RETENTION BEHAVIOR OF PTEROYL-OLIGO-γ-L-GLUTAMATES IN REVERSED-PHASE CHROMATOGRAPHY

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SUMMARY

The effect of eluent pH on the retention of pteroyl-oligo-γ-L-glutamates containing up to eight glutamyl residues is investigated in reversed-phase chromatography with octadecyl-silica column. When the carboxylic groups of the solutes are largely undissociated, at pH 2, the retention of oligoglutamates increases with the number of glutamyl residues and the elution order parallels that in anion-exchange chromatography. At sufficiently high eluent pH the carboxylic groups are dissociated and the elution order is reversed so that solute molecules having smaller number of charges, *i.e.* less glutamyl residues, are retained stronger. The logarithm of capacity factor, with the exception of folic acid, is linearly dependent on the number of glutamyl residues over a wide range of eluent pH. The dependence of the capacity factor on pH for oligoglutamates is quantitatively interpreted considering the different dissociation constants for the α- and γ-carboxyls. The results suggest that in reversed-phase chromatography the selectivity of separation for polyionogenic compounds can be drastically modulated by changing the pH of the eluent.

INTRODUCTION

Derivatives of folic acid (FA) are coenzymes essential in the synthesis of proteins and nucleic acids[1]. The FA molecule consists of a pteridine moiety linked to *p*-aminobenzoylglutamic acid. Numerous derivatives of FA, which differ in the substitution at N^5 and N^{10} positions and the state of oxidation, have been identified. In many cases more than one glutamic acid is attached via γ-carboxylic group to the rest of the molecule[2]. The general formula of such folate–oligo-γ-L-glutamates, which usually contain 2 to 9 glutamyl residues, is shown in Fig. 1 and we can see that each of such molecules contains one γ-carboxylic group and *n* α-carboxylic groups.

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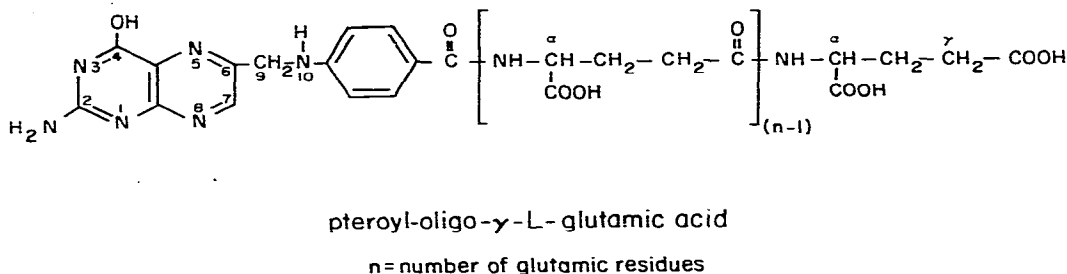
pteroyl-oligo-γ-L-glutamic acid

n = number of glutamic residues

Fig. 1. Structure of pteroyl-oligo-γ-L-glutamic acids, PtGlu$_n$, where *n* is the number of glutamyl residues. For the parent compound, FA, *n* equals one.

It was suggested that the derivatives of tetrahydrofolate–polyglutamates are biologically active coenzymes in microorganisms[3]. The reduced forms of pteroyl-tri-γ-glutamate (PtGlu$_3$) and pteroyl-hepta-γ-glutamate (PtGlu$_7$) are the principal folate-oligo-γ-glutamates found in bacteria and yeast, respectively. Recently, oligo-γ-glutamates were also isolated from mammalian cells and found to be active as co-enzymes in several folate-dependent "one carbon" enzymic reactions[4,5]. The presence of biologically active oligo-γ-L-glutamates of *p*-aminobenzoic acid (PABA) in mammalian cells in culture has also been postulated[5]. The study of folate metabolism has been hampered by the large number and low physiological concentrations of active folate derivatives and the lack of sufficiently efficient separation methods and sensitive analytical tools.

Column chromatography was first used in 1957 to fractionate a complex mixture of folates[6]. Thereafter the technique was refined and ion-exchange columns packed with DEAE-cellulose and DEAE-Sephadex have been used widely for the separation of folate coenzymes[7,8].

