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Note

Rapid separation of folic acid derivatives by paired-ion high-performance liquid chromatography

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The enzyme, dihydrofolate reductase, which catalyzes the reduction of dihydrofolic acid to tetrahydrofolic acid, is inhibited by the folate antagonists including the antineoplastic agent, methotrexate[1]. In order to determine the extent of enzyme inhibition *in vivo* by a series of antifolate analogs, a more rapid method was needed than the traditional ion-exchange chromatography with DEAE-cellulose[2].

We describe here a method for the separation of folic acid, dihydrofolic acid, and tetrahydrofolic acid, as well as N-(*p*-aminobenzoyl)-L-glutamic acid, a degradation product of the folates, by paired-ion high-performance liquid chromatography (HPLC) on a μBondapak C_{18} column. By using paired-ion[3] instead of anion-exchange[4] HPLC, such problems as pH control and short column life may be avoided.

EXPERIMENTAL

Liquid chromatography

A Waters Associates Model ALC 202 liquid chromatograph (Waters Assoc., Milford, Mass., U.S.A.) fitted with a Model U6K injector and an ultraviolet detector set at 254 nm was employed. The column used was a 4.0 mm × 30 cm μBondapak C_{18} (Waters Assoc.). The columns were prepacked with 10-μm totally porous silica particles to which octadecyl groups were bonded through a Si–O–Si bond.

An isocratic solvent system of 35% methanol–water at ambient temperature was utilized. Both the methanol and water were 0.005 *M* tetrabutylammonium phosphate solutions (PIC Reagent A from Waters Assoc.) which were de-aerated by application of a vacuum and sonication. Organic solvents were purchased from Burdick and Jackson (Muskegon, Mich., U.S.A.). Water was first passed through a Milli-R04 purification system from Millipore Corporation (Bedford, Mass., U.S.A.). A flow-rate of 1.0 ml/min at pressures up to about 2000 p.s.i. was maintained.

All injections were made with pressure-lock liquid syringes from Precision Sampling Corporation (Baton Rouge, La., U.S.A.).

Chemicals

Folic acid (F), 7,8-dihydrofolic acid (FH_2), 5,6,7,8-tetrahydrofolic acid (FH_4), and N-(*p*-aminobenzoyl)-L-glutamic acid (PABG), were purchased from Sigma (St. Louis, Mo., U.S.A.). Standard solutions were prepared by first dissolving the com-

pound in basic solution ($KHCO_3$ in de-aerated water). A trace of 2-mercaptoethanol was added and the pH was quickly brought down to about pH 7.0 with hydrochloric acid to inhibit oxidative decomposition. Standards were protected from light and kept cold at all times.

RESULTS AND DISCUSSION

We decided to investigate first the separation of the three oxidation states of folic acid and the common decomposition product, N-(*p*-aminobenzoyl)-L-glutamic acid.

Fig. 1 shows the separation of the folates and PABG using 35% methanol–water–PIC Reagent A. Standard curves were obtained for folic acid and its reduced derivatives as well as PABG at 35% methanol–water–PIC Reagent A by plotting peak height *vs.* the amount of the compound injected into the column. The UV detector sensitivity was set at 64. The curves for all four compounds were linear over the range from 1 μg to the upper limit for each compound: PABG, 1–7 μg; FH_4, 1–28 μg; FH_2, 1–20 μg; F, 1–14 μg. An estimate of the minimum levels of the four compounds

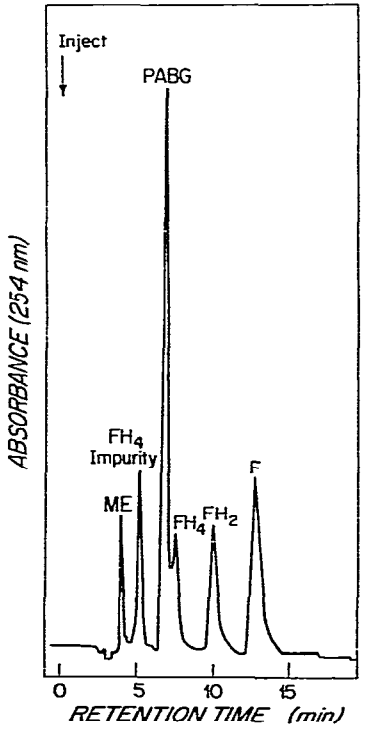

Fig. 1. Separation of the three oxidation states of folic acid and N-(*p*-aminobenzoyl)-L-glutamic acid on μBondapak C_{18}. Chromatographic conditions: column, 4.0 mm I.D. × 30 cm; mobile phase, 35% methanol–water–PIC Reagent A; flow-rate, 1.0 ml/min; column temperature, ambient; detector, Waters UV photometer (254 nm); attenuation, ×64. ME = 2-mercaptoethanol; PABG = N-(*p*-aminobenzoyl)-L-glutamic acid (7 μg); FH_4 = 5,6,7,8-tetrahydrofolic acid (7 μg); FH_2 = 7,8-dihydrofolic acid (7 μg); F = folic acid (7 μg).

that can be quantitated at a sensitivity setting of 8 is 0.2 μg of FH_4, 0.1 μg of FH_2, 0.05 μg of F, and 0.02 μg of PABG.

As depicted in Fig. 1, FH_4 samples routinely contained oxidation products of unknown structure, some of which may have remained bound to the column. The reversed-phase system used in this work allows one to flush the column easily of many such unwanted bound components with either 100% water or methanol. This step increases column lifetime and maintains resolution for successive runs and would be impractical to do by ion-exchange HPLC. We have used the same μBondapak C_{18} column for as many as 253 injections (F, FH_2, FH_4, antifolates, as well as biological samples) before high column back-pressure due to the adsorption of impurities necessitated changing to a new column.

Application of this method to determining the extent of formation of tetrahydrofolic acid in tissues of animals dosed with tritiated dihydrofolic acid and antifolate analogs[2] is now in progress in this laboratory.

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