Seperation of Folic Acid Compounds by Gel Chromatography on Sephadex G-15 and G-25*

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SUMMARY

The separation of folate compounds has been achieved by gel chromatography and the effect of buffer concentration on the elution pattern of folate derivatives was investigated by Sephadex G-15 and G-25 column chromatography.

Highly conjugated polyglutamates appear in the effluent considerably sooner when eluted with water than would be expected from their molecular weights. Monoglutamates, except 10-formyltetrahydrofolate, are retarded on G-15 and G-25 in phosphate buffer (pH 7.0) solvent system; the situation becomes more apparent as the buffer concentration is increased.

Chromatography on Sephadex G-25 with 0.1 M phosphate buffer, pH 7.0, containing 0.2 M 2-mercaptoethanol gives a satisfactory separation of pteroylpolyglutamates containing 2 to 7 glutamic acid residues.

Polyglutamate forms of folic acid are known to be the major forms of natural folates in animal tissues (1, 2), plants (3), yeast (4), and bacteria (5). The identification and separation of these compounds was based mainly on ion exchange column chromatography together with microbiological assays (1, 3). However, this method has not proved suitable for the determination of the number of glutamic acid residues in polyglutamate forms of folic acid.

Gel chromatography, commonly used for the separation of molecules of different sizes, has been neglected for the separation of polyglutamates of folic acid. Recently, Whitehead used Bio-Gel chromatography for the separation of pteroyltriglutamates from di- and triglutamates in liver (6). Another approach to determine the chain length of polyglutamates involves the degradation of natural folates to their corresponding p-aminobenzoypolyglutamyl derivatives (7).

In our study, we developed a method to separate natural folates containing 1 to 7 glutamic acid residues on Sephadex G-15 and G-25 columns and examined the influence of various buffer concentrations on the elution volume of these compounds.

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MATERIALS AND METHODS

p-Aminobenzoic acid and folic acid were purchased from Sigma. Trinitated folic acid was obtained from Amersham-Searle and 5-CHO-H,PteGlu from Lederle Laboratories. H,PteGlu was prepared by the reduction of folic acid with hydrogen with platinum as the catalyst (8). 10-CHO-H,PteGlu was prepared from 5-CHO-H,PteGlu (9) or from PteGlu (10). 5-CH,H,PteGlu was synthesized by reducing 5,10=CH-H,PteGlu with sodium borohydride (11). Pteroyl acid was obtained from Lederle. Pteroyl acid was also prepared by incubation of folic acid with a strain of Pseudomonas (12). Radioactive pteroylpolyglutamates of pteroylmonoglutamates from Sephadex G-15 and G-25 columns

| Standard compounds | 0.02 M a | 0.1 M a | 0.1 M b |
|-------------------|---------|---------|---------|
| Eluant | Water b | | |
| G-15 | G-25 | G-15 | G-25 | G-15 | G-25 |
| 10-CHO-H,PteGlu | 0.78 | 1.05 | 0.78 | 1.05 | 0.78 | 1.05 |
| 5-CHO-H,PteGlu | 0.78 | 1.05 | 1.15 | 1.27 | 1.21 | 1.52 |
| 5-CH,H,PteGlu | 0.78 | 1.05 | 1.40 | 1.52 | 1.70 | 1.74 |
| H,PteGlu | 0.78 | 1.05 | 1.56 | 1.69 | 1.70 | 1.81 |
| PteGlu | 0.78 | 1.05 | 1.56 | 1.78 | 1.78 | 1.95 |

a Eluant, distilled water with 0.2 M 2-mercaptoethanol.
b Molarity of the eluant, potassium phosphate buffer (pH 7.0) with 0.2 M 2-mercaptoethanol.

