Journal of Chromatography, 268 (1983) 237–244
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,020

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF SOME DIGITALIS GLYCOSIDES AND THEIR AGLYCONES ON CHEMICALLY BONDED PHASES

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(Received April 20th, 1983)

SUMMARY

The retention behaviour of some glycosides and their aglycones was studied as a function of the composition of the acetonitrile–water eluent. The relationship between the retention constant (log k') and the volume fraction of acetonitrile in the eluent was parabolic and of shape indicating a "dual" retention mechanism for all solutes studied. This behaviour is discussed in terms of the nature of the solute, eluent and stationary phase.

INTRODUCTION

At the present time, high-performance liquid chromatography (HPLC) using chemically bonded phases is probably the most popular chromatographic method. This is due to the high efficiency of the method, its wide range of applications and the simple eluting solvents used, despite the incomplete understanding of the mechanism of the retention. Several studies have been reported on the variation of sample capacity factor (k') values in reversed-phase liquid chromatography (RPLC), as a function of mobile phase composition[1–27]. For a wide range of sample compounds, it is observed that for a given system the k' values are related to the volume fraction, v_{org}, of organic solvent in the binary mobile phase:

$$\log k' = \log k_w - m\, v_{org} \quad (1)$$

Here k_w refers to the k' value for pure water as mobile phase, and is usually an extrapolated value. Several studies (*e.g.*, refs. 2, 7, 10, 12, 14, 16–18 and 20) have shown that eqn. 1 is not strictly linear. Schoenmakers *et al.*[25,26] suggested that eqn. 1 is generally invalid, and k' as a function of v_{org} is better represented by the quadratic expression:

$$\log k' = Av_{org}^2 + Bv_{org} + C \quad (2)$$

Eqn. 2 had been derived earlier, actually in the same way, for normal phase liquid–liquid chromatography by Jandera and Churáček[28]. Assuming that the quadratic

0021-9673/83/$03.00

term can be ignored, to a first approximation, they obtained eqn. 1. Only one paper[27] has interpreted the retention mechanism in RPLC analogously to that in liquid–solid chromatography[29–31].

The former studies of the retention mechanism in RPLC leave some questions open, *e.g.*, the nature of the relationship between k' values and mobile phase composition and the effect of solute chemical structure on retention. Considering glycosides and their aglycones which are sufficiently different in molecular structure, we examined the retention behaviour on non-polar octadecyl-silica (C_{18}) and polar cyanopropyl-silica (C_3CN) phases using acetonitrile–water eluents of different compositions.

EXPERIMENTAL

A Waters Assoc. liquid chromatograph Model ALC/GPC 244 (Waters Assoc., Milford, MA, U.S.A.) equipped with a model 440 UV detector and a R 401 differential refractometer was employed. The chromatograms were obtained with a Model M 730 Data Module.

The eluent was acetonitrile (Riedel-de Haën, Hannover, F.R.G.), pure or mixed with twice distilled water in various proportions. The flow-rate was 1 ml/min and the column temperature was maintained at 25°C. The mobile phase was prefiltered through a 0.5-μm Millipore filter (Millipore, Bedford, MA, U.S.A.).

The tridigitoxides and aglycones tested were obtained from Aldrich (Milwaukee, WI, U.S.A.), and lanatosides were prepared in our laboratory[32,33]. The solutions were prepared in dimethyl sulphoxide (DMSO) (Fluka, Buchs, Switzerland) and prefiltered through a 0.5-μm Millipore filter.

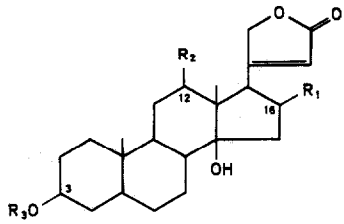
Double commercial columns (each 30 cm × 3.9 mm I.D.), 10-μm μBondapak C_{18} and μBondapak CN (Waters Assoc.), were used.

