CHROMATOGRAPHY OF PTEROYLGLUTAMIC ACID AND RELATED COMPOUNDS ON ION-EXCHANGE RESINS*

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INTRODUCTION

The high order of biological activity of pteridine compounds makes it desirable to establish the purity of the materials used in biochemical studies. In addition, it would be useful to have a method of purifying milligram quantities of these compounds.

A previous study in this laboratory[1] showed the suitability of ion-exchange chromatography for both analysis and purification of pteroylglutamic acid (folic acid, PGA) and 4-amino-PGA (Aminopterin). Subsequently this work was extended to other conjugated and unconjugated pteridines, including PGA analogs, and a preliminary report was presented[2]. The ion-exchange behavior ot these compounds has been compared under similar conditions, and the influence of several variables determined. The most useful procedure appeared to be the adsorption of pteridines from alkaline solution onto Dowex-1-chloride columns, and elution with dilute hydrochloric acid.

There have been several reports of the use of ion-exchange chromatography for pteridines, usually for the preparation of a single compound. SILVERMAN and coworkers[3, 4] have used Dowex-1 in the isolation of N^{10}-formyl-PGA and anhydroleucovorin. ZAKRZEWSKI AND NICHOL[5] separated PGA and leucovorin on Dowex-1-acetate. USDIN AND PORATH[6] studied several methods of separating PGA, leucovorin and pteroyltriglutamic acid, including a promising triethylaminoethyl cellulose anion-exchanger. The unconjugated pteridine biopterin was purified on Dowex-50 by PATTERSON, MILSTREY AND STOKSTAD[7].

MATERIALS AND METHODS

Dowex-1, -2, and -50 ion-exchange resins were obtained commercially in the 200–400 size, and in various degrees of cross-linking. Before use, a resin was washed several times by suspending it in water, allowing it to settle, and decanting the water and very fine particles. A slurry of the washed resin was poured into a glass tube containing a sintered glass disc covered by a disc of coarse filter paper.

Dowex-1 or -2 columns in the chloride form were prepared by washing with at

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least 200 ml of *N* HCl, followed by water until the washings were nearly neutral. The washing with acid and water was repeated after each column separation. In some cases new columns were washed with *N* NaOH and water prior to the acid wash, but this step was not essential. After washing was completed, excess resin was removed to give a column of the desired length, and a loose plug of glass wool was placed slightly above the resin surface.

Dowex-1-formate was prepared by washing the chloride resin with 2 *M* sodium formate until the effluent gave a negative chloride test, then with 4 *N* formic acid until the optical density of the effluent fell to less than 0.01. A final water wash removed excess formic acid. Columns were washed with 4 *N* formic acid and water after each separation run. It is important to avoid rubber connections in the system when formic acid solutions are used. Short lengths of Tygon plastic tubing, previously washed with 4 *N* formic acid, may be used.

Dowex-1-hydroxide was prepared by washing the chloride resin with several hundred ml of *N* NaOH, followed by water. Dowex-50-hydrogen form was prepared by washing with 4 *N* HCl and water.

Columns 1 cm in diameter and 5 to 14 cm in length have been used in these studies. Although these columns will adsorb large amounts of pteridines (a 7 cm long column of Dowex-1 adsorbs more than 100 mg of PGA from solution), the usable chromatographic capacity is very low. For columns of this cross-sectional area (0.8 cm^2), it appears that less than 5 mg of these compounds must be used in order to obtain complete separation of peaks. Larger amounts of material may be chromatographed on columns of correspondingly larger cross-sectional area.

The interstitial volume (holdback volume) was determined by a method similar to that of RIEMAN AND LINDENBAUM[8]. A column was washed thoroughly with 0.5 *N* HCl, and all acid above the resin removed. Washing with water to remove the acid, and titration of this wash, allowed calculation of the ml of acid in the column. Dowex-1-chloride of 4% cross-linking was found to have an interstitial volume of 51% of the total volume of the resin bed in a column.

