Interaction of Oligopeptides with Heparin

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SUMMARY: This heparin affinity chromatography study revealed some very interesting aspects with respect to oligopeptide retention. The first and most surprising effect observed was that oligopeptides with no aromatic groups were not retained on the column while those with aromatic groups were retained. The aromatic interaction was determined to be related to a charge transfer –like interaction as electron donating groups on the aromatic moiety increased retention on the column and electron withdrawing groups on the aromatic moiety deceased retention time. Column retention was dependent on oligopeptide size; the tripeptides were not retained while tetrapeptides and the larger hexapeptides were effectively retained if they contained some aromatic functionality. Retention was pH dependent; low pH's of ~2.5 were most effective while neutral pH was not effective in retaining the oligopeptides on the heparin affinity column. In addition to the aromatic interaction, retention was dependent on the hydrophobicity of the oligopeptides as well as their ability to hydrogen bond as determined by eluent solvent effects on the heparin affinity column. These results have led us design and carry out other experiments to determine more accurately the type and the degree of oligopeptide to heparin interaction.

Introduction

Macromolecular drugs, such as heparin and insulin, are easily degraded by acids and enzymes in the GI tract and do not readily penetrate the lipophilic biomembranes, resulting in low bioavailability by the oral route.[1-3] Currently, these drugs have to be administered by injection. Proteinoids, [4-8] which can form microspheres with certain macromolecular drugs, such as heparin and insulin, have been studied as an oral drug delivery system.[9-16] These proteinoid spheres can protect drugs from acid and enzyme degradation in the GI tract.[10-13,15] Recently, it was found that these proteinoids can also facilitate drug transport through the biomembranes, possibly, in the form of a proteinoid-drug complex.[9-12,17] This non-covalent complex seem to change the conformation of hydrophilic drug molecules into a more hydrophobic form that aids

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lipophilic biomembrane absorption.[9,17-18] However, these proteinoid materials are mixtures of oligopeptide dimers, trimers and tetramers with different structures and amino acid sequences.[10-11] Based on the proteinoid results, several series of structurally defined oligopeptides with specific amino acid sequences were synthesized as potential oral drug delivery carriers in order to study the mechanism of this novel oral drug delivery system.[18]

To form microspheres, the oligopeptides require a balance of hydrophilic and hydrophobic amino acids. Pyroglutamylglutamic acid (PyGluGlu) or pyroglutamylaspartic acid (PyGluAsp) seem to be important as the first two amino acid residues of the oligopeptides in order to form microspheres.[19] Since these amino acids are hydrophilic, phenylalanine, tyrosine, alanine, valine and leucine were used as the hydrophobic moieties to balance the hydrophobicity of the oligopeptides. Three series of oligopeptides were synthesized with these hydrophobic amino acids in α, γ or both the α and γ positions of the glutamic acid residue. A benzyl ester (Bz) was used to block the carboxyl functional group and a *tert*-butyloxycarbonyl (Boc) group was used to protect the amino group of the amino acids. A stepwise condensation process was used to synthesize these oligopeptides using diphenylphosphoryl azide (DPPA) as a coupling agent and triethylamine (TEA) as a catalyst. These oligopeptides were purified and characterized by ^{1}H NMR, ^{1}H-^{1}H NMR, ^{1}H-^{13}C NMR, IR, UV, ES/MS, reversed phase HPLC.[18]

In this research, the interactions of the synthesized oligopeptides with biomacromolecular heparin were evaluated by heparin affinity chromatography.

Oligopeptide Interactions with Heparin

A SigmaChrom™ AF-Heparin affinity HPLC column (2.5 mL, Sigma/Aldrich) was used in this study with different mobile phases and at different pHs. The column was prepared with highly porous 20 μm methacrylate gel particles, which have been functionalized with heparin.[20] The heparin density for this column is 5 mg/g. Each heparin molecule is covalently bonded to the polymer matrix through a bifunctional spacer such as amino or carboxyl groups by the cyanogen bromide method.[21] Multi-point coupling ensures stable attachment. Immobilized heparin has two major interaction modes with ligands: as an affinity matrix (e.g. interaction with growth factors and antithrombin III) and as a cation exchanger due to its high anionic sulfate

group content (e.g. interaction with nucleic acid-binding proteins where it mimics the similarly polyanionic structure of the nucleic acid).[20,22]

It was found that the retention of oligopeptides on the heparin affinity column was pH dependent. For example, with a phosphate buffer mobile phase at H 7, the oligopeptides were not retained. But when the mobile phase was changed to pH 4.1, using an acetate buffer, some of the oligopeptide tetramers that we synthesized, pEE(α)F(γ)F, pED(α)F(γ)F, pEE(α)Y(γ)Y and PE(α)F(γ)F, showed short retention times. However, when the mobile phase was changed to an acetate buffer with a pH of 2.27, several of the oligopeptide tetramers showed significant retention (t > 4) on the heparin affinity column. The capacity factors (k') were calculated and are depicted in Figure 1 for several of the oligopeptides that were evaluated.

