Journal of Chromatography A, 661 (1994) 77–82
Elsevier Science B.V., Amsterdam

CHROMSYMP. 2904

Determination of peptide hydrophobicity parameters by reversed-phase high-performance liquid chromatography

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ABSTRACT

The log k_w values of fourteen potential fibrinogen receptor antagonist peptides (RGDX) determined by reversed-phase HPLC were correlated to hydrophobic parameters of the amino acid side-chain log P in position X of the tetrapeptides. Comparing the polymer columns with LiChrosorb RP-8, the correlation coefficient using a polyethylene column is higher (0.94) than that for RP-8 (0.88), which demonstrates the importance of a homogeneous hydrophobic surface and makes this method very suitable for the determination of the overall hydrophobicity of shorter peptides. The hydrophobicity parameters log k_w of the RGDX peptides (−1.15 to 2.19) were used to investigate the influence of molecular parameters of X on the potency of RGDX in inhibiting platelet aggregation. The results confirm the importance of hydrophobicity for the contribution of X to the biological activity of RGDX.

INTRODUCTION

In addition to the steric and electronic properties, hydrophobicity has a strong influence on the biological activity of drugs. Many studies of quantitative structure–activity relationships (QSAR) are based on the relation between bioactivity and hydrophobicity [1,2]. As suggested by Hansch and Fujita [3], this influence may be due to the transport processes and/or hydrophobic ligand–receptor interactions. Usually, the partition coefficient between 1-octanol and water (log P) is accepted as a hydrophobic parameter and a reference system because of its analogy with biomembranes [4]. An important limitation of QSAR studies of peptides is the lack of reliable sets of structural descriptors. However, the traditional shake-flask method for log P determination causes experimental problems [5] and therefore alternative methods for estimating hydrophobicity indices have been investigated [6,7].

Reversed-phase HPLC has been used for this purpose, and several studies have described a correlation between log P (n-octanol/water) and

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SSDI 0021-9673(93)E0839-M

the logarithm of the capacity factors (log k') by using alkyl-bonded silica as the stationary phase [8]. In more recent studies the hydrophobicity parameters derived by polycratic methods have shown a better correlation with log P than chromatographic retention data obtained under monocratic conditions [9,10]. In order to suppress the effect of the organic co-solvent, one approach is to use the isocratic capacity factors (log k') extrapolated to 100% water (log k_w). Linear plots of log k' *vs.* organic modifier content were obtained by using methanol as the organic modifier [11]. However, the presence of residual surface silanols of alkyl-bonded silica phases causes interactions with basic groups, leading to additional non-hydrophobic interactions with peptides [12].

Organic polymer-based stationary phases [13,14] as well as polymer-coated silica phases [15,16] have been shown to be excellent alternatives to alkyl-bonded silica. The polymer phases are stable with eluents from pH 1 to 14, and the homogeneous non-polar surface leads to a retention behaviour different to that of the alkyl-bonded silica phases [17,18].

An attractive approach for the pharmacological inhibition of platelet aggregation is focused on small molecule antagonists derived from RGD sequences. Charon *et al.* [19] studied the influence of different amino acids of RGDX tetrapeptides on platelet aggregation. These results show an increasing inhibitory potency with an increasing hydrophobicity in position X.

The aim of the present study was to compare the chromatographic hydrophobicity parameters of RGDX tetrapeptides using alkyl-bonded silica, poly(styrene–divinylbenzene) and polyethylene [20] as stationary phases. Furthermore, a relation between the determined log k_w values and the bioactivity of the peptides in a platelet aggregation test, using multiple regression, was established.

EXPERIMENTAL

HPLC conditions

The HPLC system consisted of two pumps (Jasco Model 880), a UV detector (Shimadzu SPD-6A) operated at 220 nm and a Rheodyne Model 7125 injection valve (20 μl). Chromatograms were recorded with a data system from Nuclear Interface. For isocratic elution mixtures of methanol and 0.05 M KH_2PO_4 (pH 7.0) were used as mobile phase. The flow-rate was set at 0.5 ml/min using the polyethylene column and at 1 ml/min using PLRP-S or LiChrosorb RP-8. All measurements were made at ambient temperature. Sample concentration of peptides was 1 mg/ml.

Capacity factors (k') were calculated from the retention time, $k' = (t_R - t_0)/t_0$. Data for t_0 were obtained by injecting a liquid mixture with a volume composition different from that of the eluent.