Compounds to be chromatographed were suspended in a few ml of water, and dissolved by the addition of ammonium hydroxide to pH 8 to 9. The solution was allowed to run into the column and was washed in with a few ml of water. Eluants were added by gravity from bottles above the column, to give flow rates of 0.3–0.7 ml/min (0.4–0.9 ml/min/cm^2). The column and fraction collector were covered to exclude light. Fractions were collected on a time basis rather than with a photoelectric drop counter, to avoid exposure to light. The optical density of each fraction was determined in the Beckman DU spectrophotometer, usually at 300 mμ. Fractions to be studied further were usually neutralized; these solutions are generally of sufficient concentration to be used directly for biological studies. Fractions to be chromatographed on paper were evaporated to dryness in a vacuum desiccator at room temperature in the dark. Paper chromatography was carried out on Whatman No. 1 paper, in 0.1 *M* aqueous phosphate buffer of pH 7.0, and spots were located under ultraviolet light.

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RESULTS

Choice of resin

In preliminary tests, it was found that PGA was adsorbed from solution at pH 5 on a column of Dowex-50-hydrogen form cation-exchanger. Some of the compound was eluted with 3 *N* hydrochloric acid, but recovery was poor. Cation-exchangers were not studied further, because of the insolubility and instability of most of these compounds in the required concentrations of acid. In a single test with Dowex-2-chloride, PGA was satisfactorily adsorbed and eluted under the same conditons used with Dowex-1.

The remainder of the studies described here were carried out with Dowex-1. The percentage of cross-linking of the resin has a considerable effect on the chromatograms obtained with conjugated pteridines. A 10% cross-linked Dowex-1 (Dowex-1-X10) did not give as sharp elution peaks as 4% resin (Dowex-1-X4) under the same conditions (Fig. 1). A further difficulty experienced with 10% cross-linked resin was the incomplete elution of material in the peak fractions, with the remainder appearing in the *N* hydrochloric acid wash at the conclusion of the run. This was particularly true of experiments done at lower temperatures, or where the room temperature dropped considerably during a run. Although Dowex-1 of from 2 to 10% cross-linking has been used successfully, resin with about 4% cross-linking appears to give the most consistent results.

Temperature

The effects of temperature were studied by surrounding the ion-exchange column with a jacket through which water from a constant temperature bath was circulated. Fig. 2 illustrates the sharpening of the peak obtained when PGA is eluted at 35° (curve B) as compared with 25° (curve C), using similar flow rates. Increasing the

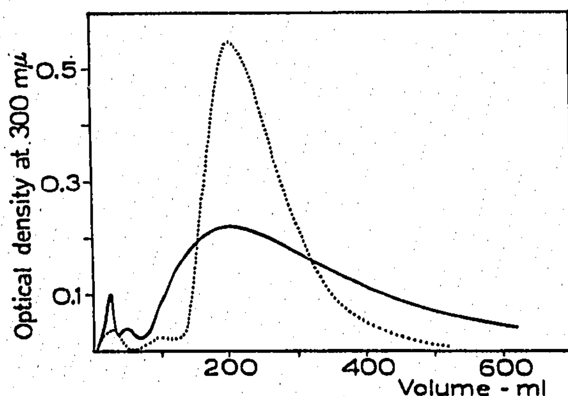

Fig. 1. Effect of resin cross-linking on elution of 2 mg PGA with 0.05 *N* HCl at 35°. 8 × 1 cm Dowex-1-chloride. —— 10% cross-linked, flow rate 0.35 ml/min; ····· 4% cross-linked, 0.45 ml/min.

