Compound	Counter Ion	Molar Ratio	log P' (±SD)
Propantheline	bromide	1:1	−1.07(0.05)
	caproate	1:50	0.64(0.01)
	deoxycholate	1:50	1.14(0.10)
Trantheline	bromide	1:1	−1.45(0.02)
	caproate	1:50	0.65(0.01)
	deoxycholate	1:50	2.14(0.05)
Homidium	bromide	1:1	−1.10(0.01)
	caproate	1:50	0.48(0.01)
	deoxycholate	1:50	2.18(0.01)
Neostigmine	bromide	1:1	<-3
	caproate	1:50	−1.38(0.01)
	deoxycholate	1:50	−0.70(0.06)

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Table 4. Binding of Deoxycholic Acid Epimers by the C_{18} Surface[a,b]

	Amount Bound, μM/g (molecule/nm^2)[a]					
	DOC		ChDOC		UDOC	
Eluent	1[b]	2	1	2	1	2
A	128.5 (0.42)	4.92 (0.017)	121.4 (0.43)	3.18 (0.011)	63.84 (0.225)	–
B	13.71 (0.048)	11.27 (0.040)	13.10 (0.47)	11.88 (0.042)	14.66 (0.052)	–

[a]For principles of calculations, see the experimental.
[b]1: first layer, 2: second layer.
A:methanol–phosphate buffer (pH = 7.4), 40:60 + 0.002 M deoxycholic acid epimer. B:methanol–phosphate buffer (pH = 7.4), 60:40 + 0.002 M deoxycholic acid epimer amount of caproic acid (CA) bound: 13.64 μM; amount of pentanesulfonic acid (PSA) bound: 13.74 μM. (from methanol-phosphate buffer (pH = 7.4), 60:40 + 0.002 M CA or PSA).

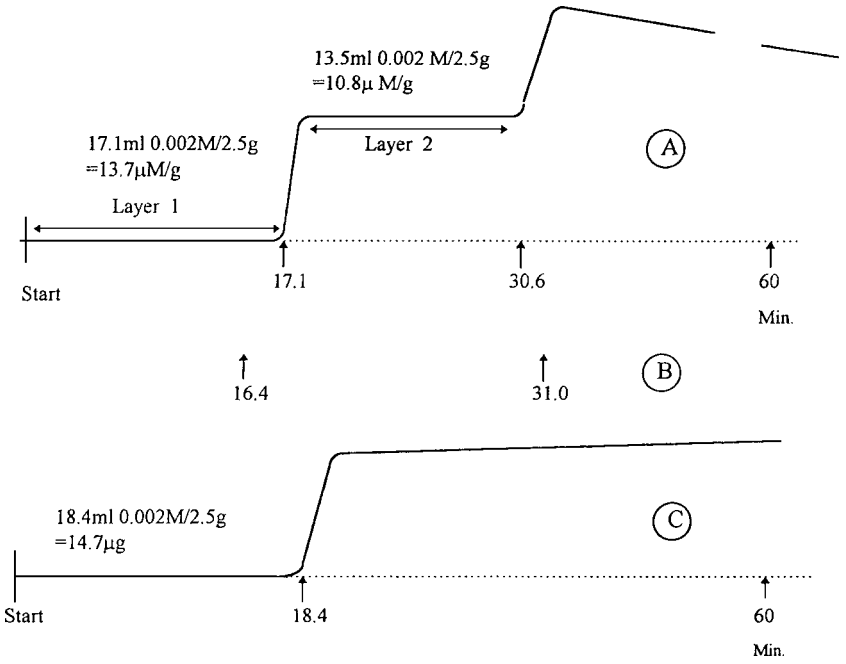

Figure 3. Break through curves of deoxycholic acid epimers. Eluent: methanol-phosphate buffer (0.025 M, pH: 7.4) 60:40 + 0.002 M A (B, C) Flow rate : 1.0 mL/min. A: DOC, B: ChDOC, C: UDOC.

