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Simultaneous Separation of Folic Acid and Related Polar Compounds on the Acclaim Mixed-Mode WCX-1 Column

INTRODUCTION

Folic acid is a B vitamin that helps the body make new, healthy cells. Folic acid is particularly important for women before and during pregnancy. Adequate folic acid in pregnant women can prevent major birth defects in the brain and spine of the fetus. Foods containing folic acid include green leafy vegetables, fruits, dried beans, peas, and nuts. Enriched breads, cereals, and other grain products also contain folic acid. Folic acid is also commonly used as a dietary supplement.

2,4,5-Triamino-6-hydroxypyrimidine sulfate (THP) synthesized from the reduction of 2,4-Diamino-5-isonitroso-6-hydroxypyrimidine (DNHP) is an important precursor for the synthesis of folic acid and acyclovir.¹ The synthetic process for folic acid is shown in Figure 1. For quality control of folic acid products, it is beneficial to determine the presence of THP, DNHP, and their degradation products. Folic acid analysis on reversed-phase silica columns (for example, C₁₈ and C₈ columns) by high-performance liquid chromatography (HPLC) has been reported.^{2,3} However, THP and DNHP are highly polar compounds with poor retention on C₁₈ and C₈ stationary phases.⁴

Here, an efficient HPLC method for the simultaneous separation of THP, DNHP, and folic acid on the Acclaim[®] Mixed-mode WCX-1 column and the Dionex UltiMate[®] 3000 system is demonstrated. The Acclaim Mixed-Mode WCX-1 column features a mixed-mode, silica-based packing material that incorporates both hydrophobic and weak cation-exchange properties, and demonstrates good potential for separating samples that contain a mixture of ionic and neutral compounds.⁵ The analytes are well-retained, based either on an ion-exchange and/or a reversed-phase mechanism, when using an appropriate mobile phase (for example, acetate buffer-acetonitrile). The solubility and stability of the compounds and their retention behaviors on the Acclaim Mixed-Mode WCX-1 column are presented here.

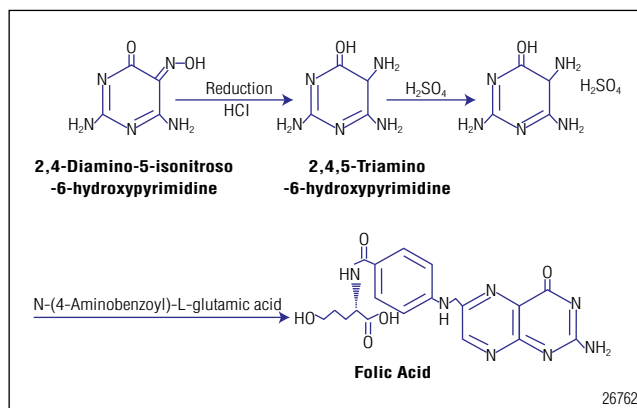


Figure 1. A synthetic process for folic acid.

EQUIPMENT:

Dionex UltiMate 3000 system consisting of:

HPG 3400A pump

WPS 3000TSL autosampler

TCC-3000 thermostatted column compartment

VWD-3400RS UV-vis detector

Chromleon[®] 6.80 SP5 Chromatography Data System

Kudos[®] SK3200LH Ultrasonic generator, Kudos

Ultrasonic Instrumental Co., Shanghai, China

Anke[®] TGL-16B centrifuge, Anting Scientific

Instrumental Factory, Shanghai, China

IKA[®] MS1 Minishaker, IKA Works, Guangzhou, China

REAGENTS AND STANDARDS

Deionized water, from Milli-Q[®] Gradient A10

Acetonitrile (CH₃CN), HPLC grade, Fisher

Ammonium acetate (NH₄Ac), analytical grade,
SCRC, China

Acetic acid (HAc), analytical grade, SCRC, China

Folic acid, ≥ 97%, HPLC, Fluka

Sodium hydroxide (NaOH), analytical grade, SCRC, China

SAMPLES

Two refined and unrefined products of THP and a refined product of DNHP were obtained from a chemical company. Folic acid tablets were purchased from a drug store in Shanghai, China.

STANDARD AND SAMPLE PREPARATION

Preparation of Folic Acid, THP, and DNHP Samples

Stock solutions

Transfer accurately weighed ~10 mg folic acid, refined DNHP product, and refined/unrefined THP products into separate 1.5 mL vials. Add 1 mL 0.1% NaOH to each vial for dissolution. The concentrations of the stock solutions are ~1000 µg/mL. The stability of the THP stock solution may be well-maintained for at least 4 h when stored at 4 °C.

Monocomponent solutions for analysis

Add suitable amounts of the stock solutions to four 10 mL flasks respectively, and dilute them to the mark with 0.5% HAc for refined or unrefined THP, and with water for folic acid and DNHP.