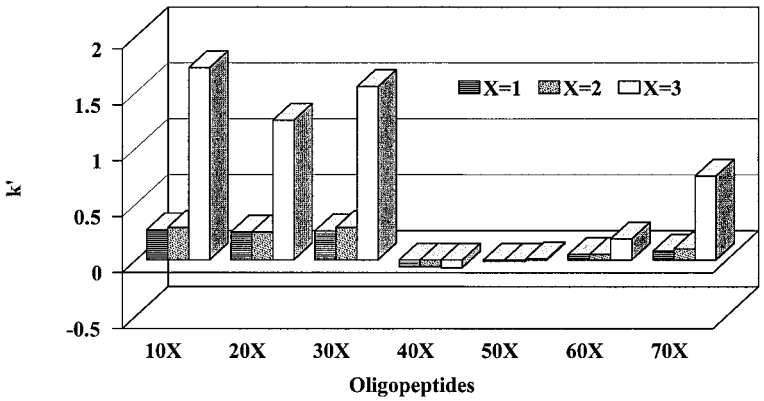

Figure 1. Retention behavior of the oligopeptides on heparin affinity column at pH 2.3 Series 10 represents pEE with F, series 20 represents pED with F, series 30 represents pEE with Y, series 40 represents pEE with A, series 50 represents pEE with V, series 60 represents pEE with L, series 70 represents PE with F; X=1 represents the α-trimer (the end group in the α position of E), X=2 represents the γ-trimer (the end group in the γ position of E), X=3 represents the α,γ-tetramer (the end groups in both α and γ positions of E).

At pH 2.27, the oligopeptides are in their molecular form, which increase the possibility for H-bonding and hydrophobic interaction and decrease electrostatic repulsion. Based on the capacity factor data for the oligopeptides at pH~2.3 shown in Figure 1, only four oligopeptide tetramers, pEE(α)F(γ)F, pED(α)F(γ)F, pEE(α)Y(γ)Y and PE(α)F(γ)F, showed significant retention (k'~1.0) on the heparin affinity column. The oligopeptide trimers, pEE(α)F, pEE(γ)F, pED(α)F, pED(α)F, pEE(α)Y and pEE(α)Y had very short and very similar retention times (k'~0.25) on this heparin affinity column. The oligopeptide tetramers pEE(α)A(γ)A, pEE(α)V(γ)V and pEE(α)L(γ)L were not retained (k'<0.2) on the heparin affinity column. The four tetrapeptides that were retained on the heparin affinity column, pEE(α)F(γ)F, pED(α)F(γ)F, pEE(α)Y(γ)Y and PE(α)F(γ)F, have aromatic groups on the terminal amino acid residues; while the tetrapeptides that were not retained on the heparin affinity column, pEE(α)A(γ)A, pEE(α)V(γ)V and pEE(α)L(γ)L, have aliphatic groups on the terminal amino acid residues. Consequently, the aromatic functionality on the amino acid residues in the oligopeptides appear to have a major effect on the retention of these oligopeptides on the heparin affinity column.

The hydrophobicity of a compound can be expressed by the partition coefficient P_{oct} between octanol and water.[23] This partition coefficient is one of the most widely used physical parameters to predict the biological activities of new drugs. Currently, high-performance liquid chromatography (HPLC) is generally being used for the determination of P_{oct}, since this method is very simple, rapid and accurate. In many cases,[24] a linear relationship between log P_{oct} and the logarithm of the capacity factor k' (k' is the ratio of the real retention volume and the void retention volume, the real retention volume is the difference between the measured retention volume and the void retention volume) in HPLC was observed. Based on this relationship, log P_{oct} values of the oligopeptides were calibrated from a standard curve developed from compounds whose log P_{oct} values are known.[24] In this research, the mobile phase was 50% methanol/water with 0.02% TFA (trifluoroacetic acid) in order to maintain the pH at 2.30. Since the pK_a values of these oligopeptides were between 3.5-4.5, as determined by titration analysis, the oligopeptide compounds were in the molecular form rather than ionic form. The log P_{oct} values for the oligopeptides are were evaluated and shown in Figure 2.

