Journal of Inclusion Phenomena and Macrocyclic Chemistry **38:** 453–465, 2000.

Effect of Gamma-Cyclodextrin, Methanol and pH on the Parameters of Reversed-Phase Thin-Layer Chromatography of Some Antisense Nucleotides

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(Received: 1 February 2000; in final form: 11 April 2000)

Abstract. The binding of twelve 8-substituted-2′-deoxyadenosine and seventeen 5-substituted-2′-deoxyuridine derivatives with gamma-cyclodextrin (γ-CD) was studied by reversed-phase thin-layer chromatography in acidic and alkaline environments. The relative strengths of the binding were calculated and compared with those determined in an ion-free environment. The binding strength of the antisense nucleosides depended considerably on both the chemical structure of the nucleosides and on the pH of the environment.

Key words: antisense nucleosides, γ-CD, pH effect.

1. Introduction

Antisense nucleosides are promising pharmaceutical agents and their use in human health care can be expected in the future [1]. Antisense nucleosides were effective versus *Herpex simplex* virus [2] displaying antiherpetic activity [3]. The synthesis and antiviral activity of L-2′-deoxy-2′up-fluoro-4′-thionucleosides [4], isodideoxy-nucleosides with a furan-ethanol sugar moiety [5], and a ring-expanded purine nucleoside with a 5:7-fused, planar aromatic, imidazo[4,5-E] [1, 3] diazepine ring system [6] have been recently reported.

In order to increase the efficacy of the active ingredient in pharmaceutical formulations a wide variety of compounds were employed as adjuvants for antiviral nucleosides. The beneficial effect of an amphiphilic peptide [7], cationic lipid particles [8] and cationic polyhexylcyanoacrylate nanoparticles [9] was demonstrated. However, the adverse effect of cationic lipids as carriers has also been observed [10]. Cyclodextrins (CDs) are cyclic oligosaccharides which can bind both inorganic and organic molecules into the CD cavity [11, 12] forming so-called inclusion complexes. The formation of inclusion complexes may considerably modify the biological efficacy and pharmacokinetic parameters of the complexed molecule. Thus, it can enhance stability [13], modify the decomposition rate

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[14], and improve the delivery [15] and penetration rate [16], etc. CDs and CD derivatives have also been used for the formulation of nucleosides [17, 18]. Chromatographic methods have been frequently used for the study of molecular interactions [19]. High performance liquid chromatography [20], free solution capillary electrophoresis [21], gas-liquid chromatography [22], and reversed-phase thin-layer chromatography [23] have all been employed for determination of molecular interactions.

The objectives of the study was the determination of the interaction of some antisense nucleosides with γ-CD, and the assessment of the effect of pH on the strength and selectivity on the formation of inclusion complexes. The study was motivated by the fact that the pH of the environment may exert a marked effect on the stability of the inclusion complexes influencing in this manner the biological efficiency of the guest molecule [24]. The elucidation of the formation of inclusion complexes between antisense nucleosides and γ-CD may promote the development of new, more effective pharmaceutical formulations with higher biological efficiencies and lower toxic side effects.