Mixture solutions

Add suitable amounts of the stock solutions of folic acid, refined THP, and DNHP to a 10 mL flask, and dilute to the mark with water.

The well-prepared sample solutions should be stored in the dark. Prior to injection, filter the solutions through a 0.2 µm filter (Millex[®]-HV).

Preparation of Folic Acid Tablet Sample

Add one ground folic acid tablet (labeled amount, 5 mg per tablet) to a 15 mL centrifuge tube, and then add 10 mL 0.05% NaOH solution. After 1 min of vortex shaking, 2 min in the ultrasonic bath, and 5 min of centrifugation (setting= rpm ≥ 3000), transfer the supernatant to a 10 mL volumetric flask, and add water to the mark. The well-prepared folic acid sample solution can be diluted with water, if necessary. Prior to injection, filter the solution through a 0.2 µm filter.

CONDITIONS

Analytical Column: Acclaim Mixed-Mode WCX-1,
5 µm, 4.6 × 150 mm, P/N 068353

Column Temperature: 30 °C

Mobile Phase: HAc-NH₄Ac buffer (mixture
of 600 mL of 30 mM HAc and
200 mL of 30 mM NH₄Ac,
~ pH 4.1 (without adjustment)–
CH₃CN (95:5, v/v)

Flow Rate: 1.0 mL/min

Inj. Volume: 5 µL

UV Detection: Absorbance at 254 nm

Table 1. Solubility and Stability in Different Solvents at Room Temperature^(1,2)

Compound	Water		Basic Solution		Acidic Solution	
	Solubility	Stability	Solubility	Stability	Solubility	Stability
2,4-Diamino-5-isonitroso-6-hydroxypyrimidine (DNHP)	X	/	✓	✓	X	/
2,4,5-Triamino-6-hydroxypyrimidine sulfate (THP)	✗	X	✓	X ^[3]	✗	✓ ^[4]
Folic Acid	X	/	✓	✓	X	/

Note: 1. ✓ = good, X = poor, ✗ = slight, and / = not evaluated

2. Room temperature, ~20 °C.

3. Unstable at room temperature; stable for at least 3 h when stored at 4 °C and in the dark.

4. Stable for at least 6 h at room temperature.

RESULTS AND DISCUSSION

Solubility and Stability

The solubility and stability of THP, DNHP, and folic acid in water, basic (0.05% NaOH), and acidic solutions (20 mM HAc) were investigated. The results are summarized in Table 1 and were used to design the procedures for sample preparation as specified in the section Standard and Sample Preparation.

The stock solutions of the three compounds are obtained by dissolving them in basic solution: DNHP and folic acid samples were diluted with water, and THP was diluted with acidic acid solution. When preparing a mixture of the three compounds, the best choice for dilution is water, despite the instability of THP in water. The mixture sample is prepared just prior to analysis.

Retention Behavior of THP and DNHP on the Acclaim Mixed-Mode WCX-1 Column

Hu et al.⁴ reported an HPLC method for the simultaneous separation of THP and folic acid on a C₁₈ reversed-phase silica stationary column. Folic acid is retained very well on the C₁₈ column; however, the highly polar compound THP has no retention on the hydrophobic stationary phase. Therefore, the Acclaim Mixed-Mode WCX-1 column, which incorporates both hydrophobic and weak cation-exchange properties, was used to separate this highly polar compound together with DNHP and folic acid.

Retention behavior of the above compounds on the Acclaim Mixed-Mode WCX-1 column was investigated using ammonium acetate/acetic acid-CH₃CN as the mobile phase. Hydrophobic retention is affected by the organic modifier composition of the mobile phase, and as expected, retention of the three compounds decreases with increasing CH₃CN in the mobile phase. Ionic strength is crucial for changing retention of charged molecules; therefore, increasing the concentration of acetate buffer results in retention decrease for THP and a slight decrease for DNHP. The pH value of the mobile phase affects the charge and hydrophobicity of the stationary phase; hence, selectivity can be altered by modifying the mobile phase pH. Decreasing the pH of the mobile phase decreases retention of THP and also slightly decreases retention of DNHP.