Column

A commercial Merck LiChrosorb RP-8 (5 μm) column (125 × 3 mm I.D.) and a PLRP-S (8 μm) column (150 × 4.6 mm I.D.) containing poly(styrene–divinylbenzene) from Polymer Laboratories were used without further treatment. Polyethylene was supplied by E. Merck, (Darmstadt, Germany), sieved to 20–40 μm irregular particles and was packed into a stainless-steel column (150 × 4.6 mm I.D.) as described previously [21].

Peptide synthesis and purification

The peptides RGD-Nal [3-(1-naphthyl)-alanine], RGD-Hph (homo-phenylalanine), RGD-Cha (cyclohexyl-alanine), RGD-Fpa (4-fluorophenylalanine), RGD-Bpa (4-bromophenylalanine), RGDS(Bzl) (O-benzylserine), RGDY(Bzl) (O-benzyltyrosine) and RGD-Cpa (4-chloro-phenylalanine) were synthesized by the classical mixed anhydride method with final deprotection by catalytic hydrogenolysis. RGDN, RGDS, RGDK, RGDH, RGDY, RGDF and RGDW were synthesized by a solid-phase method using the Fmoc strategy. Purification of crude peptides was carried out by preparative reversed-phase chromatography on a polyethylene column (450 × 25 mm, 40–60 μm). The peptides gave correct values in amino acid analysis and fast atom bombardment MS and were analysed by HPLC.

Measurement of platelet aggregation

Platelets were isolated from human venous blood drawn into 1:10 volume of acid citrate.

The platelet-rich plasma was prepared by centrifugation (10 min, 150 *g*). Briefly, platelets at a final concentration $2.5–3.0 \cdot 10^8$ /ml were incubated with varying concentrations of peptides. After 3 min aggregation was induced by the addition of ADP, adrenaline, collagen and plasma activating factor (PAF). Aggregation was measured at 37°C using a dual-channel aggregometer (Payton).

IC_{50} values were estimated from the concentration–response curves.

RESULTS AND DISCUSSION

Determination of log k_w values

The capacity factors of fourteen RGDX tetrapeptides were determined using LiChrosorb RP-8, PLRP-S and polyethylene. The measurements were performed in methanol concentrations of 0–40% (v/v). To suppress the silanol activity of the alkyl-bonded silica column, 0.05 *M* NaH_2PO_4 buffer (pH 7.0) was used as eluent. Plots of log *k'* against the methanol concentration in the eluent using polyethylene, PLRP-S and LiChrosorb RP-8 are shown in Figs. 1, 2 and 3, respectively.

The increase in log *k'* with decreasing methanol concentration was very close to linear with all stationary phases. Consequently, the log *k'* values could be extrapolated linearly to 100% water content, yielding log k_w values of the peptides shown in Table I. The elution orders of the tetrapeptides on the polymer phase and RP-8 were identical, indicating that the dominant retention mechanism was hydrophobic in nature. The deviations in terracing of the log k_w values emphasize the individual retention behaviour of each stationary phase. The capacity factors using poly(styrene–divinylbenzene) show increasing retention for peptides with aromatic amino acids in position X owing to the additional $\pi-\pi$ interactions. As expected, RGDY(Bzl) with two

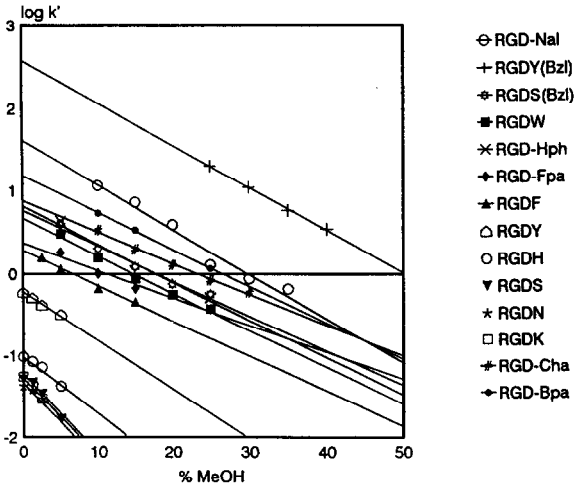

Fig. 2. Change in the capacity factors of RGDX peptides with increasing content of methanol in water (0.05 *M* phosphate, pH 7.0) on PLRP-S.