2. Experimental

Reversed-phase RP-18W/UV_{254} plates (Macherey-Nagel, Dürren, Germany) were used for the determination of the relative strength of interaction without any pretreatment. Gamma-CD was purchased from CYCLOLAB Research and Development Laboratory (Budapest, Hungary) and was used as received. The IUPAC names of the nucleosides are compiled in Table I. They were synthesized by Dr. G. Ságі at the Chemical Research Center of Hungarian Academy of Sciences (Budapest, Hungary). Their purity was checked by reversed-phase high-performance liquid chromatography and was found to be over 95% in each instance. The solutes were dissolved in methanol at a concentration of 5 mg/mL, and 4 μL of the solutions were spotted separately on the plates. As the object was the study of the interaction between nucleosides and γ-CD and not the elucidation of the influence of γ-CD on their separation, the nucleosides were separately spotted on the plates. Mobile phases were water: methanol mixtures, the methanol concentration varying between 0–95 vol.%. in steps of 5 vol.%. The use of this wide range of methanol concentration was motivated by the greatly different lipophilicity of the antisense nucleosides. Methanol was chosen as organic modifier because it forms only weak complexes with CDs [25, 26]. The concentration of γ-CD in the mobile phase varied between 0 and 50 mg/mL in steps of 12.5 mg/mL. As the biological activity of the complexes may occur in both acidic and alkaline environments each mobile phase contained either acetic acid or sodium acetate at 0.16 M end concentrations. Developments were carried out in sandwich chambers (22 $\times$ 22 $\times$ 3 cm) at room temperature, with the distance of development being about 16 cm. After development the plates were dried at 105 °C and the spots of solutes were revealed under a UV lamp. Each experiment was run in quadruplicate. The R_M value characterizing

Table I. IUPAC names of the nucleosides used

No of compound	IUPAC name
1	2′-Deoxyuridine
2	Thymidine
3	2′-Deoxy-5-ethyluridine
4	2′-Deoxy-5-*n*-propyluridine
5	2′-Deoxy-5-isopropyluridine
6	2′-Deoxy-5-*n*-butyluridine
7	2′-Deoxy-5-*n*-pentyluridine
8	2′-Deoxy-5-*n*-hexyluridine
9	2′-Deoxy-5-*n*-heptyluridine
10	2′-Deoxy-5-*n*-octyluridine
11	2′-Deoxy-5-*n*-tetradecyluridine
12	2′-Deoxy-5-ethynyluridine
13	2′-Deoxy-5-(1-pentyn-1-yl)-uridine
14	2′-Deoxy-5-(1-hexyn-1-yl)-uridine
15	2′-Deoxy-5-(1-heptyn-1-yl)-uridine
16	2′-Deoxy-5-(1-octyn-1-yl)-uridine
17	2′-Deoxy-5-(1-decy-1-yl)-uridine
18	2′-Deoxyadenosine
19	2′-Deoxy-8-ethyladenosine
20	2′-Deoxy-8-*n*-propyladenosine
21	2′-Deoxy-8-*n*-pentyladenosine
22	2′-Deoxy-8-*n*-heptyladenosine
23	(Z)-2′-Deoxy-8-(propen-1-yl)-adenosine
24	(Z)-2′-Deoxy-8-(1-penten-1-yl)-adenosine
25	(Z)-2′-Deoxy-8-(1-hepten-1-yl)-adenosine
26	2′-Deoxy-8-ethynyladenosine
27	2′-Deoxy-8-(propyn-1-yl)-adenosine
28	2′-Deoxy-8-(1-pentyn-1-yl)-adenosine
29	2′-Deoxy-8-(1-heptyn-1-yl)-adenosine

the molecular hydrophobicity in reversed-phase thin-layer chromatography was calculated for each solute in each eluent:

$$R_M = \log(1/R_f - 1). \tag{1}$$

When the coefficient of variation of the parallel determinations was higher than 5% the R_M value was omitted from the following calculations. To separate the

effects of methanol and γ-CD on the lipophilicity of the nucleosides the following equation was fitted to the experimental data:

$$R_M = R_{M0} + b_1 \cdot C_1 + b_2 \cdot C_2, \tag{2}$$

where R_M is the R_M value for a nucleoside determined at given methanol and γ-CD concentrations; R_{M0} is the R_M value extrapolated to zero methanol and γ-CD concentrations; b_1 is the decrease in the R_M value caused by a 1% increase in the methanol concentration in the eluent; b_2 is the decrease in the R_M value caused by a 1 mg/mL concentration change of γ-CD in the eluent (related to the relative strength of interaction); C_1 and $C2$ are the concentrations of methanol and γ-CD, respectively. Equation (2) was applied separately for each nucleoside in acidic and alkaline environments.