RESULTS AND DISCUSSION

The digitalis glycosides and their aglycones examined are given in Table I and the corresponding k' values are given in Table II. The effect of the solvent composition on the solute retention is shown in Fig. 1; similar behaviour was obtained for other solutes studied. As illustrated, the relationship between log k' and the volume fraction of acetonitrile, v_{MeCN}, in the eluent mixtures used was not linear and, on both bonded phases examined, showed a shape indicating the "dual" retention mechanism, explained for large solute molecules and silica-bonded hydrocarbonaceous stationary phases by Horváth and co-workers[23,24]. In pure acetonitrile and in water-lean mixtures the groups of solutes (lanatosides, tridigitoxides and aglycones) were eluted in the order characteristic for normal phase liquid–solid chromatography on "naked" silica, *i.e.*, the most polar lanatosides were mostly retained. The elution order on both octadecyl-silica (Fig. 1a) and cyanopropyl-silica (Fig. 1c) with eluent mixtures containing about 20–50% of water was typical for reversed-phase chromatography. The lanatosides were eluted first. The retention behaviour of solutes of the same series in water-rich solvent mixtures was unusual on both bonded phases, as has been mentioned earlier[34]. In general, the more polar lanatosides and tridigitoxides were retained more strongly than corresponding aglycones (Fig. 1a,c). The explanation for

TABLE I

CHEMICAL STRUCTURES OF DIGITALIS GLYCOSIDES AND AGLYCONES INVESTIGATED



Group	*Compound*	*Series*	*Substituent*		
			R_1	R_2	R_3*
Lanato-	Lanatoside A (LA)	A	H	H	G–AcD–D–D–
sides	Lanatoside B (LB)	B	OH	H	G–AcD–D–D–
	Lanatoside C (LC)	C	H	OH	G–AcD–D–D–
Tridigi-	Digitoxin (Dx)	A	H	H	D–D–D–
toxides	Gitoxin (Gx)	B	OH	H	D–D–D–
	Digoxin (Dgx)	C	H	OH	D–D–D–
Agly-	Digitoxigenin (Dx-G)	A	H	H	H
cones	Gitoxigenin (Gx-G)	B	OH	H	H
	Digoxigenin (Dgx-G)	C	H	OH	H

* D = Digitoxose; AcD = acetyldigitoxose; G = glucose.

TABLE II

CAPACITY RATIOS, k', OF GLYCOSIDES AND THEIR AGLYCONES ON C_{18} AND C_3CN PACKINGS

Compound	*Acetonitrile concentration, % (v/v)*									
	25	*30*	*35*	*40*	*45*	*50*	*55*	*60*	*80*	*100*
C_{18} phase										
Lanatoside A			11.83	6.25	3.42	2.00	1.27	0.81	0.51	1.34
Lanatoside B			4.53	2.68	1.63	1.05	0.72	0.55	0.43	1.34
Lanatoside C			1.79	1.27	0.92	0.69	0.54	0.43	0.36	1.34
Digitoxin			17.00	9.16	5.12	3.05	1.96	1.27	0.60	1.13
Gitoxin			6.40	3.78	2.30	1.48	1.02	0.72	0.52	1.13
Digoxin			2.33	1.66	1.20	0.90	0.71	0.57	0.44	1.13
Digitoxigenin			7.33	4.50	3.02	2.14	1.55	1.12	0.63	0.77
Gitoxigenin			2.88	1.97	1.43	1.09	0.84	0.66	0.55	0.77
Digoxigenin			1.33	1.05	0.85	0.69	0.58	0.51	0.48	0.77
C_3CN phase										
Lanatoside A	7.06	3.74	2.34	1.60	1.12	0.77		0.59	0.54	1.24
Lanatoside B	4.19	2.48	1.58	1.15	0.81	0.62		0.51	0.50	1.24
Lanatoside C	2.26	1.46	1.07	0.82	0.63	0.51		0.44	0.45	1.24
Digitoxin	7.19	4.11	2.90	1.90	1.32	0.96		0.68	0.58	1.03
Gitoxin	4.19	2.55	2.00	1.35	1.00	0.77		0.62	0.55	1.03
Digoxin	2.40	1.75	1.35	1.05	0.83	0.68		0.57	0.50	1.03
Digitoxigenin	4.14	2.96	2.11	1.70	1.34	1.10		0.84	0.64	0.80
Gitoxigenin	2.50	1.89	1.43	1.21	1.00	0.87		0.69	0.60	0.80
Digoxigenin	1.57	1.29	1.06	0.94	0.82	0.74		0.65	0.58	0.80