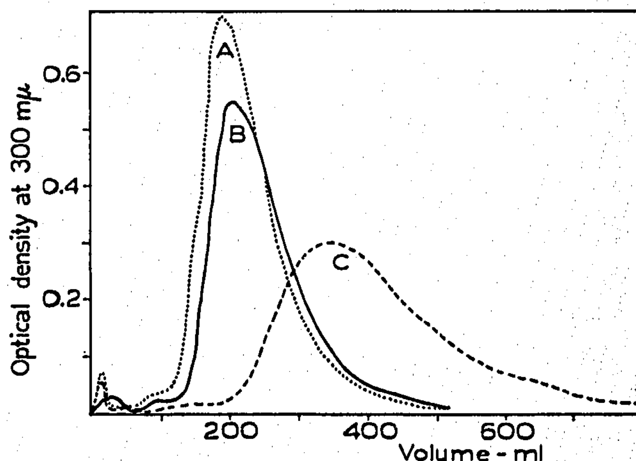

Fig. 2. Effect of temperature on elution of 2 mg PGA from 8 × 1 cm Dowex-1-X4-chloride with 0.05 *N* HCl. A: 35°, flow rate 0.73 ml/min. B: 35°, 0.45 ml/min. C: 25°, 0.53 ml/min.

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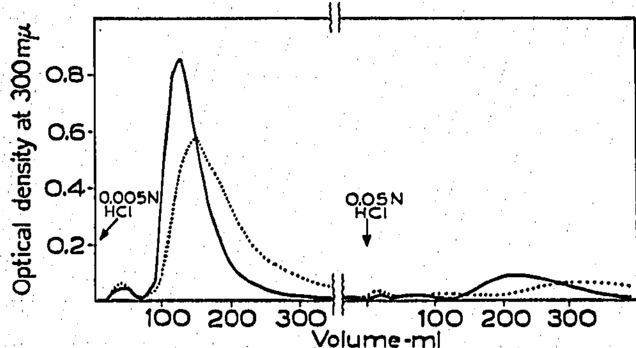

Fig. 3. Effect of temperature on elution of 2 mg Aminopterin from 8 × 1 cm Dowex-1-X4-chloride, 0.60 ml/min. —— 35°, ······ 25°.

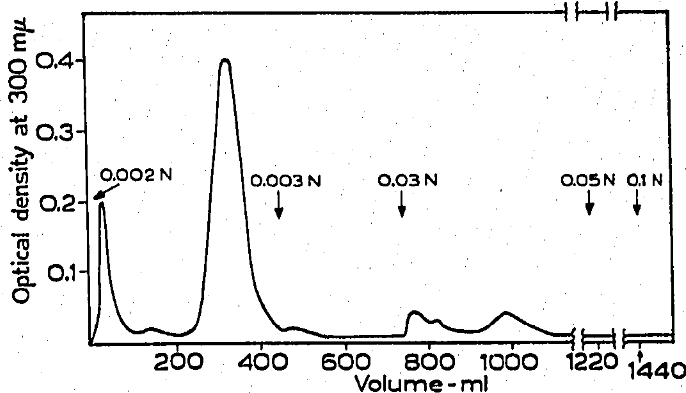

Fig. 4. Amethopterin, 1.5 mg, 7 × 0.9 cm Dowex-1-X2-chloride. Eluted with HCl, 0.50 ml/min.

flow rate at the higher temperature (curve A) results in a further slight narrowing of the peak.

The elution patterns of compounds which are eluted with more dilute acid are less markedly affected by temperature, as shown by the elution curves for Aminopterin (Fig. 3). The broad peak which is eluted with 0.05 *N* hydrochloric acid is PGA, present as an impurity. Most of the experiments described in this paper were done without temperature control, at room temperatures of approximately 25°.

Elution from Dowex-1-chloride with hydrochloric acid

The free pteridines tested were found to be eluted more readily than the conjugated pteridines. Thus xanthopterin, leucopterin, and biopterin were adsorbed from alkaline solution on Dowex-1-chloride, were not washed off with water, but were brought off with small volumes of 0.001 *N* hydrochloric acid. Among the PGA analogs, those in which an amino group replaces the 4-hydroxyl are more readily eluted. 4-Amino-N^{10}-methyl-PGA (Amethopterin) is eluted with 0.002 *N* hydrochloric acid (Fig. 4), and Aminopterin with 0.005 *N* hydrochloric acid (Fig. 3), while elution of PGA requires 0.05 *N* acid. The small peak preceding Aminopterin in Fig. 3 is a minor unidentified impurity. The fact that this material and PGA have been found in even the best samples of Aminopterin examined may reflect the instability of this analog on storage. The ultraviolet absorption spectra in acid and alkali found for Aminopterin purified on a Dowex-1-chloride column appear to be identical with those presented by SEEGER *et al.*[9].