Recently, high-performance liquid chromatography was introduced to afford rapid analysis of various FA derivatives. Initially, columns packed with micro-particulate bonded anion exchangers were used[9,10] for the separation of reduced and oxidized mono- and polyglutamates. It has been shown, however, that reversed-phase chromatography with microparticulate octadecyl-silica as the stationary phase also yields high resolution and fast analysis[11]. In fact, reversed-phase chromatography offers an excellent alternative to ion-exchange chromatography for microanalysis of folate derivatives.

In this study the effect of pH on the retention of oligoglutamates in reversed-phase chromatography is examined. In this technique hydrophobic interactions between the eluites and the non-polar stationary phase play an important role in determining the retention behavior[12]. Therefore results obtained in reversed-phase chromatography can shed light on the hydrophobic properties of the sample components.

EXPERIMENTAL

A Perkin-Elmer (Norwalk, Conn., U.S.A.) Model 601 liquid chromatograph with a Rheodyne (Berkeley, Calif., U.S.A.) Model 7010 sampling valve and a variable

wavelength UV detector Model SF-770 (Schoeffel, Westwood, N.J., U.S.A.) were used. In some experiments, a Schoeffel fluorimetric detector, Model FS-970 was also used. One experiment was carried out using a DuPont Model 830 liquid chromatograph with UV detector in the gradient elution mode.

Octadecyl-silica columns were prepared from 10 in. × 0.25 in. O.D. × 4.6 mm I.D., No. 316 stainless-steel tubing by slurry packing. Two kinds of material were used: 5-μm Partisil ODS 2 with 16% carbon load was donated by Whatman (Clifton, N.J., U.S.A.); 5-μm Spherisorb ODS with 6% carbon load was purchased from Phase Sep (Hauppage, N.Y., U.S.A.).

FA was obtained from Sigma (St. Louis, Mo., U.S.A.) and the oligo-γ-glutamates of FA and p-aminobenzoic acid were synthesized as described[11,13]. Acetonitrile was obtained from Burdick & Jackson (Muskegon, Mich., U.S.A.) and the other chemicals were reagent-grade and purchased from Fisher (Pittsburgh, Pa., U.S.A.).

Isocratic elution was carried out with a 0.1 M phosphate buffer containing a few per cent (v/v) of acetonitrile. In experiments with gradient elution the starting eluent was neat aqueous phosphate buffer and acetonitrile was used as the gradient former. Column temperature was held at 45° by using a water jacket connected to a thermostated circulator bath (Lauda Messgeräte, Lauda, G.F.R.).

Solute retention has been expressed by the dimensionless retardation factor, k, which is calculated from the formula $k = (t_R - t_0)/t_0$, where t_R is the retention time of the eluite and t_0 is the hold-up time for an unretained solute. In this study t_0 was evaluated from the retention time of sodium nitrate.

The analysis of the data was performed on a PDP 11/10 minicomputer (Digital Equipment, Maynard, Mass., U.S.A.) equipped with a floppy disk unit, a VT-55 CRT and a Decwriter. The computer program used for parameter estimation was written in BASIC language.

RESULTS AND DISCUSSION

Ionized biological substances such as FA derivatives are traditionally separated by ion-exchange chromatography. Recent results, however, clearly indicate that reversed-phase chromatography is eminently suitable for the separation of peptides[14] and has a great potential for the assay of folate–oligoglutamates[11]. The physicochemical phenomena which govern solute retention in the two chromatographic techniques are often quite different. In ion-exchange chromatography coulombic interactions between the fixed charges on the stationary phase and the oppositely charged solute molecules play an important role in determining the magnitude of retention. Consequently, the retention of the polyglutamates of FA on an anion-exchange column, which is customarily employed for their separation, increases with increasing number of glutamic acid residues, i.e., with increasing number of ionized carboxylic groups in the molecules[9,11].