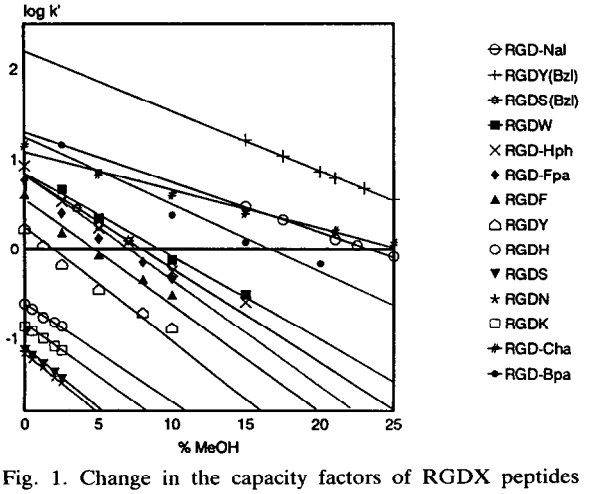

Fig. 1. Change in the capacity factors of RGDX peptides with increasing content of methanol in water (0.05 *M* phosphate, pH 7.0) on polyethylene.

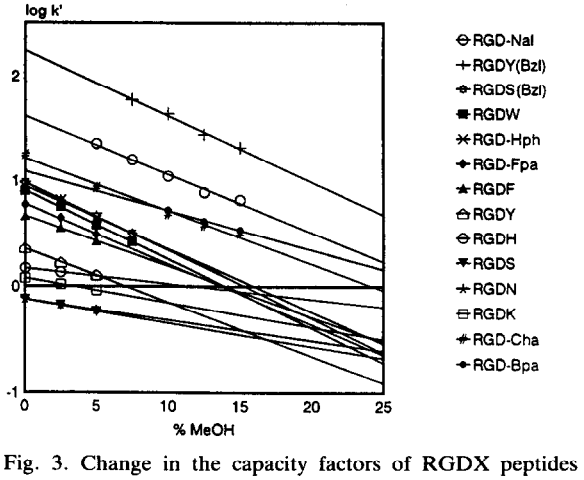

Fig. 3. Change in the capacity factors of RGDX peptides with increasing content of methanol in water (0.05 *M* phosphate, pH 7.0) on LiChrosorb RP-8.

TABLE I

LOG k_w VALUES DETERMINED BY USING DIFFERENT STATIONARY PHASES (POLYETHYLENE, PLRP-S AND LICHROSORB RP-8) AND THE CORRELATION WITH 1-OCTANOL/WATER PARTITION COEFFICIENTS LOG P [22] OF THE AMINO ACID SIDE-CHAIN IN POSITION X AND HYDROPHOBICITY SCALES $z1$ [23]

n.d., not determined.

Sequence	Log k_w polyethylene	Log k_w PLRP-S	Log k_w LiChrosorb RP-8	Log P amino acid side-chain [22]	$z1$ amino acid [23]
RGDN	−1.15	−1.33	−0.12	−0.60	2.88
RGDS	−1.13	−1.26	−0.12	−0.04	2.48
RGDK	−0.87	−1.26	0.08	−0.99	3.76
RGDH	−0.63	−1.00	0.17	0.13	3.46
RGDY	0.15	−0.23	0.35	0.96	−3.58
RGDF	0.52	0.25	0.67	1.79	−3.62
RGD-Fpa	0.70	0.37	0.78	n.d.	−4.70
RGD-Hph	0.81	0.82	0.95	2.10	n.d.
RGDS(Bzl)	0.82	0.76	0.98	2.34	−4.76
RGDW	0.84	0.67	0.91	2.25	−4.02
RGD-Cha	1.08	0.88	1.21	2.72	−5.15
RGD-Bpa	1.25	1.19	1.09	n.d.	−5.13
RGD-Nal	1.30	1.48	1.61	3.15	−6.21
RGDY(Bzl)	2.19	2.53	2.27	2.72	−6.02
$r(z1)$	0.93	0.91	0.85	0.96	1.00
r (log P)	0.94	0.93	0.88	1.00	0.96

separated aromatic rings shows the highest retention on PLRP-S.

Comparison of the overall tetrapeptide hydrophobicity (log k_w) and calculated amino acid side-chain parameters

In order to verify the determined hydrophobicity parameters of the RGDX tetrapeptides, the log k_w values were compared with calculated hydrophobicity parameters of the different natural and non-natural amino acids in position X (Table I).