Methopterin (N^{10}-methyl-PGA) is eluted with 0.03 *N* hydrochloric acid. Both PGA and 5-formyl-5,6,7,8-tetrahydro-PGA (Citrovorum factor, leucovorin) are eluted with 0.05 *N* hydrochloric acid, and it has not been possible to separate a mixture of these two compounds by hydrochloric acid elution. Since the acid lability of leucovorin[10] results in rapid decomposition even in 0.05 *N* acid (as shown by changes in ultraviolet absorption, and paper chromatograms), each fraction containing this compound would have to be neutralized immediately. A more practical eluant is

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sodium chloride, which avoids the use of acid, and separates PGA and leucovorin, as described below.

N^{10}-Formyl-PGA is eluted more rapidly than PGA with 0.05 N hydrochloric acid, and the two compounds may be separated completely by use of this solvent (Fig. 5).

Pteroic acid is very firmly adsorbed on Dowex-1, and only a fraction of it is removed with 0.5 N or 1 N hydrochloric acid. Complete elution from the resin requires 1 N hydrochloric acid at elevated temperatures, and this is not considered a practical procedure. Pteroic acids which may be present as impurities in the other compounds studied would therefore not contaminate the products purified by the above procedures involving acids of less than 0.1 N.

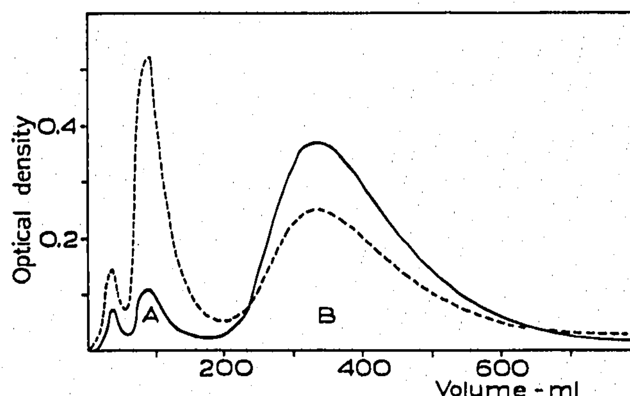

Fig. 5. Separation of 1 mg N^{10}-formyl-PGA (A) and 2 mg PGA (B). 9 × 1 cm Dowex-1-X4-chloride. Eluted with 0.05 N HCl, 0.62 ml/min. —— O.D. at 300 mμ, ------ O.D. at 250 mμ.

Gradient elution of some of the compounds has been carried out as an alternative to the step-wise change of acid concentration. Satisfactory results were obtained with 2 mg of Amethopterin on an 8 × 1 cm column, with the concentration gradient provided by running 0.005 N hydrochloric acid into a mixing flask containing 200 ml of water, and then to the column. Under similar conditions, PGA was eluted when 0.1 N hydrochloric acid was run into the mixing flask of water. In general, gradient elution appeared to be less sensitive in detecting trace impurities, and did not offer any particular advantage to offset the requirement for a more complicated experimental arrangement.

