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Note

Liquid chromatography assay of the calcium salt of citrovorum factor

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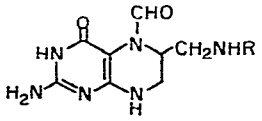
The calcium salt of citrovorum factor (1, CF·Ca) has been found to reverse the toxicity associated with a high dose of methotrexate (2) in the treatment of osteogenic sarcoma[1]. The high cost of purification of the salt by column chromatography prompted the investigation of the use of partially purified, but well characterized, samples of the salt in biological studies. Previously, a high-performance liquid chromatography (HPLC) method was described for the identification of CF and the impurities generated during its preparation[2]. These impurities included the pterins, *p*-aminobenzoylglutamic acid (PABGA), 10-formyl-7,8-dihydrofolic acid (10-CHO-DHF), and 10-formylfolic acid (10-CHO-FA). In this system, however, the CF and 10-CHO-FA peaks overlapped and limited the usefulness of the procedure for the determination of the amounts of these two components. In contrast, the use of a microparticulate reversed-phase packing with a mobile phase consisting of pH 4 citrate buffer and dioxane gave baseline separations for 10-CHO-DHF, 10-CHO-FA, and CF. This system coupled with elemental analyses provided a method for the assay of CF·Ca samples.



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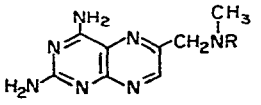

2

R = 4-$C_6H_4CONHCH(CO_2H)CH_2CH_2CO_2H$

EXPERIMENTAL

Liquid chromatography

All separations were carried out with an ALC-242 liquid chromatograph equipped with a UV detector (254 nm), an M-6000 pump, and a column (30 cm × 4 mm I.D.) packed with μBondapak C_{18} (Waters Assoc., Milford, Mass., U.S.A.) at room temperature with an eluting solvent of pH 4 citrate buffer–dioxane (94:6) (Burdick & Jackson, Muskegon, Mich., U.S.A.) at a flow-rate of 1 ml/min. All chromatograms were recorded at a chart speed of 1 cm/min and an attenuation of 16 a.u.f.s. at a pressure of about 1000 p.s.i. Solutions of the samples were injected with

a Hamilton Model 701 syringe (10 μl capacity, 0.0185 in. O.D. needle). For the preparation of the buffer, citric acid monohydrate (21.01 g) and sodium citrate dihydrate (29.41 g) were dissolved separately in distilled water (1000 ml). After filtration of each solution through a Millipore filter (type HAWP04700, 0.45 μm), a mixture of the citric acid solution (330 ml) and the sodium citrate solution (170 ml) was diluted to 1000 ml with filtered water.

Standard compounds

Pterin was purchased from Aldrich (Milwaukee, Wisc., U.S.A.), and pterin-6-methanol was prepared from 2,4,5-triaminopyrimidin-6(1H)-one and 1,3-dihydroxyacetone[3]. The calcium salts of PABGA and 10-CHO-FA were prepared in an aqueous solution and precipitated by the addition of ethanol. Reaction of 5,10-methenyl-5,6,7,8-tetrahydrofolic acid chloride with aqueous base (pH 11) at room temperature gave mainly 10-formyl-5,6,7,8-tetrahydrofolic acid and trace amounts of CF, 10-CHO-DHF, PABGA, and pterins. Further treatment of this sample in water (pH 7.4) in the presence of air resulted in a lowering of the pH to 6.7 and the precipitation in low yield of a 2:1 mixture of the calcium salts of 10-CHO-DHF and 10-CHO-FA. In contrast, treatment of a neutral, aqueous solution of the methenyl compound at 100° gave the impure CF·Ca, which was purified by Florisil chromatography[4].