In order to compare the relative strength of binding determined under different conditions linear correlations were calculated between the b_2 values measured in alkaline, acidic and ion-free environments:

$$b_{2i} = A + B \cdot b_{2j}, \tag{3}$$

where b_{2i} and b_{2j} are the relative strength of interactions determined in any two environments, A and B are the intercept and slope values. The significant deviation of the intercept (A) from zero and the slope (B) from 1 was assessed by employing the "*t*" test. The significant deviation of A from zero and B from 1 was considered as indicators that the strength of binding in the two environments are different. When no significant linear regression was found between the two sets of b_2 values, the selectivity of the systems was considered to be different. The b_2 values determined in an ion-free environment were taken from reference 27.

3. Results and Discussion

The simultaneous effect of methanol and γ-CD concentrations on the R_M values of nucleosides **2** and **10** in acidic and alkaline environments are shown in Figures 1 and 2, respectively. The R_M values of these antisense nucleosides decreased with increasing concentration of methanol in the mobile phase, i.e., the nucleosides do not show any anomalous retention behaviour that would invalidate the evaluation using Equation (2). An increase in the γ-CD concentration caused a decrease or increase in R_M values indicating the formation of inclusion complexes between γ-CD and nucleosides. The data indicate that the interaction of γ-CD with the antisense nucleosides modifies the lipophilicity of the latter: in the case of highly hydrophobic nucleosides it decreases the apparent lipophilicity and in the case of highly hydrophilic nucleosides the complexation with γ-CD increases the apparent lipophilicity. This result suggests that the pharmacological properties (adsorption, uptake, half-life, etc.) of the nucleoside – γ-CD complex may be different from

those of the uncomplexed compound resulting in modified effectivity. The parameters of Equation (2) calculated for acidic and alkaline environments are compiled in Tables II and III, respectively. Blank sites in Tables II and III indicate that in these instances the binding of the nucleosides to γ-CD cannot be proven. This result suggests that the relative strength of interaction is weak and it is under the detection limit of the method. The equation fits the experimental data well, the significance levels in each instance being over 99% (see calculated F values). The ratios of variance explained varied between 63 and 99% (see r^2 values). The interaction of antisense nucleosides with γ-CD means that in pharmaceutical formulations containing both nucleosides and γ-CD their interaction must be taken into consideration. The parameters in Tables II and III show marked variations proving that the capacity of nucleosides to form complexes with γ-CD differ considerably. This result further suggests that the complex formation may influence differently the biological activity of individual antisense nucleosides. The path coefficients (b'% values) indicate that the effect of the change of methanol and γ-CD concentrations exert a similar impact on the mobility of nucleosides under reversed-phase chromatographic conditions, indicating that the retention can be equally modified by changing either the methanol or the γ-CD concentration in the mobile phase.

No significant linear relationships were found between the relative strengths (b_2 values of Equation (2)) of nucleoside – γ-CD interaction determined in acidic and alkaline environments (r = 0.2656) and in acidic and ion-free environments (r = 0.1940). This finding indicates that the pH exerts a significant influence on the strength of interaction, the effect depending on the chemical structure of the antisense nucleoside. A significant linear relationship was found between the b_2 values determined in alkaline and ion-free environments (Figure 3), the variance explained being fairly low (16.80%). The slope and intercept values of Equation (3) significantly deviated from 1 and from zero, respectively. This finding indicates that the acidic and/or alkaline character of the environment influences not only the strength of interaction but also its selectivity.

The marked impact of pH on the strength and selectivity of binding of antisense nucleosides to γ-CD can be tentatively explained by the supposition that the dissociable polar substructures of nucleosides contribute to the formation of inclusion complexes. They probably interact with the hydrophilic hydroxyl groups of γ-CD outside of the apolar cavity by electrostatic binding forces. As the degree of dissociation of the polar substructures depends on the pH of the environment and it may depend on the chemical structure of the nucleoside, the modification of pH modifies also the strength and selectivity of interaction.

