# Chromatographic Assays for Folic Acid

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**Abstract** □ TLC and high-pressure liquid chromatographic (HPLC) assays for folic acid were developed. In the TLC procedure, the folic acid band was extracted from the silica gel after development and determined spectrophotometrically by an oxidation and Bratton–Marshall sequence. A column packed with octadecylsilane chemically bonded to microparticulate silica gel was used for the HPLC assay. Potentiometric determination of water in folic acid samples was necessary to obtain accurate purity values. In a comparison of four assay methods, a direct colorimetric method gave precise, but unspecific, results, while the USP XIX colorimetric method lacked both precision and specificity. The two chromatographic methods were both precise and specific and gave the same assay results. Both methods separated *p*-aminobenzoic acid, *N*-(*p*-aminobenzoyl)-L-glutamic acid, 2-amino-1,4-dihydro-4-oxo-6-pteridinecarboxylic acid, 2-amino-4(1*H*)-pteridinone, and several unidentified impurities from folic acid.

**Keyphrases** □ Folic acid—TLC and high-pressure liquid chromatographic analyses, bulk drug □ TLC—analysis, folic acid bulk drug □ High-pressure liquid chromatography—analysis, folic acid bulk drug □ Vitamins—folic acid, TLC and high-pressure liquid chromatographic analyses, bulk drug

Folic acid has been analyzed by direct UV spectroscopy (1), fluorometry after oxidation to 2-amino-1,4-dihydro-4-oxo-6-pteridinecarboxylic acid (1–3), polarography (4, 5), and colorimetry (6–9). The USP XIX colorimetric method (6) involves permanganate oxidation of folic acid to *p*-aminobenzoic acid, which is diazotized and coupled with the Bratton–Marshall reagent [*N*-(1-naphthyl)ethylenediamine dihydrochloride] for determination by spectrophotometry. In this standard addition method, assay values are corrected for diazotizable impurities by performing the colorimetric reaction on unoxidized material. Other colorimetric methods involve reduction of folic acid with zinc instead of oxidation (7, 9) and conversion of diazotizable impurities to phenols before permanganate oxidation (8). Experiments in this laboratory showed that commercial folic acid contains fluorescent pteridines, diazotizable amines, and compounds other than folic acid that cleave to give the functional groups measured in the colorimetric assays. Therefore, these methods are unspecific, and separation of folic acid from its impurities prior to quantitation is necessary.

A TLC method with colorimetric quantitation (10) and a similar method with UV quantitation (11) were reported, but less mobile impurities were *not* separated in the solvent systems used since folic acid did not move from the origin. Williams *et al.* (12) and, separately, investigators in this laboratory used high-pressure anion-exchange chromatography, but these systems require a solvent gradient which makes quantitation unreliable. Also, the separation of impurities from folic acid was not fully demonstrated. This paper reports the development of improved TLC and high-pressure liquid chromatographic (HPLC) assays.

## EXPERIMENTAL

**Materials**—Reference compounds for *p*-aminobenzoic acid, 2-amino-1,4-dihydro-4-oxo-6-pteridinecarboxylic acid, 2-amino-4(1*H*)-pteridinone, *N*-(*p*-aminobenzoyl)-L-glutamic acid, dihydrofolic acid, and tetrahydrofolic acid were used as received[1]. Leucovorin calcium was also used as obtained[2]. Except for commercial folic acid samples, other reagents were USP, NF, or ACS grade. Two lots of USP reference standard folic acid[3], designated A and B, were used for assay evaluations.

**Moisture Determination**—The water content of folic acid samples was determined by direct potentiometric titration[4] according to USP XIX Method I (Karl Fischer determination) (13) except that chloroform–methanol (4:1) was used as the solvent in place of methanol[5]. Sample sizes of 10–15 mg and 100 ml of solvent were used. Since folic acid is hygroscopic, the water content was determined at the time the samples were weighed.

**Chromatographic Assays**—*Standard Preparation*—Transfer 40-, 50-, and 60-mg samples of USP folic acid reference standard, accurately weighed and corrected for moisture, to individual 5-ml volumetric flasks. Then dissolve and dilute to volume with 0.3 *N* ammonium hydroxide. These solutions should be made fresh daily.

*Assay Preparation*—Prepare as directed under *Standard Preparation*, using the 50-mg sample size.