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TABLE II

\[ \text{K}_{\text{eq}} \text{ values of standard pteroylpolyglutamates from Sephadex G-15 and G-25 columns} \]

| Standard compounds | Eluant |
|--------------------|--------|
|                    | Water² | 0.02 M³ | 0.1 M³ | 0.5 M³ |
|                    | G-15   | G-15   | G-25   | G-15   | G-15   | G-25   | G-15   | G-25   |
| PteGlu₇            | 0.03   | 0.07   | 0.07   | 0.33   |
| PteGlu₆            | 0.07   | 0.11   | 0.10   | 0.25   | 0.14   | 0.43   | 0.24   |
| PteGlu₅            | 0.07   | 0.11   | 0.14   | 0.33   | 0.33   | 0.54   | 0.54   | 0.37   | 0.84   |
| PteGlu₄            | 0.24   | 0.48   | 0.31   | 0.70   |
| PteGlu₃            | 0.51   | 0.91   | 0.51   | 0.88   | 0.64   | 0.91   | 0.78   | 1.32   |
| PteGlu₂            | 0.51   | 0.68   | 1.13   | 1.12   | 1.16   | 1.10   | 1.19   | 1.71   |
| PteGlu₁            | 0.78   | 1.05   | 1.56   | 1.56   | 1.78   | 1.81   |
| 10-CHO-H₄PteGlu₃   | 0.10   | 0.45   |
| 5-CH₃-H₄PteGlu₅    | 0.65   | 0.51   |
| 5-CH₃-H₄PteGlu₇    | 0.44   |

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² Eluant, distilled water with 0.2 M 2-mercaptoethanol.

³ Molarity of the eluant, potassium phosphate buffer (pH 7.0) with 0.2 M 2-mercaptoethanol.

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**FIG. 1.** The elution diagram of folate compounds from a Sephadex G-25 column with a 0.1 M phosphate buffer eluant. The elution positions and the sample sizes are as follows: A, PteGlu, 250 nmoles; B, PteGlu₆, 200 nmoles; C, PteGlu₅-[U-¹⁴C]Glu-Glu 4,000 dpm (300 μCi per mM); D, PteGlu₄-[U-¹⁴C]Glu-Glu 1,500 dpm (600 μCi per mM); E, PteGlu₃-[U-¹⁴C]Glu-Glu 5,000 dpm (300 μCi per mM); F, PteGlu₅, 350 nmoles; G, [¹⁴C]CH₃-H₄PteGlu 8,000 dpm (50 mCi per mM); H, PteGlu 200 nmoles. Column dimension, 0.75 X 200 cm; sample volume, 2.0 ml; flow rate, 14 ml per hour; eluant, 0.1 M phosphate buffer, pH 7, with 0.2 M 2-mercaptoethanol.

**FIG. 2.** Sephadex G-25 column chromatography of folic acid derivatives in rat kidney. Rats, maintained on a nutritionally complete diet, were injected with 20 μCi of [²⁵³]PteGlu (40 μCi per mM). After 24 hours, the animal was killed by decapitation. The kidneys were removed immediately and folate derivatives were extracted (2). The elution pattern was obtained from chromatography of an equivalent of 1.0 g of fresh kidney together with 6,000 dpm of PteGlu₅-[U-¹⁴C]Glu-Glu and 4,000 dpm of [¹⁴C]CH₃-H₄PteGlu. Peak I was eluted at the same position as 10-CHO-H₄PteGlu₅; Peak II, at the position of H₄PteGlu₅ or CH₃-H₄PteGlu₅; Peak III, at the position of derivatives of H₄PteGlu₅; Peak IV, at the position of derivatives of H₄PteGlu₆; Peak V, at the position of 10-CHO-H₄PteGlu₆; Peak VI, at the position of derivatives of H₄PteGlu₆; Peak VII, 5-CHO-H₄PteGlu₈; Peak VIII, at the position of CH₃-H₄PteGlu₈; Peak IX, at the position of H₄PteGlu. Column dimension, 0.75 X 200 cm; sample volume, 2.0 ml; flow rate, 14 ml per hour; eluant, 0.1 M phosphate buffer, pH 7, with 0.2 M 2-mercaptoethanol.