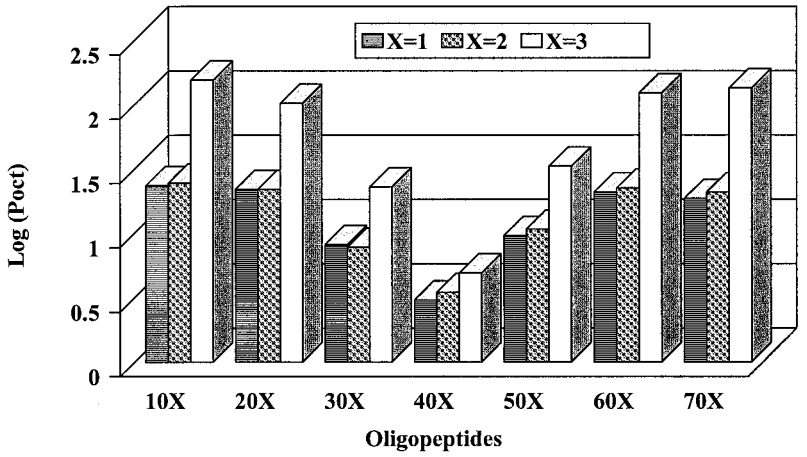

Figure 2. Apparent hydrophobicity (log P_{oct}) of the oligopeptides
Series 10 represents pEE with F, series 20 represents pED with F, series 30 represents pEE with Y, series 40 represents pEE with A, series 50 represents pEE with V, series 60 represents pEE with L, series 70 represents PE with F; X=1 represents the α-trimer (the end group in the α position of E), X=2 represents the γ-trimer (the end group in the γ position of E), X=3 represents the α,γ-tetramer (the end groups in both α and γ positions of E).

A correlation of the hydrophobicity log P values of the tetrapeptides with their capacity factors (k') on the heparin affinity column was carried out and the results are depicted in Figure 3. Based on the data in Figure 3, it is clear that tetrapeptide pEE(α)L(γ)L was not retained on the heparin affinity column even though this tetrapeptide had a comparable log P value as pEE(α)F(γ)F.

It seems that hydrophobicity alone is not directly related with the retention of the oligopeptides on the heparin affinity column. However, the oligopeptide trimers containing an aromatic-ring, which have lower hydrophobicity log P values than their corresponding tetrapeptides, also had much shorter retention times on the heparin affinity column than their corresponding tetrapeptides. Furthermore, when the sodium chloride concentration in the mobile phase was increased from 0.1 M to 0.3 M, the retention (k') of the tetrapeptide pEE(α)F(γ)F was also increased from 1.72 to 1.86. Since salts have been reported to increase hydrophobic interactions,[20,25] it appears that hydrophobic interactions may be involved in the retention of the

oligopeptides on the heparin affinity column. Consequently, the aromatic amino acid residues on the oligopeptides as well as hydrophobic character have a significant effect (k' > 1.0) on the retention of the oligopeptide heparin affinity column.

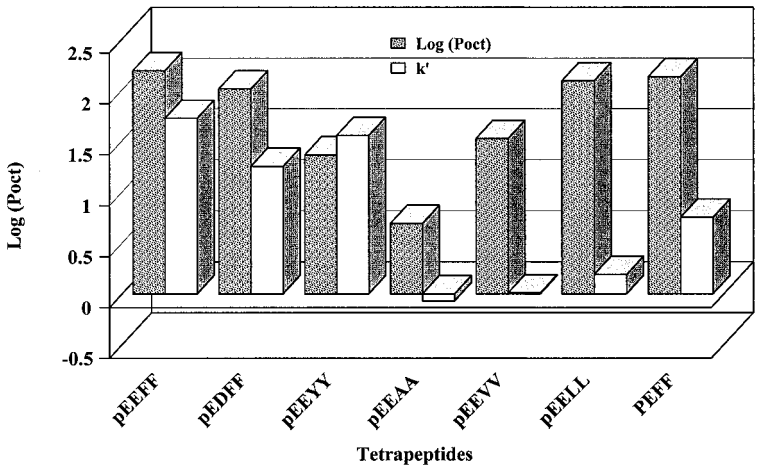

Figure 3. Correlation of apparent hydrophobicity (log P) with heparin affinity column retention Behavior (k') of the tetrapeptides.