Elution from Dowex-1-chloride with hydrochloric acid and ethanol

The principal disadvantage of hydrochloric acid eluants is the low solubility of the pteridines in acid. When it is necessary to increase the capacity of the chromatographic system, alcohol may be added to the eluants. In the case of PGA, the presence of alcohol reduces the volume of solution required to elute the compound, and sharpens the peaks. With 5, 10, and 20% ethanol in 0.05 N hydrochloric acid, the peak fraction of PGA occurred at 240, 180, and 80 ml, respectively. When 20% ethanol was used, it was possible to reduce the acid concentration by one-half, without impairing resolution

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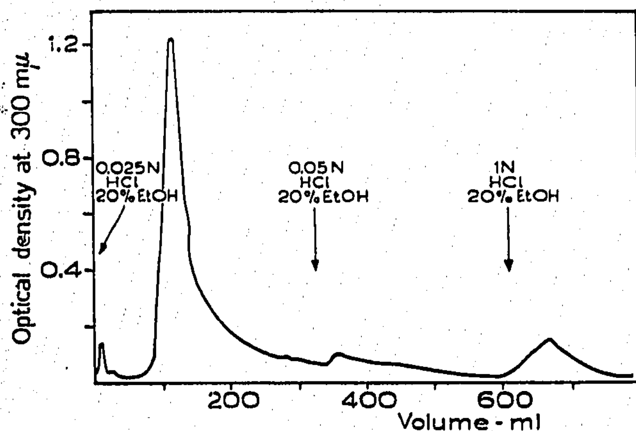

Fig. 6. Elution of 3 mg PGA with HCl–ethanol. 7 × 0.9 cm Dowex-1-X7.5-chloride, 0.12 ml/min.

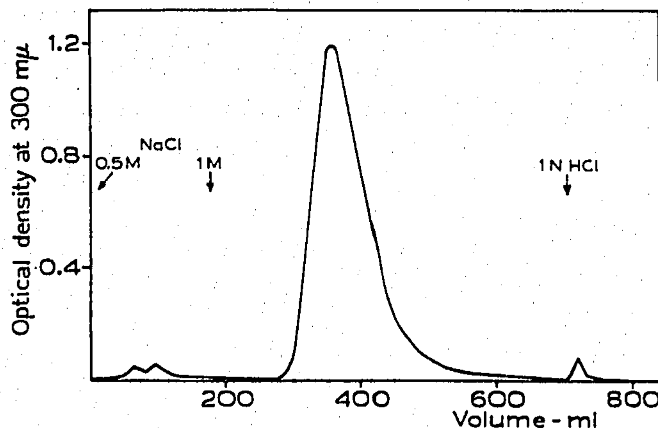

Fig. 7. Elution of 3 mg PGA with NaCl. 7 × 0.9 cm Dowex-1-X7.5-chloride, 0.27 ml/min.

or recovery of PGA (Fig. 6). When Aminopterin was chromatographed with 20% ethanol added to the 0.005 N hydrochloric acid usually used for elution, the compound was removed too rapidly for good resolution. Eluting with 10% ethanol in 0.005 N acid was more satisfactory. These solvents were not studied further.

Elution from Dowex-1-chloride with sodium chloride

The removal of pteridine compounds from anion-exchangers with acid requires solutions of pH 1.5 to 3. In some cases, notably leucovorin, this pH is low enough to cause chemical changes, and make it difficult to recover the separated compounds. An alternative to desorbing the pteridines by lowering the pH is the use of solutions of higher ionic strength. Thus it is possible to elute leucovorin with 0.5 M sodium chloride with no acid added, while PGA is eluted with larger volumes of 0.5 M or smaller volumes of 1 M sodium chloride (Fig. 7). A mixture of these two compounds is separated completely by use of these eluants. Aminopterin is eluted with larger volumes of 1 M sodium chloride than are required for PGA elution.

Elution from Dowex-1-formate with formic acid

It has been found that the formic acid solvents will separate the monoamino- from the diamino-folic acid compounds, but there is no resolution within each group. Aminopterin and Amethopterin were eluted with 0.05 N formic acid. When a mixture of these two compounds was chromatographed on a column of Dowex-1-X4-formate 8 cm in length, both compounds appeared in a single peak. Paper chromatography showed the first fractions of the peak to contain more Amethopterin than Aminopterin, and the last fractions to have the opposite proportion, but there was no separation. In a similar manner Methopterin and PGA are eluted with 1.5 N formic acid, but are not separated when a mixture of the two is chromatographed.