Standard curves

Stock solutions were prepared in volumetric flasks by dissolving weighted samples of the calcium salts of PABGA, 2:1 10-CHO-DHF:10-CHO-FA, 10-CHO-FA, and CF in distilled water. Aliquot portions from each stock solution were diluted in volumetric flasks to give a series of concentrations from which the chromatograms were determined on 10-μl portions. These operations were carried out as rapidly as possible to minimize errors resulting from oxidative decomposition of the samples. Standard curves were obtained by plotting either peak height (PABGA) or peak area (peak height $\times$ peak width at one-half peak height) *versus* the equivalent amount of the *anhydrous* calcium salt injected. In the preparation of the standard curve for 10-CHO-DHF, the amount of 10-CHO-FA in each dilution was determined from its standard cuvre and subtracted from the weight of the mixture. The standard curves were linear in the following concentration ranges: PABGA·Ca (0–0.6 μg), 10-CHO-DHF·Ca (0–0.5 μg), 10-CHO-FA·Ca (0–0.4 μg), and CF·Ca (0–7 μg). Before an assay, the standard curves were checked by injection of a known amount of a fresh solution of the standards. Over a two-day period, an error of less than 5% was observed.

RESULTS AND DISCUSSION

A sample of unpurified CF·Ca was dried to constant weight *in vacuo* over P_2O_5 and analyzed for C, H, N, Ca, ash (CaO). This sample dissolved in water gave the chromatogram shown in Fig. 1. The amount of each identifiable component was determined from a chromatogram in which the sample weight was 10.23 μg (Table I). The results indicated that this sample contained about 78% CF·Ca, which on an anhydrous basis corresponded to about 86% CF·Ca and 14% impurities. The only major unidentified component ($<3\%$) appeared as a shoulder on the side of the

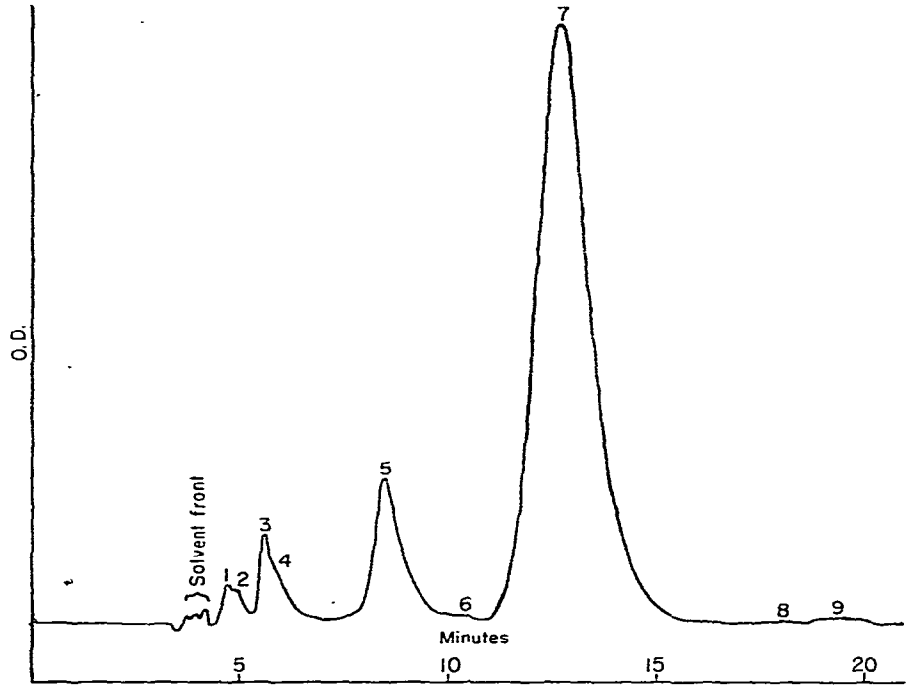

Fig. 1. HPLC of unpurified CF·Ca. Solvent: pH 4 citrate buffer–dioxane (94:6); sample weight, 8.26 μg (in water). 1 = Pterin-6-methanol; 2 = pterin; 3 = *p*-aminobenzoyl-L-glutamic acid; 4 = unidentified; 5 = 10-formyl-7,8-dihydrofolic acid; 6 = 10-formylfolic acid; 7 = CF; 8 and 9 = unidentified.

TABLE I

HPLC ASSAY RESULTS

This assay represents an analysis of a solution of the sample because the presence of either 10-formyl-5,6,7,8-tetrahydrofolic acid or 5, 10-methenyl-5,6,7,8-tetrahydrofolic acid in the solid would probably be detected as 10-formyl-7,8-dihydrofolic acid in the solution.