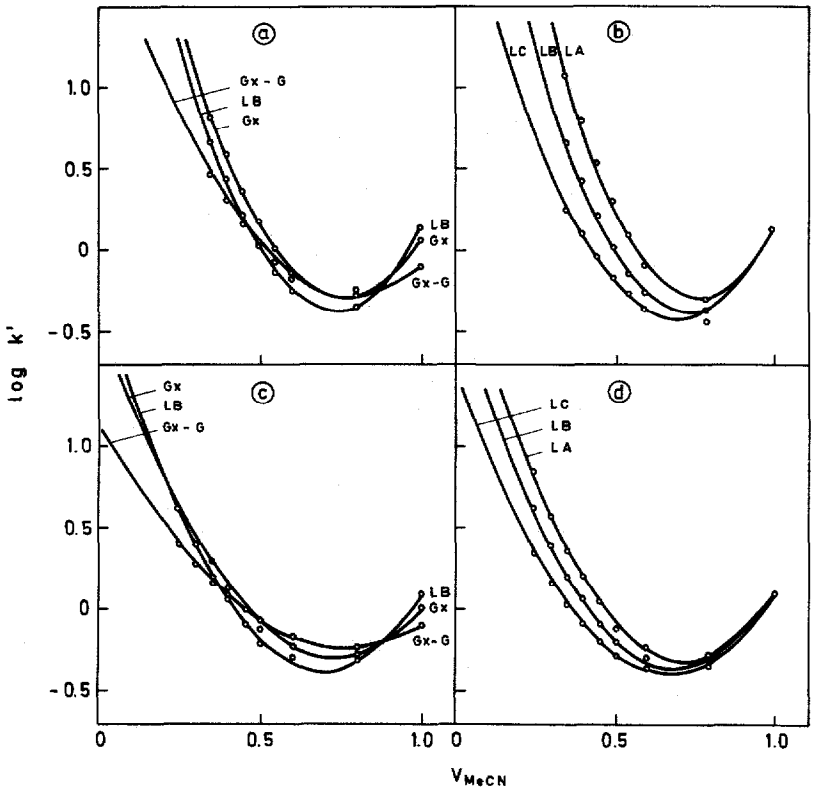


Fig. 1. Plots of the logarithmic retention factor, log k', against the volume fraction of acetonitrile, v_{MeCN}, in the acetonitrile–water mixture obtained on octadecyl-silica (a,b) and cyanopropyl-silica (c,d) stationary phases. Designation of samples as in Table I.

this behaviour could lie in the hydrophobicity of the carbohydrate function of glycosides. Obviously, the hydrophobic moiety of digitoxose units in the carbohydrate function of glycosides is responsible for their enhanced retention in water-rich eluent mixtures in comparison with corresponding aglycones. Therefore, tridigitoxides were retained more strongly on both bonded phases than corresponding lanatosides containing a glucose unit in the carbohydrate function. In each group of compounds, particular solutes were eluted in the sequence characteristic for reversed-phase chromatography over the whole concentration range studied; in pure acetonitrile, solutes of this group were eluted as one peak (Fig. 1b,d).