**TLC Assay**—On suitable TLC plates coated with a 250-μm layer of fluorescent silica gel[6], predeveloped with ethyl acetate and air dried, make a 2-cm streak with 10 μl of the assay preparation. Apply a 2-cm, 10-μl streak of spotting solvent to serve as a blank. Similarly streak 10-μl portions of the standard preparations. Develop the chromatograms about 13 cm in 1.48 *N* ammonium hydroxide–1-propanol (1:3). Remove the plate from the developing chamber, dry it under a nitrogen stream for 5–10 min, and view it under long and short wavelength UV light.

Mark the folic acid main band, taking care to exclude adjacent impurity bands. Quantitatively and separately remove the silica gel mixture containing each of the main spots of the standard and assay preparations. Remove a corresponding area of silica gel from the blank, spotting at the same $R_f$ as the folic acid main spot. Transfer the material to separate 25-ml test tubes with polytef-lined screw caps, add exactly 15 ml of 0.17 *M* dibasic potassium phosphate to each, and shake mechanically for 15 min. Centrifuge the tubes for 10 min at 1000 rpm (10-cm radius) or until clear.

Pipet a 10.0-ml aliquot of each preparation into separate 25-ml conical flasks, add 1.0 ml of 0.4% (w/v) aqueous potassium permanganate solution (freshly prepared and filtered), and allow to stand for 2–3 min. Add 1.0 ml of 2% (w/v) sodium nitrite solution and 1.0 ml of 5 *N* hydrochloric acid, mix, and allow to stand for 2 min. Add 1.0 ml of 5% (w/v) ammonium sulfamate solution, and mix with swirling until the nitrogen dioxide has been dispelled. Then add 1.0 ml of 0.1% (w/v) *N*-(1-naphthyl)ethylenediamine dihydrochloride solution (freshly prepared and filtered), mix, and allow to stand for 10 min.

Determine the absorbance of each solution at the maximum at about 550 nm, with a suitable spectrophotometer[7], using 0.17 *M* dibasic potassium phosphate as the blank. Calculate the quantity, in milligrams, of folic acid in each milliliter of the assay preparation by the formula $C(A_U - A_B/A_S - A_B)$, in which $C$ is the concentration, in milligrams per milliliter, of folic acid in the standard preparations; $A_U$ and $A_S$ are the absorbances of the solutions from the assay and standard preparations, respectively; and $A_B$ is the absorbance from the blank.

**HPLC Assay**—*Chromatographic System*—The chromatograph[8] was equipped with a fixed wavelength (254 nm) UV detector and a 30-cm × 4-mm i.d. stainless steel column packed with siliceous microbeads, 5–10 μm in diameter, to which octadecylsilane[9] was chemically bonded.

[1] Sigma Chemical Co.
[2] Lederle Laboratories.
[3] USP Reference Standards, Rockville, MD 20852.
[4] Radiometer, Copenhagen, Denmark.
[5] M. M. Tuckerman, Temple University School of Pharmacy, Philadelphia, Pa., personal communication.
[6] Merck silica gel F-254 precoated glass plates.
[7] Cary model 14.
[8] DuPont model 820.
[9] The method was developed using μBondapak $C_{18}$, Waters Associates. In this or in other laboratories, comparable separations were obtained with the following columns: Spherisorb 10 μm ODS, Spectra-Physics; Zorbax ODS, du Pont; and Partisil-10 ODS-2, Whatman.

**Table I—Assay Results**

| Day[a] | USP Colorimetric Assay Lot B/A, % | Lot A/B, % | Lot A/A, % | Direct Colorimetric, Lot B/A, % |
|---|---|---|---|---|
| 1 | 108.8 | 103.2 | 101.9, 105.2 | 101.6 |
| 2 | 103.3 | 105.0 | 108.0, 108.0 | 103.3 |

TLC Assay

| Day[a] | Lot | Mean, $\frac{\text{Absorbance}}{\mu g \text{ Spotted}} \times 100$ | $n$ | *RSD*, % | B/A + 95% C.I.[b] |
|---|---|---|---|---|---|
| 1 | B | 0.476 | 3 | 1.63 | 101.9 ± 1.6 |
| 1 | A | 0.467 | 3 | 0.98 | |
| 2 | B | 0.468 | 3 | 0.47 | 102.2 ± 0.5 |
| 2 | A | 0.458 | 3 | 0.40 | |