On the other hand, in reversed-phase chromatography the stationary phase is non-polar and it has been shown[15,16] that an enhancement of electrostatic interactions between the solute and solvent, e.g., that which occurs upon solute ionization in aqueous

buffer solutions, leads to a decrease in retention. In other words, the hydrophobic effect which largely affects the magnitude of retention in reversed-phase chromatography is attenuated by increasing the charge on the solute molecule. It follows then that the elution order of the oligo-γ-glutamates in reversed-phase chromatography, when the pH of the eluent is sufficiently high for the carboxylic groups to be ionized, should be the opposite of that observed in anion-exchange chromatography. Indeed the chromatogram of pteroyl-γ-L-glutamates in Fig. 2 demonstrates that retention decreases with increasing number of glutamyl residues at pH 6 where the carboxylic groups are almost completely dissociated. As the retention order of oligo-γ-glutamates in reversed-phase chromatography under such conditions is just the opposite of that observed in anion-exchange chromatography, the two methods complement each other. The identification of polyglutamate peaks can be greatly facilitated by using both techniques concomitantly[11].
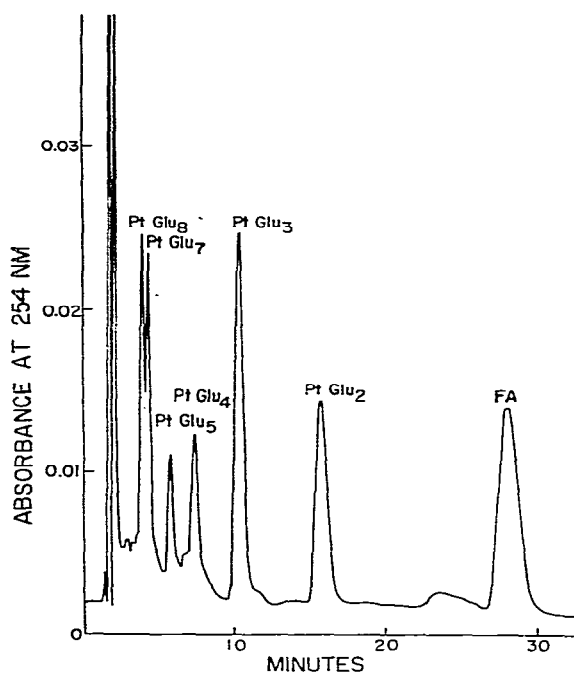

Fig. 2. Reversed-phase chromatogram of pteroyl-oligo-γ-L-glutamates obtained by isocratic elution at pH 6. Column, 5-μm Spherisorb ODS, 250 $\times$ 4.6 mm; eluent, 0.1 *M* phosphate buffer, pH 6.0, containing 1 % (v/v) acetonitrile; flow-rate, 1.5 ml/min; inlet pressure, 11.42 MN/m²; temperature, 45°; sample size, approx. 10 μg of each component.

As expected from the preceding discussion when oligoglutamates of *p*-amino-benzoic acid are chromatographed at sufficiently high eluent pH on an octadecyl-silica column retention also decreases with increasing number of glutamyl residues. How-
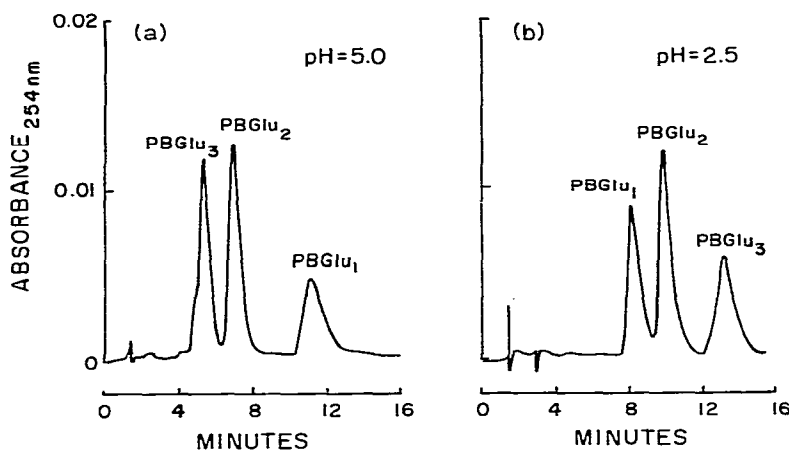
Fig. 3. Reversal of elution order for p-aminobenzoyl-oligo-γ-L-glutamates upon changing the pH of the eluent in reversed-phase chromatography. Column, 5-μm Partisil ODS-2, 250 × 4.6 mm; eluent (a) 0.1 M plain aqueous phosphate solution, pH 5.0, (b) 0.1 M phosphate buffer, pH 2.5, containing 3% (v/v) acetonitrile; flow-rate, 1.5 ml/min; inlet pressure, 11.5 MN/m²; temperature, 45°; sample size, approx. 10 μg of each component.