The experimental k' values (Table II) fit very well eqn. 2 over the whole range of eluent concentrations used. The solid lines in Fig. 1 drawn through the data points represent a quadratic relationship between log k' and v_{MeCN}, from which the coefficients A, B and C, collected in Table III, were derived. In practice, good separation of solute compounds could be expected for k' values between 1 and 10. In our experiments, such k' values were obtained with both bonded phases at water concentrations higher than 40%. For example, complete separation of all solutes on the C_{18} phase was attained when the water content was 60% (Fig. 2), and on the C_3CN phase the best separation was obtained with 68% water (Fig. 3). In this region the

TABLE III

COEFFICIENTS FOR THE QUADRATIC RELATIONSHIP BETWEEN log k' AND ν_{MeCN} (EQN. 2)

Compound	*C_{18} phase*				*C_3CN phase*			
	A	*B*	*C*	*S.D.**	*A*	*B*	*C*	*S.D.*
Lanatoside A	7.84	−12.08	4.37	0.019	5.29	−7.56	2.38	0.019
Lanatoside B	7.18	−10.55	3.49	0.010	4.93	−6.83	2.00	0.015
Lanatoside C	5.88	− 8.24	2.46	0.024	4.34	−5.77	1.52	0.008
Digitoxin	6.72	−10.94	4.26	0.013	4.53	−6.76	2.25	0.013
Gitoxin	6.43	− 9.85	3.48	0.013	4.02	−5.82	1.81	0.020
Digoxin	5.06	− 7.39	2.36	0.006	3.39	−4.75	1.37	0.009
Digitoxigenin	4.27	− 7.27	2.89	0.005	2.63	−4.22	1.50	0.009
Gitoxigenin	3.96	− 6.21	2.14	0.012	2.34	−3.57	1.14	0.008
Digoxigenin	3.20	− 4.70	1.38	0.007	1.82	−2.67	0.75	0.004

* Standard deviation $= \sqrt{\frac{\Sigma[\log k' (\text{exp}) - \log k' (\text{calc})]^2}{n}}$

TABLE IV

COEFFICIENTS FOR THE LINEAR RELATIONSHIP BETWEEN log k' AND ν_{MeCN} (EQN. 1)

Compound	*C_{18}*			*C_3CN*		
	m	*log k_w*	*S.D.**	*m*	*log k_w*	*S.D.*
Lanatoside A	4.61	2.64	0.025	3.74	1.73	0.037
Lanatoside B	3.70	1.91	0.034	3.27	1.39	0.034
Lanatoside C	2.47	1.10	0.019	2.53	0.94	0.027
Digitoxin	4.49	2.76	0.029	3.45	1.68	0.025
Gitoxin	3.80	2.10	0.027	2.90	1.31	0.026
Digoxin	2.46	1.20	0.019	2.19	0.91	0.013
Digitoxigenin	3.21	1.95	0.023	2.29	1.16	0.022
Ditoxigenin	2.54	1.32	0.018	1.83	0.83	0.020
Digoxigenin	1.68	0.70	0.015	1.31	0.50	0.014

* Standard deviation calculated as in Table III.

TABLE V

EXPERIMENTAL (REF. 34)* AND CALCULATED (EQNS. 1 AND 2) k' VALUES

Compound	*k'*		
	Ref. 34	*Eqn. 1*	*Eqn. 2*
Lanatoside A	8.60	8.60	9.30
Lanatoside B	3.10	3.50	3.70
Lanatoside C	1.30	1.50	1.64
Digitoxin	13.80	12.55	13.50
Gitoxin	4.90	4.95	5.20
Digoxin	1.95	1.95	2.10
Digitoxigenin	7.15	5.80	6.03
Gitoxigenin	2.65	2.40	2.45
Digoxigenin	1.30	1.20	1.21

* Stationary phase: octadecyl-silica. Mobile phase: 37% acetonitrile in water.