It can be concluded from the data that nucleosides readily form complexes with γ-CD. The pH of the environment exerts a marked impact on both the strength and selectivity of binding. As the complex formation may modify the physicochemical parameters of the guest nucleoside molecule the effect of pH has to be taken into consideration as an important factor influencing the biological efficiency of the inclusion complex.

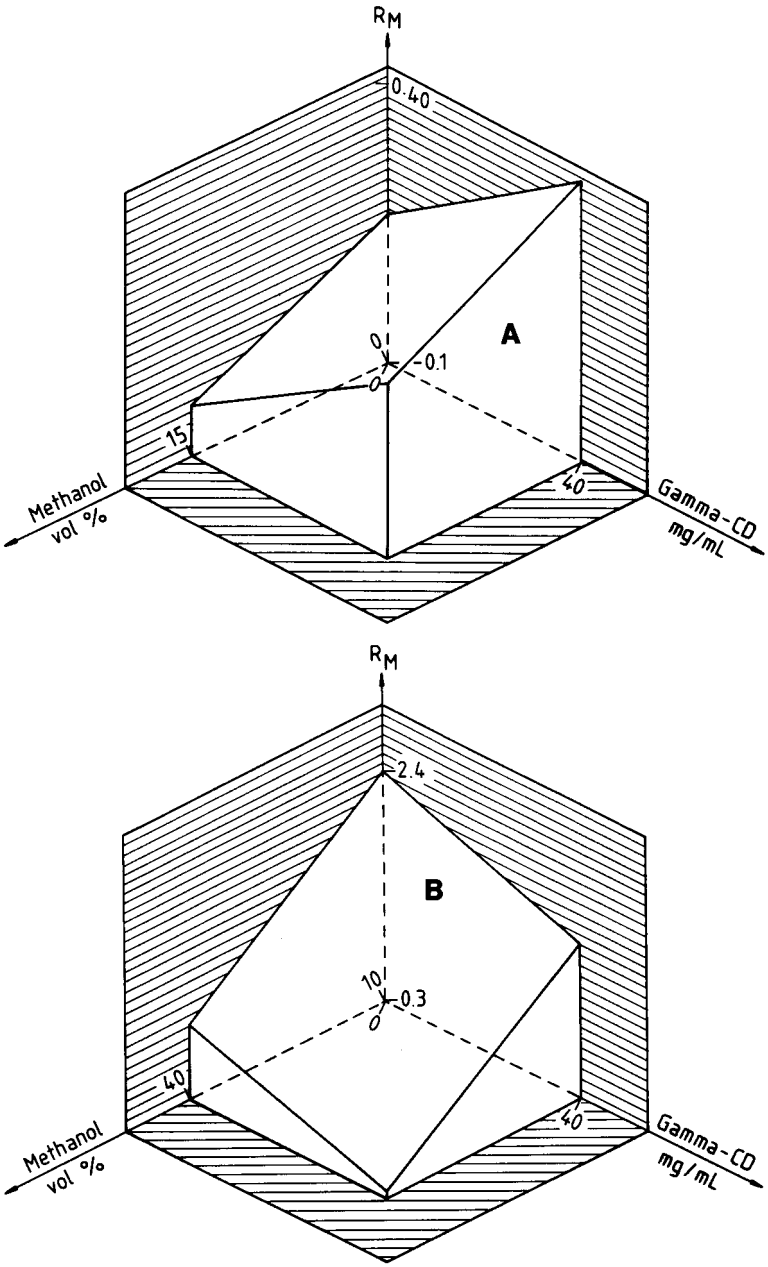

Figure 1. Effect of methanol and gamma-cyclodextrin (γ-CD) concentrations on the R_M value of nucleoside **2** in alkaline (A) and acidic environments (B).