The second layer is thin, relative to that of camphorsulfonic acid (CSA), reported earlier (42). It may be assumed with very high probability, that the second layer of DOC or ChDOC is formed through intermolecular H-bonding (dipole-dipole interactions) between the alcoholic OH groups and/or the OH and COO^- groups of the molecules. The much denser character of the layers of CSA (42) can be interpreted in terms of molecular size and shape differences and special interaction abilities. As expected, increase of the methanol content of the eluent leads to a very sharp reduction in the amount of ion-pairing acid bound (cf. Tab. 4/A, B). At the same time, the relative thickness of the second layer increases. While the extents of total binding of DOC and ChDOC are practically the same, the amount UDOC bound is much lower and very similar to the amounts of PSA and CA bound.

This seems to indicate, that UDOC,PSA, and CA are bound by the same sites on the C_{18}-surface, and the same appears to be true for the first layer of bound ChDOC and DOC.

These results suggest, that not only the 3α-, but also the 12α- or 7α-OH groups of the epimers are involved in the intermolecular interactions needed for bilayer formation. When the dielectric constant of the eluent is lowered, the amountof UDOC bound does not decrease so dramatically as is observed with DOC or ChDOC (Tab. 4). This indicates that UDOC molecules are bound quite strongly by the C_{18}-surface. The differences observed between the surface-binding (Tab. 4) and ion-pairing chromatographic behavior of UDOC and its two epimers (Tab. 5), are presumably caused by the different structural positions of the 7α- and 7β-OH groups (Fig. 4). The relatively strong polarity of UDOC appears to be

Table 5. Comparison of Ion-Pairing Abilities of Deoxycholic Acid Epimers[a]

	Retention, log k'			
	Deoxycholic Acid in Eluent, 2 mM			
Compound		DOC	ChDOC	UDOC
Methylhomatropine	−0.268	0.241	0.253	0.143
Pyridostigmine	−0.027	0.303	0.312	0.212
Neostigmine	−0.122	0.303	0.312	0.225
14-Methylrutecarpine	0.148	0.520	0.528	0.554
	0.553	0.615	0.618	0.658
N-Methylquinidine	0.396	0.820	0.871	0.868
	0.945	1.226	1.265	1.336
Homidium	0.371	0.828	0.808	0.825
Propantheline	1.056	1.463	1.413	1.426
Trantheline	0.992	1.359	1.394	1.384

[a]Eluent:methanol–phosphate buffer (pH = 7.4), 60:40 v/v %.

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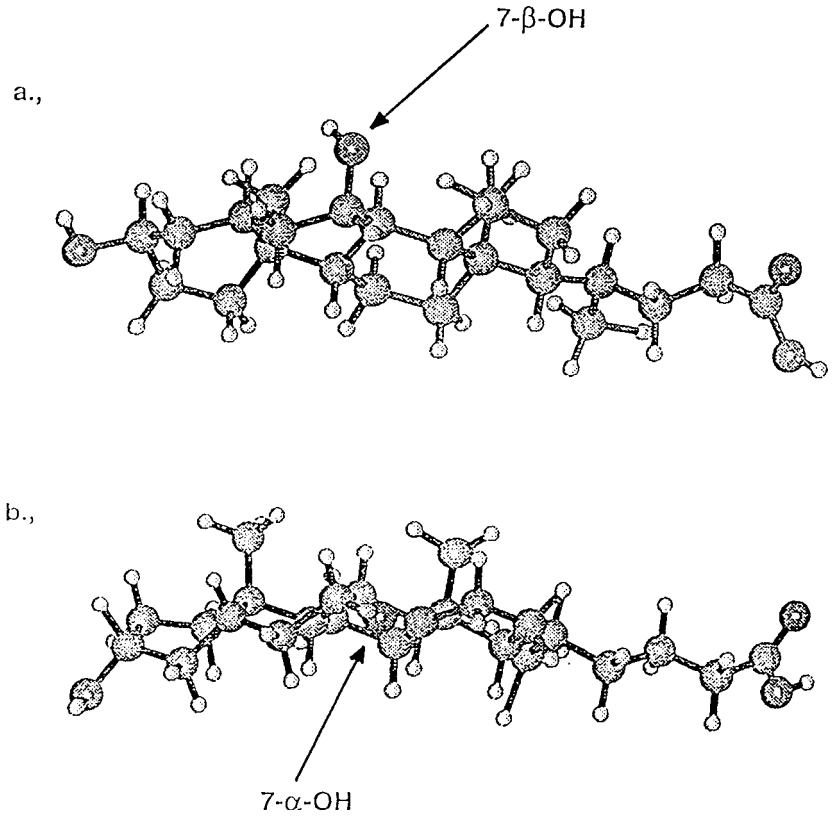