ever, a dramatic reversal of the retention order is observed when the dissociation of the carboxylic acids is suppressed by lowering the pH of the eluent. Similar effects have been observed with folate–oligoglutamates as well. The comparison of the chromatograms obtained at pH 5.0 and 2.5 as shown in Fig. 3, clearly demonstrates this phenomenon.

Therefore, we investigated the effect of eluent pH and the number of glutamyl residues in pteroyl-oligo-γ-L-glutamates on retention in reversed-phase chromatography under a wide range of conditions. The pH was varied between 2.2 and 6.0 and the retention of eluites having 1, 3, 5 and 7 glutamyl residues was measured. The results of this study are illustrated in Fig. 4. The graph shows the capacity factor (on a logarithmic scale) as a function of the number of glutamyl residues with the eluent pH as the parameter. The straight lines in Fig. 4 demonstrate that log k is proportional to the number of glutamyl residues. This behavior is in agreement with that postulated by the solvophobic theory[15] for solute retention in reversed-phase chromatography. FA ($n = 1$) shows "irregular" behavior in the pH range 3.5–5.0 where it is partially protonated.

Changes in the elution order manifest themselves in the changing slopes on the graph in Fig. 4. At low pH the slope is positive indicating that retention increases with the number of glutamyl residues. Then the line becomes horizontal between pH 3.0 and 3.8 so that all sample components elute together and no separation occurs. At high pH the slope is negative and the capacity factor decreases with increasing number of glutamyl residues. In reversed-phase chromatography, therefore, the elution order of polyglutamates can be the same as in ion-exchange chromatography when the ionization of the carboxylic groups is suppressed at low eluent pH. However, the elution order of the polyglutamates with dissociated carboxylic groups, i.e.,

at sufficiently high eluent pH, is the reverse of that observed in ion-exchange chromatography.

The dependence of the capacity factor on the hydrogen ion concentration, $[H^+]$, in the pH domain investigated is evidently related to the degree of dissociation of the carboxylic groups in the glutamyl moiety. For a diprotic acid, such as FA, it has been shown[15] that the capacity factor can be expressed by

$$k = \frac{k_0 + k_1 \dfrac{K_1}{[H^+]} + k_2 \dfrac{K_\gamma K_1}{[H^+]^2}}{1 + \dfrac{K_1}{[H^+]} + \dfrac{K_\gamma K_1}{[H^+]^2}} \tag{1}$$