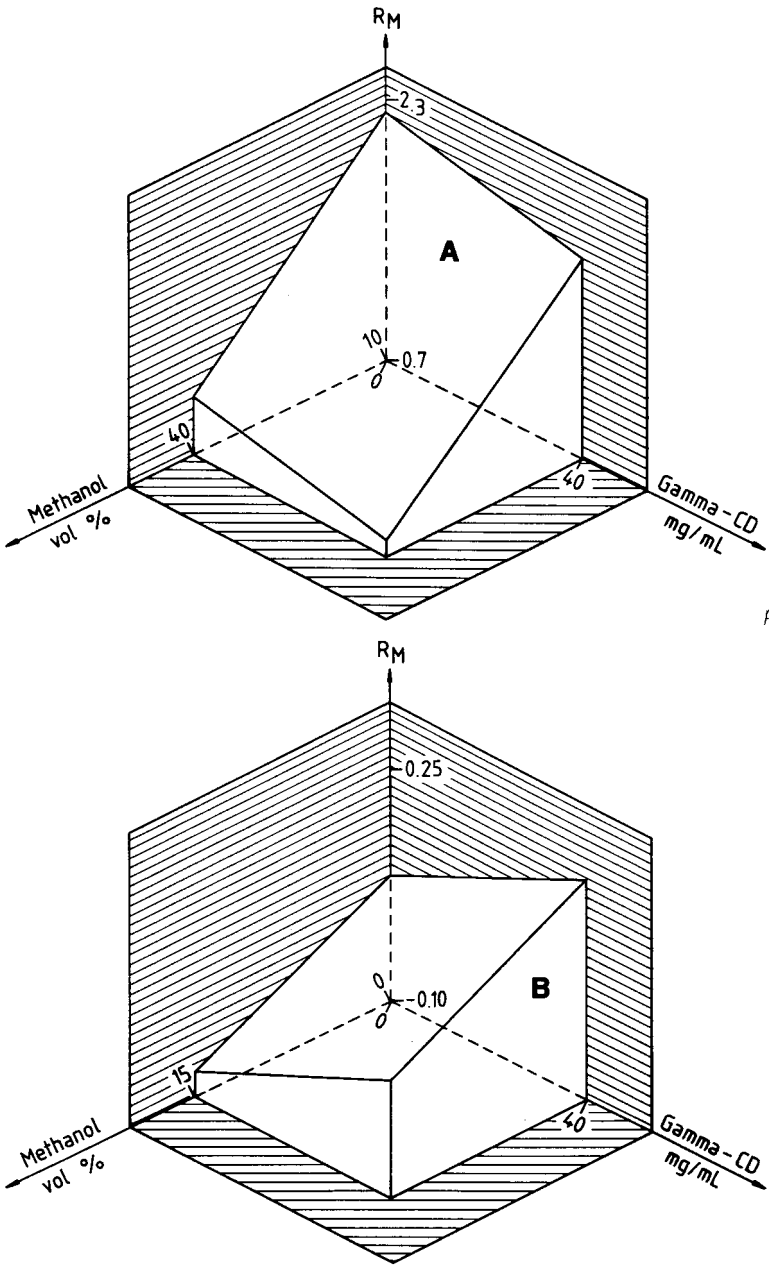

Figure 2. Effect of methanol and gamma-cyclodextrin (γ-CD) concentrations on the R_M value of nucleoside **10** in alkaline (A) and acidic environments (B).