HPLC Assay

| Day[a,c] | Lot[d] | $n$ | *RSD*[e], % | % A + 95% C.I.[b] |
|---|---|---|---|---|
| 1 | B | 5 | 1.56 | 101.7 ± 1.2 |
| 1 | A | 4 | 0.93 | |
| 2 | B | 5 | 1.20 | 101.6 ± 1.2 |
| 2 | A | 8 | 1.47 | |
| 3 | B | 5 | 1.18 | 101.2 ± 0.7 |
| 3 | A | 5 | 0.67 | |
| 4 | B | 5 | 0.87 | 101.0 ± 0.9 |
| 4 | A | 3 | 0.36 | |
| 3 | C | 4 | 1.18 | 93.8 ± 0.8 |
| 4 | C | 3 | 0.83 | 95.3 ± 0.7 |
| 4 | D | 3 | 0.60 | 96.5 ± 0.6 |
| 5 | D | 4 | 1.78 | 96.7 ± 1.4 |
| 5 | A | 4 | 1.46 | |
| 4 | E | 3 | 0.87 | 96.7 ± 0.7 |
| 5 | E | 3 | 1.17 | 97.5 ± 1.7 |

[a] Values for Days 1 and 2 are based on the same moisture determinations for each USP lot, A and B. [b] TLC and HPLC values for Days 1 and 2 are based on sampling from the same solutions. [c] Values for Days 3–5 are based on the same moisture determinations for each lot. Lot A and B material used for Days 3 and 4 was drawn from different sublots ($A_2$ and $B_2$ in Table III) than those used for Days 1 and 2 ($A_1$ and $B_1$ in Table III) and had different moisture levels. [d] Commercial lots of folic acid are designated by C, D, and E. [e] Precision of multiple, $n$, injections of the same sample preparation.

Prepare the mobile phase by adding 35.1 g of sodium perchlorate monohydrate, 1.36 g of monobasic potassium phosphate, 6.94 ml of 1 *N* potassium hydroxide, and 40 ml of methanol to a 1-liter volumetric flask and diluting to volume with water. If necessary, adjust the pH to 7.2 with 1 *N* potassium hydroxide. The amount of methanol may be varied by several percent to give a suitable retention time for folic acid as long as system suitability requirements are met. Degas and membrane filter[10] the mobile phase. During chromatography, maintain a pressure of about 76 atm and ambient temperature so that the flow is about 1.0–1.5 ml/min.

*System Suitability Preparation*—Prepare a solution containing about 5 mg/ml each of folic acid and leucovorin calcium in 0.3 *N* ammonium hydroxide. Filter before use.

*System Suitability Test*—Chromatograph six to 10 injections of the 50-mg standard preparation, and measure the peak response[11] as directed under *Procedure*. The relative standard deviation, 100 × (standard deviation/mean response), for the peak response does not exceed 2%. When 5 μl of the system suitability preparation is injected, the resolution factor (14) between leucovorin calcium and folic acid is not less than 3.6.

*Procedure*—Introduce equal volumes (about 3 μl) of the assay preparation and of the 50-mg standard preparation into the high-pressure liquid chromatograph by means of a suitable sampling valve or microsyringe. Measure the response of the peaks, at identical retention times, obtained with the assay and standard preparations. Then calculate the quantity, in milligrams, of folic acid in each milliliter of the assay preparation by the formula $C(A_U/A_S)$, in which $C$ is the concentration in milligrams per milliliter of folic acid in the standard preparation, and $A_U$ and $A_S$ are the peak responses of the solutions from the assay and standard preparations, respectively.

**Direct Colorimetric Assay**—*Standard Preparation*—Dissolve about 50 mg of USP folic acid reference standard, accurately weighed, in a mixture of 50 ml of water and 2 ml of 6 *N* ammonium hydroxide in a 100-ml volumetric flask. When solution is complete, dilute with water to volume; mix. Dilute a measured portion of this stock solution with 0.17 *M* dibasic potassium phosphate to give a concentration of about 10 μg of folic acid/ml, calculated on the anhydrous basis.

*Assay Preparation*—Prepare as directed under *Standard Preparation*.

*Procedure*—Add 5 ml of the standard and assay preparations to separate 25-ml conical flasks. Continue as directed under *TLC Assay*, beginning with "add 1.0 ml of 0.4% (w/v) aqueous potassium permanganate solution . . . ." Calculate the quantity, in micrograms, of folic acid in each milliliter of the assay preparation by the formula $C(A_U/A_S)$, in which $C$ is the concentration in micrograms per milliliter of folic acid in the standard preparation, and $A_U$ and $A_S$ are the absorbances of the solutions from the assay and standard preparations, respectively.