where k_0, k_1 and k_2 are the capacity factors of FA when none, one and both of the carboxylic groups are dissociated, respectively. The dissociation constants of the α-
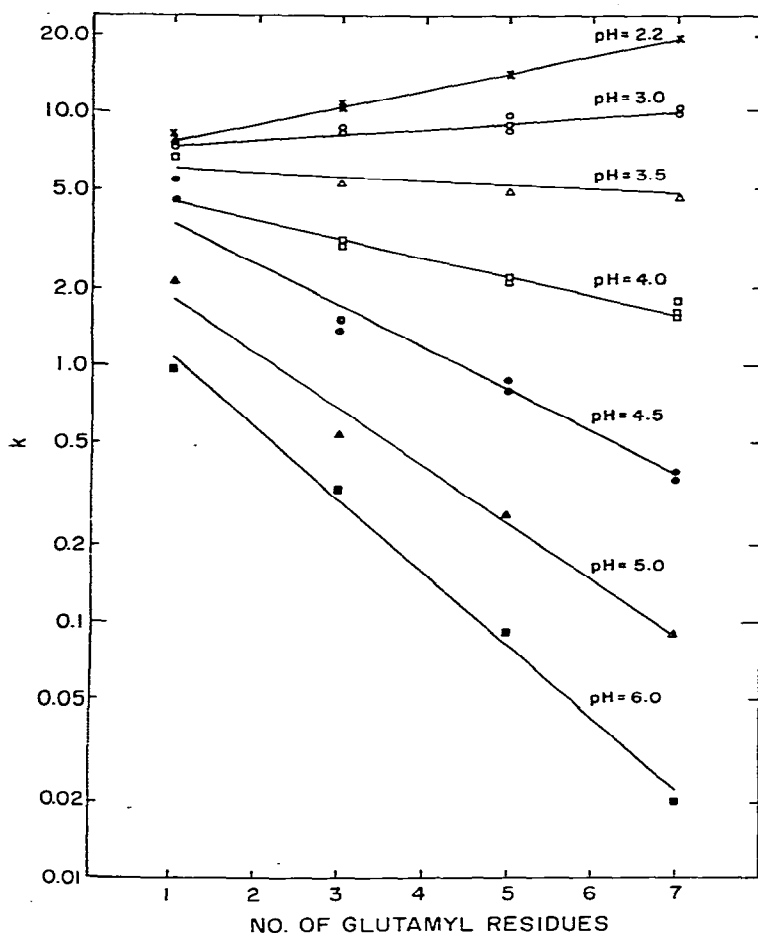

Fig. 4. Plots of the capacity factor (on a logarithmic scale) against the number of the glutamyl residues in pteroyl-oligo-γ-glutamates at different pH of the eluent. Column, 5-μm Partisil ODS-2; eluent, 0.1 M phosphate solution containing 6% (v/v) acetonitrile; temperature, 45°.

and γ-carboxylic groups in the glutamyl residue are given by K_1 and K_γ, respectively.

The α- and γ-carboxylic groups in FA are expected to have significantly different pK_a values although a literature search failed to reveal any pK_a values. The low solubility of FA in aqueous solutions at pH below 6 makes the determination of the pK_a values difficult. Nevertheless for tetrahydrofolic acid the pK_a values of the α- and γ-carboxylic groups have been reported[17] as 3.5 and 4.8, respectively, and we may assume that the corresponding pK_a values are similar for FA. Therefore the degree of dissociation of the carboxylic group will change in the pH domain from 2 to 6. On the other hand, the amino functions in the pteridine moiety are largely ionized under such conditions.

With the pteroyl-oligo-γ-glutamates the situation is similar except that the number of α-carboxylic groups, which is equal to n, increases with the number of glutamyl residues. For these substances eqn. 1 can be generalized as

$$k = \frac{k_0 + \sum\limits_{i=1}^{n} k_i \dfrac{\prod\limits_{j=1}^{i} K_j}{[H^+]^i} + k_{n+1} \dfrac{K_\gamma \prod\limits_{i=1}^{n} K_i}{[H^+]^{n+1}}}{1 + \sum\limits_{i=1}^{n} \dfrac{\prod\limits_{j=1}^{i} K_j}{[H^+]} + \dfrac{K_\gamma \prod\limits_{i=1}^{n} K_i}{[H^+]^{n+1}}} \tag{2}$$

where k_i and K_j are the capacity factor of the molecule containing i dissociated α-carboxylic groups and the acid dissociation constant of the ith α-carboxylic groups, respectively. On the other hand, k_{n+1} and K_γ are the capacity factors of the fully dissociated species and the dissociation constant for the γ-carboxylic group, respectively.