Table II. Parameters of linear correlations between the R_M values of antisense nucleosides and the concentrations of methanol (C_1) and gamma-cyclodextrin (C_2) in the eluent (0.16 M end concentration of acetic acid). Numbers refer to the antisense nucleosides in Table I. ($R_M = R_{M0} + b_1.C_1 + b_2.C_2$)

Parameter	No of antisense nucleosides				
	1	**2**	**3**	**4**	**5**
R_{M0}	−0.15	0.09	0.48	0.67	0.68
$-b_1.10^2$	−0.70	−0.98	−0.96	−2.43	−2.73
$s_{b1}.10^3$	0.11	0.12	0.15	0.34	0.26
$-b_2.10^2$	0.33	0.35	−0.39	−0.39	−0.29
$s_{b2}.10^3$	0.10	0.12	0.11	0.13	0.10
b_1'%	66.14	73.38	71.26	70.37	78.38
b_2'% 33	86.26	62.28	74.29	63.21	67.
r^2	0.8105	0.8685	0.8127	0.8908	0.9420
$F_{\text{calc.}}$	21.38	36.33	30.37	28.56	56.86
Parameter	No of antisense nucleosides				
	6	**7**	**8**	**9**	**10**
R_{M0}	0.90	1.32	1.72	2.11	2.43
$-b_1.10^2$	−2.66	−3.11	−3.45	−3.67	−3.51
$s_{b1}.10^3$	0.27	0.22	0.19	0.20	0.22
$-b_2.10^2$	−0.55	−0.98	−1.05	−1.26	−1.66
$s_{b2}.10^3$	0.24	0.26	0.23	0.23	0.28
b_1'%	82.98	76.09	76.75	74.50	67.86
b_2'%	17.02	23.91	23.25	25.50	32.14
r^2	0.9215	0.9545	0.9708	0.9767	0.9743
$F_{\text{calc.}}$	52.85	115.42	182.65	210.09	151.94
Parameter	No of antisense nucleosides				
	11	**12**	**13**	**14**	**15**
R_{M0}	4.54	0.18	1.01	1.37	1.87
$-b_1.10^2$	−5.52	−2.42	−0.89	−2.82	−3.80
$s_{b1}.10^3$	0.22	0.19	0.13	0.12	0.29
$-b_2.10^2$	−0.33	–	−1.06	−1.17	−1.52
$s_{b2}.10^3$	0.13	–	0.18	0.12	0.35
b_1'%	94.40	–	53.96	70.73	71.38
b_2'%	5.60	–	46.04	29.27	28.62
r^2	0.9841	0.9188	0.9299	0.9824	0.9428
$F_{\text{calc.}}$	340.77	158.40	53.09	278.26	90.74

Table II. Continued.

Parameter	No of antisense nucleosides				
	16	**17**	**18**	**19**	**20**
R_{M0}	2.31	2.17	0.63	1.26	1.46
$-b_1.10^2$	−3.99	−0.83	−0.81	−2.75	−2.88
$s_{b1}.10^3$	0.26	0.24	0.15	0.12	0.15
$-b_2.10^2$	−1.91	−0.90	−0.26	−0.82	−1.02
$s_{b2}.10^3$	0.31	0.14	0.11	0.12	0.14
$b_1'\%$	67.67	47.92	76.01	77.09	73.90
$b_2'\%$	32.33	52.08	23.99	22.91	26.10
r^2	0.9713	0.8725	0.6962	0.9819	0.9751
$F_{\text{calc.}}$	152.01	23.95	17.19	271.10	195.74
Parameter	No of antisense nucleosides				
	21	**22**	**23**	**24**	**25**
R_{M0}	1.82	2.53	1.80	2.51	1.32
$-b_1.10^2$	−3.04	−3.41	−3.04	−3.74	−2.88
$s_{b1}.10^3$	0.24	0.32	0.10	0.22	0.17
$-b_2.10^2$	−1.06	−1.62	−1.02	−1.59	−1.02
$s_{b2}.10^3$	0.28	0.38	0.10	0.26	0.15
$b_1'\%$	74.23	67.74	74.88	70.22	73.78
$b_2'\%$	25.77	32.26	25.12	29.78	26.22
r^2	0.9428	0.9444	0.9894	0.9867	0.9688
$F_{\text{calc.}}$	90.65	67.94	468.75	260.10	155.08
Parameter	No of antisense nucleosides				
	26	**27**	**28**	**29**	
R_{M0}	1.24	1.36	2.05	2.43	
$-b_1.10^2$	−3.10	−3.07	−3.56	−3.64	
$s_{b1}.10^3$	0.11	0.12	0.24	0.37	
$-b_2.10^2$	−0.92	−0.93	−1.05	−1.60	
$s_{b2}.10^3$	0.11	0.15	0.23	0.46	
$b_1'\%$	77.12	76.67	77.27	69.50	
$b_2'\%$	22.88	23.33	22.73	30.50	
r^2	0.9876	0.9845	0.9665	0.9540	
$F_{\text{calc.}}$	399.38	348.47	129.90	83.04	