To further investigate the interactions involved in the retention of oligopeptides on the heparin affinity column, isopropanol was added to the above pH 2.27 mobile phase (10%, v/v). Isopropanol is commonly used as an organic modifier to disrupt the hydrophobic interaction between the solute and the matrix surface in the chromatography.[25] It was found that the retention of the tetrapeptides on the heparin affinity column was decreased ~50% (Figure 4), which implies that hydrophobic interactions are involved in the interaction forces of the retention of the oligopeptides on the heparin affinity column. As shown in Figure 4, when ethylene glycol (9%, v/v), which is also commonly used as an organic modifier in the chromatography to reduce the H-bonding interaction between the solute and the matrix surface,[20,25] was added to the pH 2.27 acetate buffer eluent, the retention of the tetrapeptides on the heparin affinity column decreased ~25%.

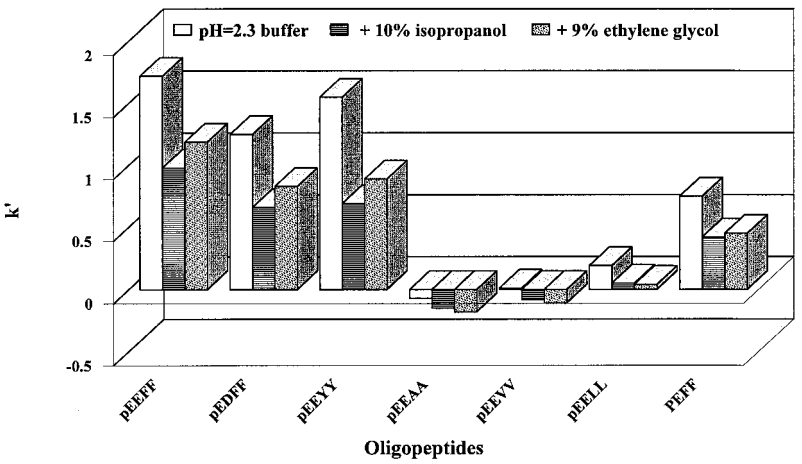

Figure 4. Heparin affinity column retention behavior of the tetrapeptides in different eluents with organic modifiers at pH 2.

Based on the data in Figure 4, it seems that hydrophobic interactions are more important with respect to oligopeptide-heparin association than H-bonding since the k' values with isopropanol were less than those with ethylene glycol. However, since no quantitative relation has been reported between the amount of the organic modifier added and the value of hydrophobic interaction and/or H-bonding or the capacity factor values. Consequently, one can only conclude that both hydrophobic interaction and H-bonding are involved in the retention of oligopeptides on the heparin affinity column in addition to the aromatic interaction, which appears to be dominant. One possible interaction mode of the immobilized heparin with the solute is that it could act as a cation exchange agent due to the numerous sulfate groups. However, the oligopeptides used in this study did not contain any free amine group {except PE(α)F(γ)F}, which excludes the ionic bonding. In addition, the high concentration of sodium chloride (0.1 M NaCl) should prevent ionic the heparin interaction.

To further evaluate the influence of the aromatic moiety on affinity on the heparin column two oligopeptide hexamers were synthesized pEE(α)FF(γ)FF and pEE(α)FY(γ)FY. Both had a much longer retention time on the heparin affinity column than the tetrapeptides pEE(α)F(γ)F and pEE(α)Y(γ)Y, respectively (Figure 5). This corroborated the specificity of the aromatic moiety on the retention of oligopeptides on the heparin affinity column.