**Correlation of Impurity Separations**—Commercial folic acid (300 μg) was analyzed by HPLC, using 0.1% (w/v) ammonium carbonate as the elution solvent. Fractions containing individual impurities were collected and reduced under a nitrogen stream to approximately 20 μl. Ten microliters was examined by TLC using the assay system, and 10 μl was subjected to HPLC using the perchlorate–phosphate–methanol system.

## RESULTS AND DISCUSSION

**TLC**—Chromatographic systems for folic acid and related compounds were evaluated. In many of these systems, folic acid remained at the origin and/or the impurities did not separate. None of the published systems separated impurities as well as the system described in this report (Fig. 1).

When the sample was extracted from the silica gel with 0.3 *N* ammonium hydroxide, silicon dioxide interfered with the UV quantitation of folic acid at 255, 283, and 365 nm. However, no interference was observed using the colorimetric determination at 550 nm; precise results (Table I) were obtained. Three brands of commercial silica gel plates[12] yielded the same results.

A plot of absorbance *versus* weights of folic acid spotted gave a linear regression coefficient of 0.999 for amounts between 50 and 200 μg. Upon extrapolation below 50 μg, the line did not pass through the origin; however, a blank determination gave a zero absorbance reading. Although the curve was not linear below the 50-μg level, the assay was made well within the linear region of the curve.

[10] Millipore HA, 0.45 μm.

[11] Infotronics CRS digital integrator, Columbia Scientific.

[12] E. Merck, Brinkmann; Analtech; and Quantum.

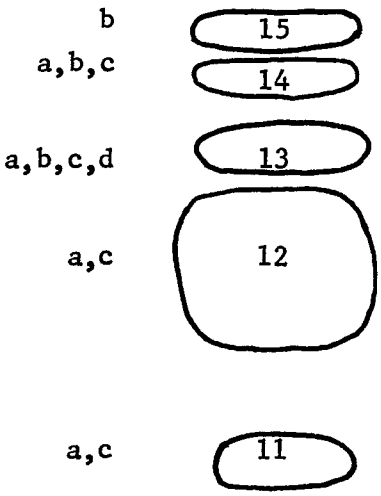


**Figure 1**—*Thin-layer chromatogram of folic acid (100 μg). Visualization was by shortwave UV light (a), longwave UV light (b), potassium permanganate followed by diazotization and Bratton–Marshall reagents (c), and diazotization and Bratton–Marshall reagents only (d). The indicated bands cochromatographed with the following compounds: 12, folic acid; 13, 2-amino-1,4-dihydro -4-oxo-6- pteridinecarboxylic acid and N-(p-aminobenzoyl) -L- glutamic acid; and 15, 2-amino-4(1H)-pteridinone.*

Duplicates of the standards, blank, and two samples can be determined in 1 day, allowing 2.5 hr for plate development. The use of autopipets is recommended for the colorimetric sequence. It may not be necessary to have all standard preparations for each testing, preparing only the 50-mg/5-ml standards and performing the standard curve only at set intervals. The standard curve procedure given here is designed to ensure accurate assay values for samples that may be considerably different from the standard.

**HPLC**—Based on reported use in the assay of methotrexate (15) and other acidic compounds (16), columns packed with octadecylsilane bonded to 5–10-μm microparticulate silica gel were investigated. Water–methanol solvent systems resulted in poor retention and tailing of folic acid. A 0.1% ammonium carbonate mobile phase gave resolution of several impurities, but the reproducibility of this system was poor. Phosphate buffers, pH 6–8, were tried; a pH near 7.2 gave optimum results.

The perchlorate–phosphate–methanol system described under *Chromatographic System* gave the best balance between resolution of impurities and retention times (Fig. 2). The system gave reproducible separations on columns from several different manufacturers[9], except that occasionally an individual column did not adequately resolve unidentified component 4 (Fig. 2). Anticipated variations in column retentiveness were overcome by altering the methanol concentration by several percent. After several months of use, no column degradation peculiar to this mobile phase was noted. However, after several months of general use, some commercial columns showed signs of degradation, resulting in unsymmetrical peaks with two maxima. This phenomenon may be due to compression of the column packing, which leads to dead space at the column inlet.