Table III. Parameters of linear correlations between the R_M values of antisense nucleosides and the concentrations of methanol (C_1) and gamma-cyclodextrin (C_2) in the eluent (0.16 M end concentration of sodium acetate). Numbers refer to antisense nucleosides in Table I. ($R_M = R_{M0} + b_1.C_1 + b_2.C_2$)

Parameter	No of antisense nucleosides				
	1	**2**	**3**	**4**	**5**
R_{M0}	0.55	0.16	0.63	0.78	0.82
$-b_1.10^2$	−2.36	−1.22	−2.54	−2.48	−2.52
$s_{b1}.10^3$	0.16	0.54	0.21	0.27	0.28
$-b_2.10^2$	–	0.61	−0.37	−0.52	−0.67
$s_{b2}.10^3$	–	0.21	0.16	0.21	0.24
b_1'%	–	44.46	83.68	78.58	76.24
b_2'%	–	55.54	16.32	21.42	23.76
r^2	0.9340	0.6345	0.9252	0.8433	0.8334
$F_{\text{calc.}}$	226.51	10.41	80.34	43.06	42.54
Parameter	No. of antisense nucleosides				
	6	**7**	**8**	**9**	**10**
R_{M0}	1.10	1.48	1.95	2.21	2.58
$-b_1.10^2$	−2.75	−3.06	−3.49	−3.36	−3.73
$s_{b1}.10^3$	0.25	0.27	0.28	0.34	0.19
$-b_2.10^2$	−0.84	−1.08	−1.16	−0.97	−0.73
$s_{b2}.10^3$	0.26	0.29	0.29	0.35	0.27
b_1'%	76.68	73.94	75.00	77.51	83.53
b_2'%	23.32	26.06	25.00	22.49	16.47
r^2	0.9384	0.9373	0.9496	0.9512	0.9603
$F_{\text{calc.}}$	68.58	67.28	84.81	68.30	205.54
Parameter	No. of antisense nucleosides				
	11	**12**	**13**	**14**	**15**
R_{M0}	4.69	0.23	1.31	1.67	1.97
$-b_1.10^2$	−5.56	−1.27	−3.00	−3.14	−3.34
$s_{b1}.10^3$	0.25	0.15	0.29	0.29	0.31
$-b_2.10^2$	−0.52	0.35	−0.76	−0.68	−0.68
$s_{b2}.10^3$	0.18	0.12	0.28	0.27	0.29
b_1'%	88.46	78.17	79.72	82.25	83.11
b_2'%	11.54	21.83	20.28	17.75	16.89
r^2	0.9881	0.8971	0.9114	0.9280	0.9383
$F_{\text{calc.}}$	290.57	61.03	61.70	64.44	68.45

Table III. Continued.