During the course of the work with formic acid solvents, it was noticed that fractions containing PGA, when evaporated and chromatographed on paper, showed

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the presence of another compound with light blue fluorescence at R_F 0.81. This compound was identified as N^{10}-formyl-PGA by its R_F, its spectrum in acid and alkali, and by the change in spectrum to that of PGA upon standing 24 h in 0.1 N sodium hydroxide[11]. It is not surprising that there was no evidence of formylation of the analogs, since Aminopterin is eluted with much more dilute formic acid, and the other compounds are methylated in the 10-position.

In a series of test tube experiments, it was found that approximately 23% of the PGA was formylated after standing 24 h at room temperature in 2 N formic acid, and evaporation in a vacuum desiccator. In 4 N formic acid there was 44% formylation after 2 h, and 48% after 24 h. The presence of Dowex-1-formate in the reaction mixture appeared to inhibit the formylation to some extent, and there was no formylation by the resin itself without the presence of formic acid.

DISCUSSION

The adsorption of pteridine compounds on the chloride form of an anion-exchanger, and elution with dilute hydrochloric acid, has proved to be the most satisfactory of the methods tested in this laboratory. When used with the proper regard for the low capacity of the solvents, and the influence of temperature, this procedure gives highly reproducible results. It is a sensitive method for detecting impurities, readily showing the presence of a component which contains 0.1 % of the total optical absorption of the starting material, if the impurity is separated from other peaks. The use of sodium chloride (also described by SILVERMAN *et al.*[3]) as eluant for compounds sensitive to acid is similar to the method of ZAKRZEWSKI AND NICHOL[5], using acetate buffers, and provides an alternative solvent system.

The recovery of known compounds adsorbed on Dowex-1-chloride columns has ranged from 60 to 100%, depending on experimental conditions. Low temperature appears to be the principal reason for incomplete elution, and recovery was essentially complete in experiments done at controlled temperatures. The presence of pteroic acid as an impurity would also contribute, since little or none of it is eluted from the resin.

Most of the compounds separated on the columns may be recovered unchanged in the effluent. It was shown previously[1] that Aminopterin is not deaminated to PGA during chromatography on Dowex-1-chloride, since purified Aminopterin may be rechromatographed without showing the presence of PGA. In the present study PGA, too, has been found to be stable to chromatography in this system. As noted above, leucovorin is rapidly destroyed by an acid solvent, but may be chromatographed with sodium chloride if the high salt concentration is not objectionable. The formylation of PGA when it is chromatographed in the formic acid system is an obvious objection to this procedure. In view of the biological activity of formylated pteridines, formate chromatography should be used with caution.

A number of solvents in addition to those discussed were tested briefly, and found to be unsatisfactory. Aminopterin was not eluted from Dowex-1-X2-chloride

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with 0.2 *M* ammonium hydroxide–0.025 *M* ammonium chloride buffer of pH 11.3. PGA was not eluted from the hydroxide form of Dowex-1 with 1 *N* ammonium hydroxide, nor from the chloride resin with 1 *M* ammonium carbonate or 0.5 *M* sodium citrate.

It has not proved possible to use this chromatographic method directly on crude extracts of natural materials. The presence of nucleotides, amino acids, and other compounds which are adsorbed and eluted under these same conditions makes impossible the detection of the much smaller amounts of pteridines usually present. The method should be applicable to material which has gone through some preliminary purification, such as charcoal adsorptions. The sensitivity and specificity of the method could be increased by the use of a biological assay of effluent fractions, in addition to optical density measurements.

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SUMMARY

Techniques are described for analysis and purification of free and conjugated pteridines by anion-exchange chromatography. The following compounds are adsorbed on Dowex-1-chloride and eluted with very dilute HCl or with NaCl: biopterin, leucopterin, xanthopterin, PGA, leucovorin, N^{10}-formyl-PGA, Aminopterin, Amethopterin, Methopterin. During chromatography in the formic acid system, appreciable amounts of PGA are formylated to N^{10}-formyl-PGA.

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