*Components**	*Amount (%)*
Anhydrous calcium salts	
p-Aminobenzoyl-L-glutamic acid	3.1
10-Formyl-7,8-dihydrofolic acid	4.6
10-Formylfolic acid	<0.5**
Citrovorum factor	78
Pterins	
Pterin-6-methanol	0.6**
Pterin	0.4**
*Other components****	
Ethanol	4.1
Water	5.7
Unidentified material	
Peaks 4, 8, and 9 (Fig. 1) and undetected material	3.0

* Elemental analysis of the impure CF sample gave the following results: Calcd. for $C_{20}H_{21}N_7O_7 \cdot Ca \cdot 0.5C_2H_6O \cdot 1.8H_2O$ (MW 567): C, 44.49; H, 4.91; N, 17.29; Ca, 7.07; ash (CaO), 9.89. Found: C, 44.49; H, 4.97; N, 17.29; Ca, 7.18; ash (CaO), 9.65.

** Estimated from peak height.

*** Calculated from elemental analyses.

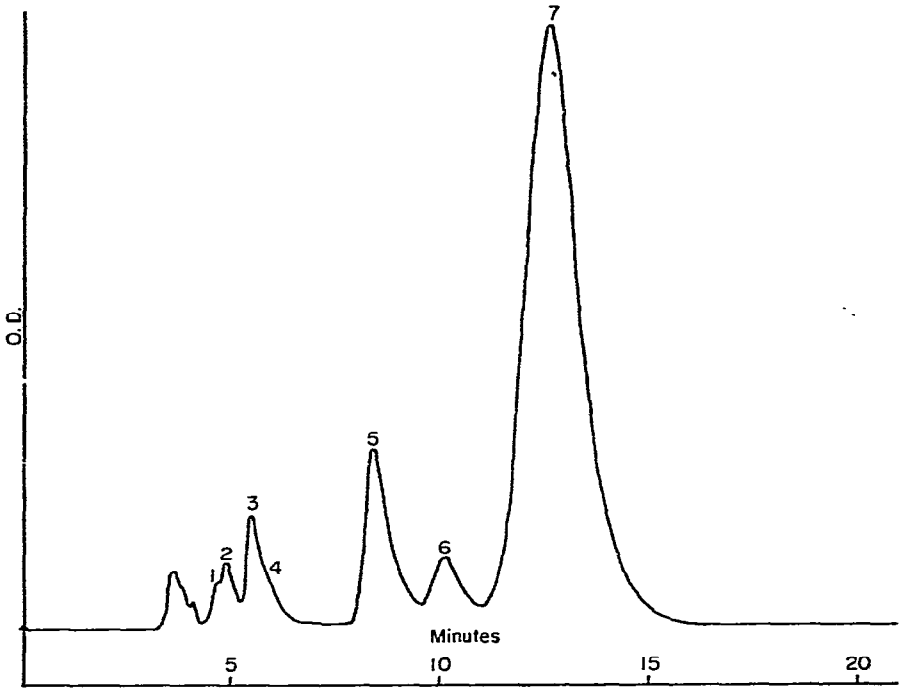

Fig. 2. HPLC of a mixture of components at concentrations similar to those found in Fig. 1. Solvent: pH 4 citrate buffer–dioxane (94:6). 1 = Pterin-6-methanol (0.059 μg); 2 = pterin (0.056 μg); 3 = calcium *p*-aminobenzoyl-L-glutamate (0.197 μg); 4 = unidentified; 5 = calcium 10-formyl-7,8-dihydrofolate (0.403 μg); 6 = calcium 10-formylfolate (0.207 μg); 7 = CF·Ca (6.82 μg).

PABGA peak. Although the error for each component is considered to be within ±5%, a lower error should be obtainable if the assay were used routinely.

Peak assignments were confirmed by the determination of the chromatogram of a mixture of the components (Fig. 2) at concentrations similar to those found in the assayed sample (Fig. 1). In the chromatogram of the known mixture, the peak area was 27% high for PABGA probably because this compound was present in small amounts in many of the other components of the mixture. This CF sample was as effective as a commercial sample in reversing the toxicity of a high dose of methotrexate in mice.

ACKNOWLEDGEMENT

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