Parameter	No. of antisense nucleosides				
	16	**17**	**18**	**19**	**20**
R_{M0}	2.23	3.34	1.01	1.75	1.88
$-b_1.10^2$	−3.26	−4.68	−2.91	−3.73	−3.68
$s_{b1}.10^3$	0.36	0.09	0.20	0.17	0.13
$-b_2.10^2$	−0.71	−1.21	−0.70	−0.89	−0.99
$s_{b2}.10^3$	0.29	0.10	0.21	0.18	0.14
$b_1'\%$	82.15.79	50.80	58.80	80.78	86.
$b_2'\%$	17.85.20	50.19	42.19	20.21	14.
r^2	0.8597	0.9971	0.9629	0.9834	0.9957
$F_{\text{calc.}}$	42.88	1714.17	116.94	266.04	460.35
Parameter	No. of antisense nucleosides				
	21	**22**	**23**	**24**	**25**
R_{M0}	2.11	3.21	2.16	3.01	1.73
$-b_1.10^2$	−3.01	−4.40	−3.47	−4.25	−3.50
$s_{b1}.10^3$	0.25	0.11	0.19	0.12	0.17
$-b_2.10^2$	−0.48	−0.63	−0.65	−0.68	−0.57
$s_{b2}.10^3$	0.22	0.11	0.16	0.12	0.16
$b_1'\%$	86.22	87.54	84.20	86.19	85.95
$b_2'\%$	13.78	12.46	15.80	13.81	14.05
r^2	0.9269	0.9917	0.9664	0.9905	0.9625
$F_{\text{calc.}}$	82.43	962.10	172.57	834.84	205.18
Parameter	No. of antisense nucleosides				
	26	**27**	**28**	**29**	
R_{M0}	1.58	1.68	2.36	2.91	
$-b_1.10^2$	−3.72	−3.41	−4.05	−4.10	
$s_{b1}.10^3$	0.21	0.28	0.12	0.15	
$-b_2.10^2$	−0.93	−0.86	−0.44	−0.49	
$s_{b2}.10^3$	0.22	0.26	0.08	0.15	
$b_1'\%$	80.01	79.81	90.20	89.35	
$b_2'\%$	19.99	20.19	9.80	10.65	
r^2	0.9761	0.9421	0.9925	0.9842	
$F_{\text{calc.}}$	183.90	81.39	595.58	499.66	

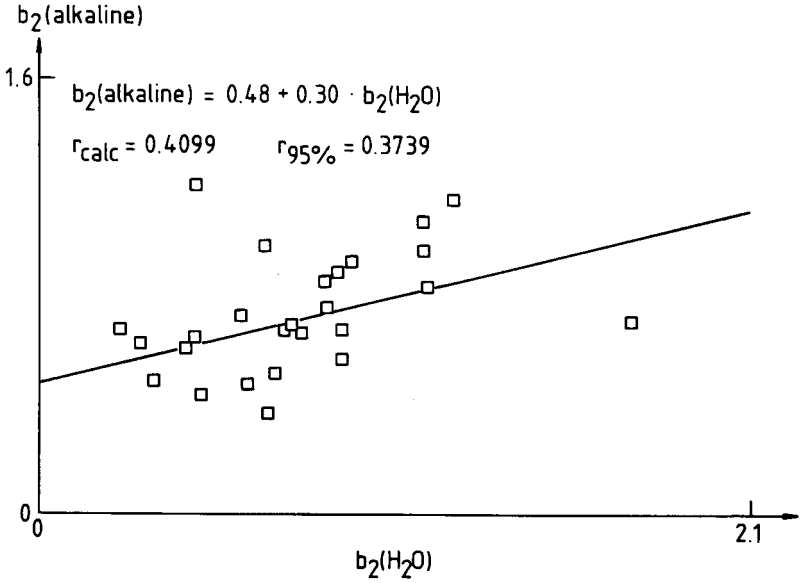

Figure 3. Linear relationship between the relative strength of nucleoside – gamma-cyclodextrin interactions determined in alkaline (b_2(alkaline)) and ion-free ($b_2(H_2O)$) environments.

4. Acknowledgement

This work was supported by the grant OTKA T 023422.

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