It is seen from eqn. 2 that the calculation of the capacity factors as the function of pH for these compounds necessitates a large number of parameters whose evaluation would require a large amount of very precise data obtained at narrow pH intervals. The number of parameters can be drastically reduced, however, by using certain assumptions. First, the capacity factor ratio of the homologues differing in one ionized α-carboxylic group can be considered constant, so that

$$C = \frac{k_{i+1}}{k_i} \tag{3}$$

This assumption can be justified by the results of a previous study on the pH dependence of the retention of ionogenic substances in reversed-phase chromatography[16]. Substituting eqn. 3 into eqn. 2 we obtain

$$k = \frac{k_0 + \sum\limits_{i=1}^{n} k_0 C^i \dfrac{\prod\limits_{j=1}^{i} K_j}{[H^+]^i} + k_0 C^{n+1} \dfrac{K_\gamma \prod\limits_{i=1}^{n} K_i}{[H^+]^{n+1}}}{1 + \sum\limits_{i=1}^{n} \dfrac{\prod\limits_{j=1}^{i} K_j}{[H^+]} + \dfrac{K_\gamma \prod\limits_{i=1}^{n} K_i}{[H^+]^{n+1}}} \tag{4}$$

In order to evaluate the K_i's, another assumption is made. It is based on widely accepted thermodynamic consideration that the energy required for the ionization of a carboxylic group increases with the number of already dissociated carboxyl functions in the molecule. In the simplest case the energy is proportional to the number of ionized groups and the dissociation constant of the *i*th α-carboxylic group in the oligo-γ-glutamate moiety, K_i, can be expressed by

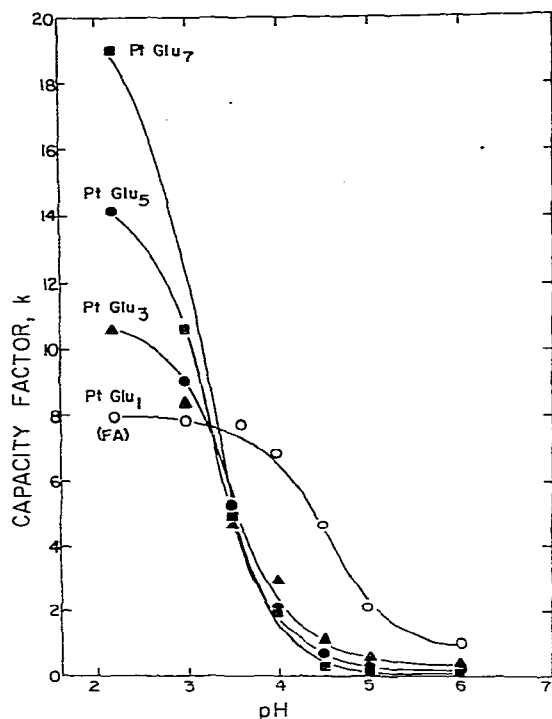$$K_i = K_1 \exp \left(-f \sum_{j=1}^{i} j \right) \tag{5}$$

Fig. 5. Capacity factors of pteroyl-oligo-γ-glutamates as a function of the eluent pH in reversed-phase chromatography. The solid lines were calculated from eqn. 4 with the parameters given in Table I. The symbols denote experimental data points obtained under conditions stated in Fig. 4.

TABLE I

PARAMETERS IN EQNS. 4 AND 5 FOR THE CALCULATION OF THE THEORETICAL CURVES IN FIG. 5

The value of pK_γ was taken as 4.8.

Eluite	pK_1	C	f	n
PtGlu₁(FA)	4.5	0.40	0.3	1
PtGlu₃	3.5	0.41	0.3	3
PtGlu₅	3.1	0.48	0.3	5
PtGlu₇	3.0	0.52	0.25	7

where K_1 is the dissociation constant of the α-carboxylic group in FA and f is the above mentioned proportionality factor. Knowing the values of k_0, k, K_γ and K_1, we can evaluate C and f from the experimental data by using eqns. 4 and 5. The parameters used to obtain the theoretical curves shown for the pH dependence of the pteroyl-oligo-γ-glutamates in Fig. 5 were evaluated by using this approach and are listed in Table I.