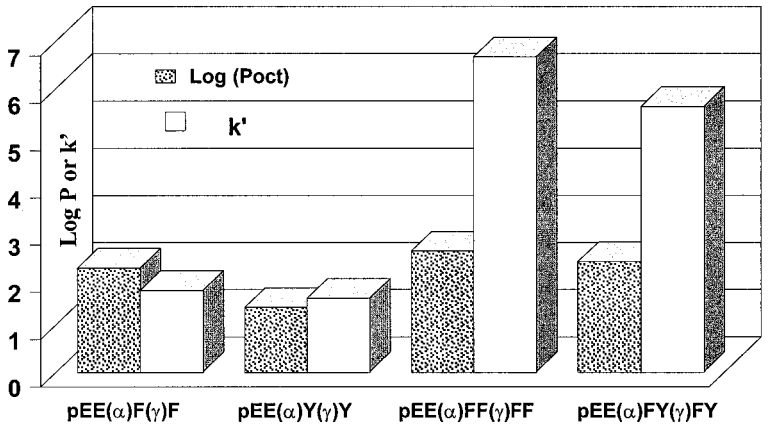

Figure 5. Correlation of Hydrophobicity (log P) with Heparin Affinity Column Retention Behavior of the Oligopeptide Hexamers.

In an effort to determine the reason for this aromatic phenomenon, the tyrosine moiety, which has a phenol group, was derivatized with electron donating and electron withdrawing groups. The tetrapeptide pEE(α)Y(γ)Y has two phenol groups which were derivatized with methyl, acetyl or phthalic acid-ester groups, respectively, by reacting the -OH group on the tyrosine phenol group with methyl iodide, acetic anhydride and phthalic anhydride, respectively, under alkaline conditions. The reverse phase HPLC log P data from 50% methanol/water at pH 2.27 and heparin affinity chromatography k' data from acetate buffer at pH 2.27 for these derivatives are represented in Figure 6.

Based on these data, the hydrophobicity increased when the tetrapeptide was derivatized with methyl, acetyl and the phthalic acid ester group. The di(O-methyl) derivative and the di(O-phthalic acetyl) derivative had much higher retentions on the heparin affinity column than the parent tetrapeptide pEE(α)Y(γ)Y while the di-(O-acetyl) derivative had a lower retention time than the parent tetrapeptide pEE(α)Y(γ)Y on the heparin affinity column. It is known that the methoxyl group is an electron-donating group while the acetyl group is an electron-withdrawing group. Thus the electron density of the aromatic ring is increased for the di-(O-methyl) derivative while the electron density of the aromatic ring is decreased for the di-(O-acetyl)

derivative. Based on this data, a charge transfer-like complex [26] may be involved in the retention of these derivatives on the heparin affinity column since the derivative with electron richer aromatic groups (O-Me) is retained on the heparin affinity column longer than that with electron poorer aromatic groups (O-Ac).

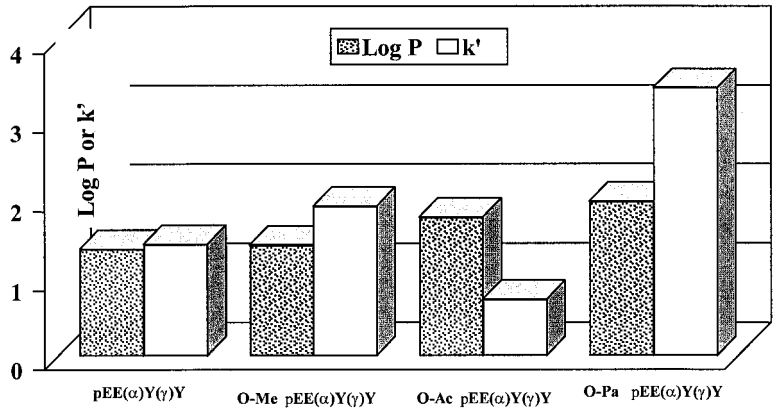

Figure 6. Correlation of Hydrophobicity (log P) with Heparin Affinity Column Retention
Behavior (k') for the Derivatives of the Tetrapeptide pEE(α)Y(γ)Y
O-Me pEE(α)Y(γ)Y is di(O-methyl) derivative, O-Ac pEE(α)Y(γ)Y is di(O-acetyl) derivative and O-Pa pEE(α)Y(γ)Y is di(O-phthalic acetyl) derivative.

In the case of the di(O-phthalic acetyl) derivative, two additional aromatic rings were added to the compound so that the total number of aromatic groups was increased from 2 to 4. This may be the cause for the longer retention of this derivative on the heparin affinity column relative the log P value for this oligopeptide. This explanation was supported by the fact that oligopeptide hexamers pEE(α)FF(γ)FF and pEE(α)FY(γ)FY both had a much longer retention on the heparin affinity column than the tetrapeptides pEE(α)F(γ)F and pEE(α)Y(γ)Y.

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