Numerous compounds were investigated as potential internal standards, but due to the number of impurities normally present in folic acid, it was difficult to find a suitable internal standard. To compensate for syringe sample size errors, either multiple syringe injections of folic acid sample and standard preparations or a more precise valve loop system were used. Leucovorin is not well resolved from several minor unidentified folic acid impurities, but its resolution from folic acid enables it to be used in a system suitability test (Fig. 3). This test specifies a minimum resolution between folic acid and leucovorin which, consequently, defines a system capable of separating impurities from folic acid. Systems with a resolution less than 3.6 gave an inadequate separation of impurities. Systems with resolution greater than 3.6 gave adequate separation of impurities, except that occasionally a column did not adequately separate component 4 (Fig. 2).

A comparison of chromatographic parameters at two temperatures is given in Table II. A 35–40° temperature may be used to reduce retention times of folic acid; however, component 4 (Fig. 2) was not completely resolved under these conditions. Resolution between leucovorin and folic acid was also reduced from approximately 4.0 to 3.0, and there was a decrease in the number of theoretical plates. This decrease indicated that absorption rather than true partition chromatography was being observed and that folic acid was chromatographing as a surfactant rather than partitioning as a solute. This finding was also consistent in part with the TLC results, where adsorption chromatography was observed. Impurities with thin-layer mobilities greater than folic acid also exhibited lower HPLC retention times, while an unidentified impurity [HPLC component 6 (Fig. 2), TLC band 11 (Fig. 1)] was more retentive than folic acid in both systems.

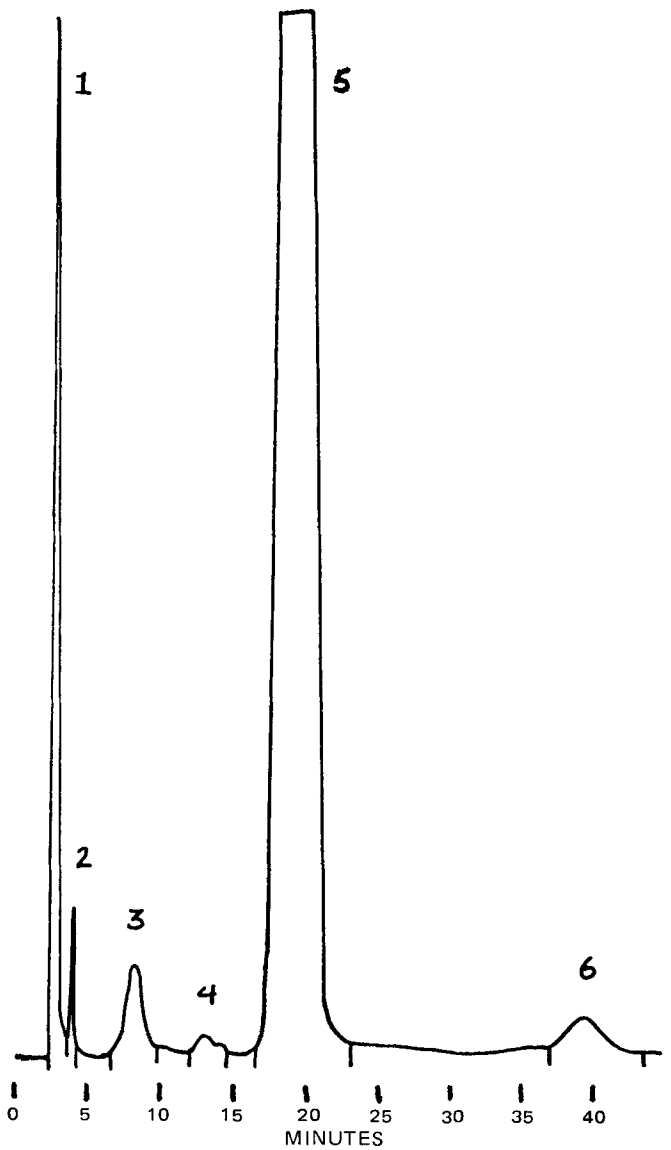


**Figure 2**—*High-pressure liquid chromatogram of folic acid (30 μg) using the assay system and a 254-nm photometer at 0.08 aufs. The indicated peaks cochromatographed with the following compounds: 1, p-aminobenzoic acid and N-(p-aminobenzoyl)-L-glutamic acid; 2, 2-amino-1,4-dihydro-4-oxo-6-pteridinecarboxylic acid; 3, 2-amino-4(1H)-pteridinone; and 5, folic acid.*

Plots of both peak heights and integrator counts *versus* amount injected by microsyringe gave a linear regression coefficient of 0.999 for amounts between 15 and 60 μg. Injections in amounts up to 140 μg were made. Electronic measurements remained linear, but a saturation phenomenon was indicated above 60 μg by a gradual leveling in the slope of the peak height *versus* amount curve. A standard and four samples with at least triplicate injections of each can be determined in 1 day.