It is seen from Fig. 5 that this approach offers a quantitative interpretation for the observed pH dependence of retention for oligo-γ-glutamates. The results demonstrate again that the behavior of FA is different from that of higher oligoglutamates. The difference is due to the effect of the α-carboxylic groups whose pK_a is relatively low. The physiological significance of this difference is not known, however. In fact, the physiological role of oligo-γ-glutamates has not yet been fully elucidated.

The results depicted in Figs. 4 and 5 can be interpreted by considering the effect of the number of glutamyl residues on the size of, and, at sufficiently high pH, on the charge carried by the oligoglutamate molecules. The conjugate bases, present at low pH, are retarded in the order of increasing molecular size in agreement with the prediction of the solvophobic theory that the free energy of the reversible association process between the solute and the hydrocarbonaceous ligands of the stationary phase is linear in the contact area between the two species provided all other factors remain the same[15]. This process of separation by size is contrasted with separation by charge which occurs when the carboxylic groups are dissociated. Under such conditions an increase in the number of glutamyl residues is tantamount to an increase in the number of charges in the solute molecules. According to the theory[16] the energy of the electrostatic interaction between the solute and eluent increases with the number of charges and results in a concomitant decrease in chromatographic retention on non-polar stationary phases. Consequently, the dissociated species elute in the order of decreasing n in reversed-phase chromatography.
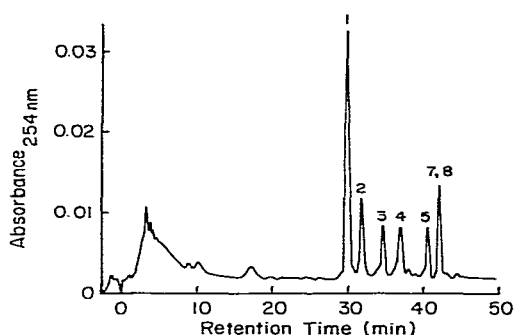

Fig. 6. Reversed-phase chromatogram of pteroyl-oligo-γ-L-glutamates at low pH and with gradient elution. Column, 5-μm Spherisorb ODS, 250 $\times$ 4.6 mm; starting eluent, 0.1 M phosphate buffer, pH 2.2, containing 4% (v/v) acetonitrile; gradient former, the same buffer with 10% (v/v) acetonitrile at No. 4 concave setting on DuPont 830; inlet pressure, 11.72 MN/m²; temperature, 45°; sample size, 3–10 μg of each component. The peak numbers correspond to the number of glutamyl residues, n, in the eluite molecules (see Fig. 1).

In contradistinction, retention in anion-exchange chromatography is due to the interaction between the negatively charged oligo-γ-glutamates and the fixed positive charges on the stationary phase[9]. Consequently, the elution order for separation by charge is the opposite of that observed with the dissociated oligo-γ-glutamates in reversed-phase chromatography and corresponds to that observed with separation by size.

In general the efficiency of ion-exchange chromatography with bonded phases such as Partisil SAX (Whatman) appears to be higher than that of reversed-phase chromatography at low pH on octadecyl-silica at the same particle size and column dimensions. It is recommended, therefore, that ion-exchange chromatography be used when an elution order in the increasing number of glutamyl residues is desired. On the other hand, reversed-phase chromatography is eminently suitable for separations with reversed elution order as at high eluent pH adequate resolution can be obtained by using isocratic (see Fig. 2) or in the case of complex mixtures, gradient elution. Experiments with a variety of octadecyl-silica columns gave very similar results and demonstrated the analytical usefulness of this approach.

Reversed-phase chromatography at low pH however, also can give satisfactory results when gradient elution is used. It is shown in Fig. 6 by the chromatogram of FA-oligoglutamates obtained at pH 2.2 by using gradient elution with exponentially increasing acetonitrile concentration. A caveat is necessary when working at low eluent pH because the elution order of the first three members of the oligoglutamate series was found to be PtGlu$_2$, PtGlu$_3$ and PtGlu$_1$ with certain other types of octadecyl-silica columns when the concentration of acetonitrile in the eluent was greater than 6% (v/v). The observed increase in the retention of FA with the concentration of the organic modifier in the eluent is likely to arise from changes in the dielectric properties of the eluent.

ACKNOWLEDGEMENTS

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