Figure 4. Stereoview of chenodeoxycholic acid (3α,7α-dihydroxy derivative) and ursodeoxycholic acid (3α,7β-dihydroxy derivative).

caused by the axial 7β-OH (Fig. 4), which, at least in the present chromatographic system, makes it unable to undergo association (i.e. double layer formation) and may also be responsible for the differences in therapeutical applicability of the deoxycholic acid epimers (6,9).

CONCLUSIONS

The results of the two indirect methods (the retention time increase in RP-HPLC system, and the partition coefficient increase in the octanol/water system) indicated a strong ion-pairing ability of these deoxycholic acids. These results allow the assumption that deoxycholic acids in vivo may significantly modify the

biokinetic behavior of certain cationic medicinal and endogenous agents. Further, the bilayer binding of DOC and ChDOC onto the C_{18}-surface suggests that these latter compounds may exert their biological functions (transport, membrane and surface activity, etc.) in associated form. The data in Table 4 shows that the double-layer forming ability of DOC and ChDOC depends on the polarity of the medium which increases strongly when the dielectric constant of the eluent is decreased.

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REFERENCES

1. Bateson, M.C. Lancet **1997**, *349*, 5.
2. Swobodnik, W.; Kuhn, K.; Janowitz, P.; Wenk, H.; Teckentrup, K.; Classen, M.D. Med. Wochenschr. **1992**, *117*, 201.
3. Fromm, H.; Malavolti, M. Bail. Clin. Gastroenterol. **1992**, *6*, 689.
4. Pereira, S.P.; Veysey, M.J.; Kennedy, C.; Hussaini, S.H.; Murphy, G.M.; Dowling, R.H. Dig. Dis. Sci. **1997**, *42*, 1775; C.A. **1997**, *127*, 257396 r.
5. Tazuma, S.; Kajiyama, G.; Mizuno, T.; Yamashita, G.; Miura, H.; Kajihara, T.; Hattori, Y.; Miyake, H.; Nishioka, T.; Hyogo, H.; Sunami, Y.; Yasumiba, S.; Ochi, H.; Matsumoto, T.; Abe, A.; Adachi, K.; Omata, F.; Ueno, F.; Sugata, F.; Ohguri, S.; Shibata, H.; Kokubu, S. J. Clin. Gastroenterol. **1998**, *26*, 287.
6. Saksena, S.; Tandon, R.K. Postgrad. Med. J. **1997**, *73*, 75.
7. Poupon, R.E.; Bonnand, A.M.; Chretien, Y.; Poupon, R. Hepatology **1997**, *26*, 936.
8. Heuman, D.M. Gastroenterology **1993**, *104*, 1865.
9. Levy, S.; Capron, J.P.; Erlinger, S. Gastroenterol. Clin. Biol. **1996**, *20*, 549.
10. Lamcharfi, E.; Cohen-Solal, C.; Parquet, M.; Lutton, C.; Dupre, J.; Meyer, C. Eur. Biophys. J. **1997**, *25*, 285.
11. Kano, K.; Tatemoto, S.; Hashimoto, S. J. Phys. Chem. **1991**, *95*, 966.
12. Jou, W.S.; Chun, P.W. J. Mol. Graphics **1991**, *9*, 237.
13. Chiu, Y.C.; Yu, K.M. J. Disp. Sci. Technol. **1992**, *13*, 587.
14. Ahlheim, M.; Hallensleben, M.L. Makromol. Chem.-Macromol. Chem. Phys. **1992**, *193*, 779.
15. Limmatvapirat, S.; Yamaguchi, K.; Yonemochi, E.; Oguchi, T. Acta Crstallogr. Section C. **1997**, *53*, 803.
16. Lessinger, L.; Low, B.W. J. Crystallogr. Spectroscop. Res. **1993**, *23*, 85.