**Folic Acid Injection**—The HPLC system probably can be adapted for assay of folic acid injection. The product examined[13] contained 5 mg of folic acid/ml in pH 9 sodium hydroxide with edetate sodium[14] and benzyl alcohol added. A 6-μl aliquot was injected directly into the instrument without prior sample preparation. In addition to the benzyl alcohol, $k' = 4.4$, two unidentified components ($k' = 0.75$ and 10.1) were separated that were not observed in the lots of folic acid examined.

**Identity of Impurities**—Where authentic samples of reported impurities could be obtained, direct chromatography of them in both TLC

[13] Folvite, Lederle.
[14] Sequestrene Sodium, Lederle.

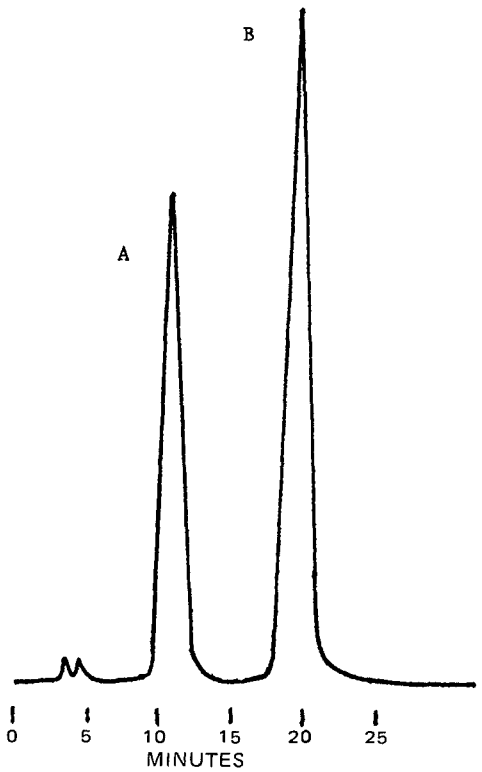


**Figure 3**—*High-pressure liquid chromatogram of leucovorin calcium (A) and folic acid (B) using the assay system.*

and HPLC systems demonstrated that all were separated by both methods. There was no rank-order correlation between $R_f$ and $k'$ values.

Figure 1 shows a typical chromatogram of folic acid, $R_f$ 0.50, well separated from its impurities at $R_f$ 0.28, 0.66, 0.74, and 0.80. Plates were sprayed with colorimetric reagents as directed under *TLC Assay*. The unidentified impurities in bands 11 and 14 reacted with colorimetric reagents only after potassium permanganate treatment, indicating that these impurities are measured along with folic acid in the USP XIX and similar colorimetric methods.

The presence of *N*-(*p*-aminobenzoyl)-L-glutamic acid in band 13 was indicated by $R_f$ comparison with reference material and by its reaction to the colorimetric reagent sprays both before and after the potassium permanganate spray. The $R_f$ and fluorescent character of band 13 indicated that it also contained 2-amino-1,4-dihydro-4-oxo-6-pteridinecarboxylic acid.

The $R_f$ and fluorescent character of band 15 indicated the presence of 2-amino-4(1*H*)-pteridinone. Although the $R_f$ of band 15 also corresponded to *p*-aminobenzoic acid, it did not give the characteristic color reaction. Thus, *p*-aminobenzoic acid was not an impurity in the folic acid lots examined.

Extraction of thin-layer bands and subsequent HPLC was not entirely satisfactory in correlating TLC and HPLC separations. The HPLC analysis of the 0.3 *N* ammonia extract of the TLC folic acid band demonstrated the presence of several impurities known to be separated by TLC, indicating folic acid decomposition during isolation. Likewise, the high phosphate–perchlorate concentrations in fractions collected from HPLC interfered with subsequent TLC analysis. When 0.1% ammonium carbonate was employed as an HPLC mobile phase, collected fractions could be analyzed by TLC.