The log P values represent the 1-octanol/water partition coefficients of the amino acid chains determined by Fauchêre *et al.* [22] using the shake-flask method of acetyl-amino acid amides. The z-scales [23] of the amino acids were derived by principal component analysis of a matrix, consisting of twelve properties (NMR, TLC, Van der Waals volume, MW) for 55 coded and non-coded amino acids and are related to hydrophobicity ($z1$), bulk ($z2$) and electronic properties ($z3$).

The data for the correlation coefficient r show a sufficient correlation (0.91–0.94) between the log k_w values, measured on polymer-based phases, and the hydrophobic side-chain parameters in position X, especially for the polyethylene stationary phases (0.93–0.94). However, the correlation coefficients obtained with LiChrosorb RP-8 (0.85–0.88) were lower than those of polyethylene and PLRP-S.

The good correlation of log k_w values with the peptide hydrophobicity demonstrates that the polyethylene stationary phase is a useful tool for assessing the overall hydrophobicity of shorter peptides. Polyethylene has a homogeneous hydrophobic adsorption surface and therefore does not require additional inhibition of polar peptide–solid phase interactions.

Quantitative structure–activity relationship (QSAR) studies using HPLC log k_W values

A set of eleven RGDX peptides with different side-chain hydrophobicity in position 4 was synthesized and used in QSAR studies. The biological activity of these peptides was investigated by measuring the inhibition of platelet aggregation. The inhibitory potencies of the peptides were expressed as the IC_{50} values and are shown in Table II.

Modelling using the multiple regression of the set resulted in a parabolic model in the biological test. The important factor in this model is the hydrophobicity parameter log k_W of the amino acid side-chain in position X, estimated on polyethylene:

$$\log IC_{50} = -0.398 \log k_W + 0.157 (\log k_W)^2 + 1.931$$
$$(0.059) \quad (0.048) \quad (0.074)$$
$$n = 11, SE = 0.17, r^2 = 0.85, F = 22.7 \quad (1)$$

Eqn. 1 gives the best fit with observed bioactivities, assuming that a parabolic dependence of log IC_{50} on the hydrophobicity of amino acids in position X of RGDX peptides contributes to the activity. Obviously, a hydrophobic amino acid side-chain in the C-terminal position of RGDX peptides enhances the binding to platelet glycoprotein GPIIb/IIIa receptors. Correlation coefficients of log P, $z1$ and log k_W determined on C_{18} PLRP-S are in each case lower than that of polyethylene ($r^2_{C8} = 0.82$, $r^2_{PLRP\text{-}S} = 0.82$, $r^2_{z1} = 0.77$, $r^2_{\log P} = 0.73$, $r^2_{polyethylene} = 0.85$).

The results demonstrate that the incorporation of amino acids with a hydrophobicity similar to tryptophan leads to an optimized inhibitory activity. Incorporation of amino acids exhibiting both lower and higher hydrophobicity resulted in analogues with diminished receptor binding. On the basis of these results further improvements of the antagonistic potency were achieved by preventing the enzymatic degradation. Thus, des-NH_2-RGDW (Table II) was the most active compound in this series.

TABLE II

IC_{50} VALUES DETERMINED BY PLATELET AGGREGATION TEST

Sequence	IC_{50} (μmol/l) platelet-aggregation assay
RGDN	322 ± 38
RGDS	447 ± 45
RGDH	203 ± 30
RGDF	38 ± 13
RGD-Fpa	53 ± 14
RGD-Hph	60 ± 20
RGDS(Bzl)	90 ± 18
RGDW	32 ± 8
RGD-Bpa	80 ± 25
RGD-Nal	33 ± 12
RGDY(Bzl)	63 ± 19
des-NH_2-RGDW	1.4 ± 0.35

CONCLUSION

Polymer-based stationary phases can be used for the determination of the overall hydrophobicity of RGDX peptides just like silica-based reversed-phase columns. The measured log k_W values of the tetrapeptides correlate with calculated hydrophobic amino acid side-chain parameters (log P, $z1$), especially for using polyethylene as stationary phases. Both the absence of residual polar groups (silanols) and the homogeneous hydrophobic adsorption surface of polyethylene contribute to the improved compatibility between log k_W values and hydrophobicity parameters.

Furthermore, a parabolic model based on peptide log k_W of the tetrapeptides was established to describe the structure–activity relationship of RGDX peptides.

The results demonstrate the applicability of reversed-phase HPLC capacity factors as hydrophobic parameters for quantitative structure–activity relationship of shorter peptides.

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