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RESULTS AND DISCUSSION

The chromatographic behavior of the compound studied is expressed by its $K_v$ value (16), which is calculated as $K_v = V_v - V_0 / V_v$, where $V_v$ is the exclusion volume, $V_0$ the elution volume, and $V_v$ the volume of the gel bed. $V_0$ was determined as the elution volume of blue dextran and $V_v$ as the effluent volume at which the concentration of the eluted substance is maximum.

Table I shows the results of chromatography of pteroylmonoglutamates on Sephadex G-15 and G-25. No difference in the $K_v$ values of different derivatives of folic acid was obtained when distilled water was used as eluant. However, all monoglutamate forms of pteric acid except 10-CHO-H$_2$PteGlu are retarded considerably on G-15 or G-25 with 0.02 M phosphate buffer (pH 7.0) as eluant. The $K_v$ values of 5-CHO-H$_2$PteGlu, H$_2$PteGlu, and PteGlu are similar, whereas 5-CHO-H$_2$PteGlu is eluted between 10-CHO-H$_2$PteGlu and PteGlu (Table I). The same $K_v$ values as with 0.1 M potassium phosphate buffer (pH 7.0) are obtained with 0.1 M NaCl solution or 0.1 M potassium phosphate buffer, pH 6.0 or pH 8.0, as eluants. p-Aminobenzoic acid has a $K_v$ value similar to 5-CHO-H$_2$PteGlu and pteric acid is retarded even when distilled water is used as eluant.

There are in essence three different types of interactions in gel chromatography: first, interactions between ion-charged regions in the gel; second, Van der Waals interactions; third, adsorption of the substance onto the structure which forms the gel matrix. The interaction between charged groups can easily be eliminated by increasing the salt concentration of the buffer. This is not the case with folic acid, since an increase in the buffer strength decreases by 0.02 to 0.03 in comparison with PteGlu$_5$.

Polyglutamates appear in the eluent considerably sooner than would be expected from their molecular weight in pure aqueous solvent system. This phenomenon remains largely unexplained. However, it seems to be related to the negative charges of the gel. The glutamic acid moiety of folic acid also diminishes this adsorption. Whereas pteric acid is retarded considerably even in a distilled water solvent system, the elution volume of polyglutamate forms of folic acid is much less affected by an increase in the buffer strength, as shown in Table II. Polyglutamates appear in the eluent considerably sooner than would be expected from their molecular weight in pure aqueous solvent system. This phenomenon remains largely unexplained.

The interaction between charged groups can easily be eliminated by increasing the salt concentration of the buffer. This is not the case with folic acid, since an increase in the buffer strength decreases by 0.02 to 0.03 in comparison with PteGlu$_5$.

Table II shows the results of chromatography of pteroylmonoglutamates on Sephadex G-15 and G-25. No difference in the $K_v$ values of different derivatives of folic acid was obtained when distilled water was used as eluant. However, all monoglutamate forms of pteric acid except 10-CHO-H$_2$PteGlu are retarded considerably on G-15 or G-25 with 0.02 M phosphate buffer (pH 7.0) as eluant. The $K_v$ values of 5-CHO-H$_2$PteGlu, H$_2$PteGlu, and PteGlu are similar, whereas 5-CHO-H$_2$PteGlu is eluted between 10-CHO-H$_2$PteGlu and PteGlu (Table I). The same $K_v$ values as with 0.1 M potassium phosphate buffer (pH 7.0) are obtained with 0.1 M NaCl solution or 0.1 M potassium phosphate buffer, pH 6.0 or pH 8.0, as eluants. p-Aminobenzoic acid has a $K_v$ value similar to 5-CHO-H$_2$PteGlu and pteric acid is retarded even when distilled water is used as eluant.

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