Figure 2 shows a typical HPLC separation of folic acid ($k'$ = 6.5) from its impurities at $k'$ = 0.12, 0.35, 2.2, 4.4, and 14.3. HPLC component 1 cochromatographed with *N*-(*p*-aminobenzoyl)-L-glutamic acid and *p*-aminobenzoic acid. HPLC component 2 cochromatographed with 2-amino-1,4-dihydro-4-oxo-6-pteridinecarboxylic acid. Components 1 and 2 collected from the ammonium carbonate HPLC system gave an $R_f$ value comparable to band 13.

HPLC component 3 ($k'$ = 2.2) cochromatographed with 2-amino-4(1*H*)-pteridinone. After collection of component 3 (HPLC ammonium carbonate system) and subsequent TLC, bands 14 and 15 were detected by longwave UV light. TLC band 15 cochromatographed with 2-amino-4(1*H*)-pteridinone, while TLC band 14 has not yet been identified.

**Table II—HPLC Parameters**

| Peak | Retention Time, min | | Capacity Factor, $k'$[a] | |
|---|---|---|---|---|
| | Ambient[b] | 35° | Ambient[b] | 35° |
| 1 | 2.9 | 2.7 | 0.12 | ~0 |
| 2 | 3.5 | 2.7 | 0.35 | ~0 |
| 3 | 8.2 | 5.7 | 2.2 | 1.1 |
| 4 | 13.9 | —[c] | 4.4 | —[c] |
| Folic acid[d] | 19.4 | 12.1 | 6.5 | 3.5 |
| 6 | 39.8 | 23.2 | 14.3 | 7.6 |

[a] $k' = (T_r - T_0)/T_0$, where $T_r$ = folic acid retention time, and $T_0$ = retention time of unretained component. [b] Approximate room temperature, 25°. [c] Peak not resolved. [d] The number of theoretical plates, $N = 5.54\ (T_r/W_{1/2})^2$, where $T_r$ = retention time and $W_{1/2}$ = peak width at half height, was 1950 at ambient temperature and 670 at 35°.

The correspondence of HPLC component 4 and TLC band 14 has not been confirmed. Both appeared to be a composite of at least two impurities. HPLC component 4 ($k'$ = 4.4) was not separated in the ammonium carbonate system where it may have chromatographed with component 3 or with folic acid (component 5). When component 3 was collected (HPLC ammonium carbonate system) and rechromatographed in the perchlorate–phosphate–methanol system, component 4 as well as component 3, 2-amino-4(1*H*)-pteridinone, was detected.

The folic acid peak was collected from the ammonium carbonate system in two portions. Chromatography of both the first half and the second half of the collected folic acid peak in the perchlorate–phosphate–methanol system yielded detectable amounts of components 1, 2, 3, and 4 ($k'$ = 0.12, 0.35, 2.2, and 4.4, respectively) as well as folic acid itself. TLC of both portions of the collected folic acid gave bands 14 and 15 as well as folic acid. These results indicate some decomposition of folic acid during collection and preparation for rechromatography, making it difficult to substantiate the purity of the folic acid peak.

TLC band 11 corresponded to HPLC component 6 ($k'$ = 14.3). Both were present in the present USP reference standard (designated A) and commercial lots but were absent in a previous USP reference standard (designated B).

An unidentified trace impurity (0.05%, $k'$ = 2.5) eluting between components 3 and 4 was detected in 1-day-old solutions of folic acid but not in fresh solutions. When a standard preparation (10 mg/ml) was heated with aeration at 80° for 2 hr, this decomposition product increased along with HPLC components 1–4. Decomposition of aerated and heated solutions was also shown by TLC; an additional fluorescent band above band 15 ($R_f$ 0.87) was detected.

Tetrahydrofolic acid and dihydrofolic acid were chromatographed to aid in impurity identification. Both gave a $k'$ = 7.6 and were resolved from folic acid ($k'$ = 6.5) when present at the 30% level. In addition to the dihydrofolic acid and tetrahydrofolic acid main peaks, numerous peaks, including one cochromatographing with folic acid, were present, indicating sample impurity and/or rapid decomposition. Likewise, numerous thin-layer bands were detected by shortwave and longwave light as well as a shortwave absorbing band cochromatographing with the folic acid main band. TLC results indicated ongoing sample decomposition. With either compound, the number of bands present made it difficult to determine which were attributable to dihydrofolic or tetrahydrofolic acid.