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17. Gyimesi, J.; Barcza, L. Magy. Kém. Folyóir. **1991**, *97*, 206; C.A. **1991**, *115*, 92702 x.
18. Boonkerd, S.; Detaevernier, M.R.; Michotte, Y.; Vindevogel, J. J. Chromatogr. A **1995**, *704*, 238.
19. Shim, C.K.; Nishigaki, R.; Iga, T.; Hanans, M. Int. J. Pharm. **1981**, *8*, 143.
20. Neubert, R.; Fischer, S. J. Pharm. Pharmacol. **1991**, *43*, 204.
21. Gasco, M.R.; Trotta, M.; Eandi, M. J. Pharm. Biomed. Anal. **1984**, *2*, 425.
22. Polli, J.E.; Amidon, G.L. J. Pharm. Sci. **1995**, *84*, 55.
23. Shalaby, A.; Budvári-Bárány, Z.; Szász, G. J. Liq. Chromatogr. **1985**, *8*, 1093.
24. van de Waterbeemd, H.; Kansy, M.; Wagner, B.; Fischer, H. In *Lipophilicity in Drug Action and Toxicology*; Pliska, V., Testa, B., van de Waterbeemd, H., Eds.; VCH: Basel, 1996.
25. Kaliszan, R.; Nasal, A.; Bucinski, A. Eur. J. Med. Chem. **1994**, *29*, 163.
26. Roda, A.; Piazza, F.; Baraldini, M. J. Chromatogr. B **1998**, *717*, 263.
27. Scalia, S. J. Chromatogr. B - Biomed. Appl. **1995**, *671*, 299.
28. Roda, A.; Cerre, C.; Simoni, P.; Polimeni, C.; Vaccari, C.; Pistillo, A. J. Lipid Res. **1992**, *33*, 1393.
29. Scalia, S.; Guarneri, M.; Menegatti, E. Farmaco **1991**, *46*, 713.
30. Roda, A.; Gatti, R.; Cavrini, V.; Cerre, C.; Simoni, P. J. Pharm. Biomed. Anal. **1993**, *11*, 751.
31. Scalia, S.; Giunchedi, P.; Conte, U.; Pazzi, P. Arch. Pharm. **1995**, *328*, 363.
32. Giunchedi, P.; Scalia, S.; Maggi, L.; Conte, U. Int. J. Pharm. **1996**, *130*, 41.
33. Bosquet, E.; Santagati, N.A.; Tirendi, S. J. Liq. Chromatogr. & Rel. Technol. **1997**, *20*, 757.
34. van Straelen, P.; de Jong, H.A.P.; Jansen, F.A. Lancet **1974**, 1518.
35. Bhattacharyya, P.K.; Bankawala, Y.G. Anal. Chem. **1978**, *50*, 1462.
36. Bailletguffroy, A.; Baylocq, D.; Pellerin, F.; Rabaron, A. J. Pharm. Sci. **1984**, *73*, 847.
37. Knox, J.H.; Hartwick, R.A. J. Chromatogr. **1981**, *204*, 3.
38. Bartha, Á.; Vigh, G. J. Chromatogr. **1983**, *260*, 337.
39. Löre, A.; Budvári-Bárány, Z.; Szász, G.; Shalaby, A. J. Liq. Chromatogr. **1991**, *14*, 2065.
40. Hancock, W.S.; Bishop, B.L.; Prestidge, R.L.; Harding, D.R.K.; Hearn, M.T.W. J. Chromatogr. **1978**, *153*, 391.
41. Takács-Novák, K.; Szász, G. Pharm. Res. **1999**, *16*, 1637.
42. Löre, A.; Budvári-Bárány, Z.; Szász, G. J. Liq. Chromatogr. **1991**, *14*, 2065.

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