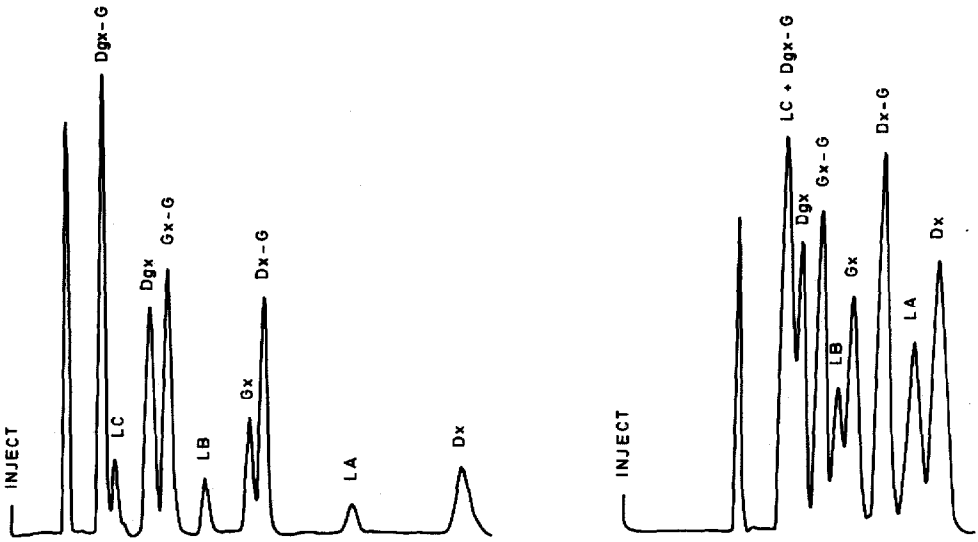


Fig. 2. Separation of glycosides and aglycones tested. Column: 10-μm μBondapack C_{18} (60 cm × 3.9 mm I.D.). Mobile phase: 40% acetonitrile in water; flow-rate, 1 ml/min. Designation of samples as in Table I.

Fig. 3. Separation of glycosides and aglycones tested. Column: 10-μm μBondapak CN; (60 cm × 3.9 mm I.D.), mobile phase: 32% acetonitrile in water; flow-rate 1 ml/min. Designation of samples as in Table I.

experimental k' values also fit eqn. 1 (Table IV). The standard deviations (Tables III and IV) between the experimental data and those calculated by means of eqns. 1 and 2 are very low, as can also be seen from Fig. 1. Comparing experimental RPLC k' data[34] with those calculated by eqns. 1 and 2, excellent agreement is obtained (Table V).

For analysis of the effect of solute molecular structure on retention, the use of eqn. 1 is more simple and convenient than that of eqn. 2. Therefore, we used the slope m values from Table IV for this purpose. Dolan *et al.*[19] generally concluded

TABLE VI

SLOPE DIFFERENCES, Δm, FOR FUNCTIONAL GROUP CONVERSION

Phase	*Solute group*	Δm		*Solute series*	Δm	
		12H → *12OH*	*16H → 16OH*		*3OH → 3OD₃*	*3OH → 3OD₂AcDG*
C_{18}	Lanatosides	−2.14	−0.91	A	1.28	1.40
	Tridigitoxides	−2.03	−0.69	B	1.26	1.16
	Aglycones	−1.53	−0.67	C	0.78	0.79
C_3CN	Lanatosides	−1.21	−0.47	A	1.16	1.45
	Tridigitoxides	−1.26	−0.55	B	1.07	1.44
	Aglycones	−0.98	−0.46	C	0.88	1.22