**Moisture Determination**—Determination of folic acid moisture content is nearly as important in determination of purity as the assay procedure. Commercial folic acid samples usually contain 7–9.5% moisture, but they readily equilibrate with atmospheric humidity. Because the moisture content of a sample so quickly changes, it is necessary to determine the moisture content at the same time that the sample is weighed for the assay determination.

The British Pharmacopoeia (9) specifies drying at 100° in vacuum to constant weight. Samples that had lost 6–7% in weight at these conditions still contained sufficient moisture by Karl Fischer titration to indicate predominance of the monohydrate (3.9% moisture) after drying. The dried material rapidly picked up moisture to return to the 7–9% range. Since the drying conditions do not remove all moisture and the dried material is hygroscopic, this procedure is not satisfactory.

Attempts at moisture determination using the USP XIX direct titrimetric procedure (13) resulted in unreliable moisture values, which varied by several percent. The poor results were due to the low solubility of folic acid in the methanolic media usually used. Even if the medium was stirred during folic acid addition, large clumps formed with entrapped moisture. Similar difficulties were reported[5] with the direct determination in

**Table III—Moisture Determination**[a]

| Lot[b] | Moisture, % ± *SD*[c] |
|---|---|
| $A_1$ | 7.68 ± 0.18 |
| $B_1$ | 9.05 ± 0.20 |
| $A_2$ | 8.46 ± 0.15 |
| $B_2$ | 8.55 ± 0.19 |
| C | 7.75 ± 0.15 |
| D | 8.48 ± 0.14 |
| E | 8.48 ± 0.03 |

[a] Values were determined potentiometrically (Karl Fischer determination) using chloroform–methanol (4:1) as the solvent. [b] Subscripts denote different sublots of the USP reference standard Lots A and B. Commercial lots are denoted by C, D, and E. [c] Values are the means of at least three determinations.

methanol, and a back-titration procedure (13) was complex and tedious, but a mixture of chloroform–methanol (4:1) dispersed the folic acid particles and allowed the release of water for titration. In this laboratory, use of chloroform–methanol (4:1) eliminated the problem of particle aggregation and led to reproducible values for the lots of folic acid tested (Table III). Increasing the chloroform beyond the specified amount led to poor electrode response.

**Assay Comparison**—Results for direct colorimetric, USP XIX colorimetric, TLC, and HPLC methods are given in Table I. Confidence limits at 95% were calculated by the range method (17). The values in Table I are all corrected for moisture by the same titrimetric determinations for each lot made at the time samples were weighed. This correction allowed a comparison of results without concern about significant variations in moisture content and its determination. Furthermore, TLC and HPLC assays were conducted using subsampling from the same solutions.

The USP XIX colorimetric assay gave anomalous results. For example, assay of Lots B *versus* A, Lots A *versus* B, and Lots A *versus* A all gave >100% (101–109%). This result may have been due to the fact that sample and standard were not treated similarly. Also, the calculation procedure concentrates measurement uncertainties, making achievement of precision and accuracy most difficult. For example, a realistic 0.5% difference in true absorbance for "tube 1" (6) leads to a difference of 1.8% in the final assay value. Also, values for "tubes 3 and 4" (used to subtract amounts of *p*-aminobenzoic acid and other diazotizable impurities) are sufficiently low to make accurate determination difficult, yet they have a considerable effect on the final purity value when taken through the calculation. Another problem is that the method is unspecific for folic acid in the presence of any impurities that oxidize to diazotizable compounds.

In contrast, the direct colorimetric determination results for relatively pure reference standard material are more consistent with the chromatographic assays. However, direct colorimetry lacks the control of specific impurities offered by the chromatographic assays and could give misleading purity values for commercial folic acid. This method would be suitable for tablet content uniformity determination.

The two chromatographic assays gave comparable results on separate days for reference standard (B *versus* A) evaluation. This result was not unexpected, since the precision, linearity, and specificity of both methods were demonstrated. The choice of one procedure over the other depends on the resources of a particular laboratory. Unlike the HPLC method, the TLC method does not require a substantial investment in equipment or the availability of reproducible and dependable columns. Since fewer manipulative steps are required with HPLC, precision is less chemist dependent, the method is less work intensive, and less elapsed time is required for completion of an assay.

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