that the coefficient m is related to the strength of a pure organic modifier as mobile phase, and that for typical samples and a given reversed-phase system little variation in m among the constituents of the sample is to be expected. They pointed out that when the organic modifier is either methanol or acetonitrile the value of m is usually about 3. The mean m values for the C_{18} and C_3CN phases are 3.22 and 2.61, respectively, but from Table IV it is evident that m varies markedly with the number, type and position of substituents in the solute molecule on both bonded phases. In each solute group a decrease in solute polarity was followed by an increase in slope m. 12β-Hydroxy derivatives are more polar and have lower slopes than 16β-hydroxy derivatives due to the steric hindrance of the 16β-hydroxy function with the lactonic ring. The effect of a particular substituent on m is presented in Table VI. Substitution of the 12β-hydrogen by a hydroxyl group decreases the slope m of solutes containing a carbohydrate function more than for aglycones. The presence of a carbohydrate function in the 3β-position of lanatosides and tridigitoxides causes a decrease in polarization of the 12β-hydroxyl group in comparison with the corresponding aglycones, due to the effect of the hydrophobic moiety of the digitoxose unit. The following observation can be explained by the same phenomenon. Namely, the introduction of a carbohydrate function in the 3β-position results in an increase in m in comparison with the corresponding aglycones. For solutes in series A and B, this increase is equal or higher than for solutes in series C on both bonded phases.

On the basis of the above discussion, one can consider the similar retention behaviour of solute compounds studied on both octadecyl and cyanopropyl silica, but careful inspection of Fig. 1 and Tables IV and VI shows that different retention mechanisms operate on these phases. Namely, the coefficients m of lanatosides and tridigitoxides eluted on the non-polar octadecyl phase are the same which means that a change in the mobile phase composition is not followed by a significant change in the stationary phase composition. However, the constant m of lanatosides obtained on the polar cyanopropyl bonded phase is about 0.3 units higher than for tridigitoxides, *i.e.*, with increasing water content in the mobile phase the retention of lanatosides also increases due to the permanent change in the composition of the stationary phase. Consequently, by binding water the stationary phase becomes more polar, but still retains all of its original hydrophobic properties. By increasing the polarity of the stationary phase, the less polar tridigitoxides overtake lanatosides, and in pure water the elution order could be expected to be the same as in pure acetonitrile.

Finally, we can conclude that in RPLC on chemically bonded phases the shape of the relationship between log k' and v_{org} is generally parabolic in accordance with eqn. 2. Only the part of the parabola, usually lying in the water-rich concentration region, can be considered as linear according to eqn. 1. The position of the parabola minimum along the concentration axis is determined for a defined chromatographic system by the nature of the solute compound. For solutes of high hydrophobicity, *e.g.*, alkanes[12], higher alcohols[7,12], higher monocarboxylic acids[12], various aromatic compounds[15,25,26], the parabola minimum usually lies out of the real eluent concentration range, but for solutes having a high content of both hydrophilic and hydrophobic constituents, *e.g.*, crown ethers[23,24], peptides[20], glycosides, the parabola minimum occurs in the real eluent concentration range. The movement of the parabola minimum depends on the ratio of hydrophilic to hydrophobic constituents in a solute molecule, *i.e.*, a higher content of hydrophilic constituents shifts the minimum toward

pure water. This is obvious from $(v_{org})_{min} = -B/2A$. For example, $(v_{org})_{min}$ of lanatoside A, B and C on the octadecyl-silica phase is equal to 0.77, 0.73 and 0.70, respectively; on the polar cyanopropyl-silica phase the corresponding values are 0,71, 0.69 and 0.66, *i.e.*, increasing the stationary phase polarity shifts the parabola minimum closer to pure water. This behaviour is established for all groups and series of solutes studied. On the basis of the experiments of Schoenmakers *et al.*[25] we can conclude that the position of the parabola minimum for a defined solute depends also on the total polarity of the eluent system. By decreasing the total eluent polarity the parabola minimum shifts toward pure water, contrary to the behaviour in normal phase chromatography[35], *e.g.*, $(v_{org})_{min}$ values of phenol for aqueous methanol, ethanol and propanol on the octadecyl-silica phase were 1.47, 1.19 and 0.86, respectively. The first two values lie out of the real eluent concentration range (see ref. 25, Fig. 2). Thus, the polarity of the solute, the stationary phase and the eluent determine the position of the parabola minimum in RPLC on chemically bonded phases.

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