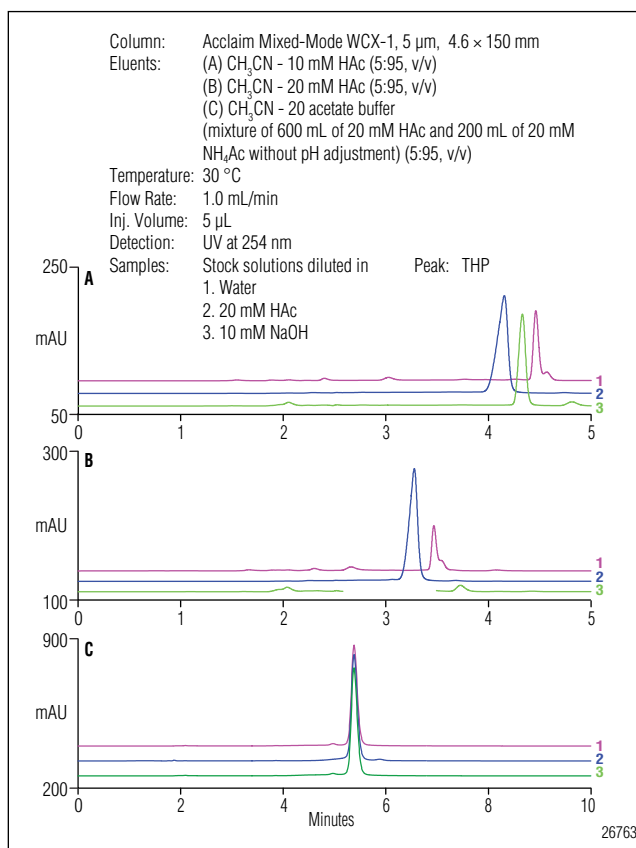


Figure 2. Overlay of chromatograms of refined THP product using (A) 10 mM HAc, (B) 20 mM HAc, and (C) 20 mM NH₄Ac-HAc (~pH 4.2) in the mobile phase.

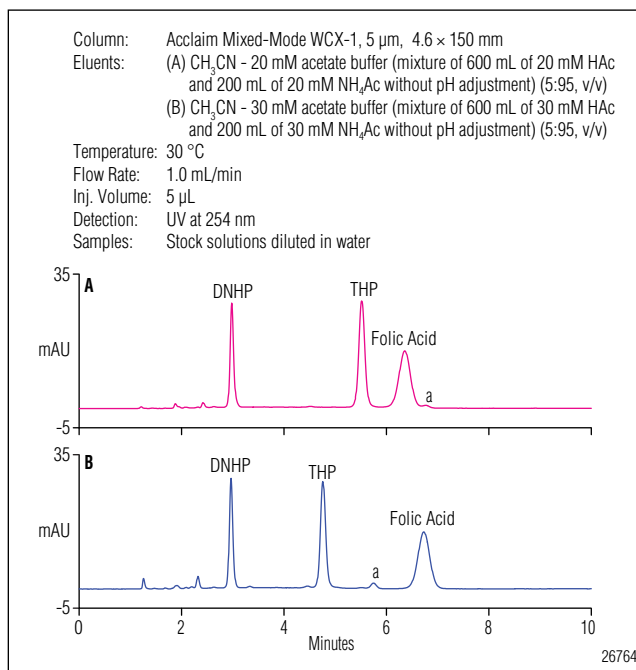


Figure 3. Chromatograms of a mixture stock solution diluted with water using (A) 20 mM NH₄Ac-HAc (~pH 4.3) and (B) 30 mM NH₄Ac-HAc (~pH 4.0) buffer in the mobile phase.

Effects of Sample Dilution

Although good retention can be obtained using either 10 or 20 mM HAc in the mobile phase, the retention time of the refined THP product varies depending on whether the sample is diluted with water, HAc, or NaOH (Figure 2). This demonstrates that the buffer capacity of the mobile phase is inadequate. Changing HAc to 20 mM NH₄Ac-HAc buffer (pH 4.2) delivers good retention time reproducibility, regardless of the sample diluent (Figure 2C).

Separations on the Acclaim Mixed-Mode WCX-1 Column

Separation of a mixture of folic acid, THP, and DNHP

As seen in Figure 3A, the three compounds are well-separated on the Acclaim Mixed-Mode WCX-1 column using 20 mM NH₄Ac-HAc buffer (pH 4.3)-CH₃CN (95:5, v/v) as the mobile phase. However, there is an unknown peak (peak *a*) very close to the folic acid peak. The selectivity of this column can be altered by changing the 20 mM NH₄Ac-HAc buffer (pH 4.3) to 30 mM NH₄Ac-HAc buffer (pH 4.0), as seen in Figure 3B; this decreases the retention of THP and the unknown peak, and increases the retention of folic acid, while only slightly changing the retention of DNHP. This produces a much better separation of these three compounds and unknown peak *a*.

The unknown peaks *a-f* come from the degradation of THP in basic solution. Comparing the chromatograms of a mixture solution to a chromatogram obtained 90 min later, reveals that the peak areas of the unknown peaks increase concomitantly with a significant decrease of THP peak area. The peak areas of DNHP and folic acid exhibit little change (Figure 4).

Separation of unrefined THP product and folic acid tablet

The unrefined THP product and folic acid tablet sample were prepared as specified above. Figure 5 shows the chromatography of both samples. No DNHP was found in the unrefined THP product and no THP was found in the folic acid tablet.

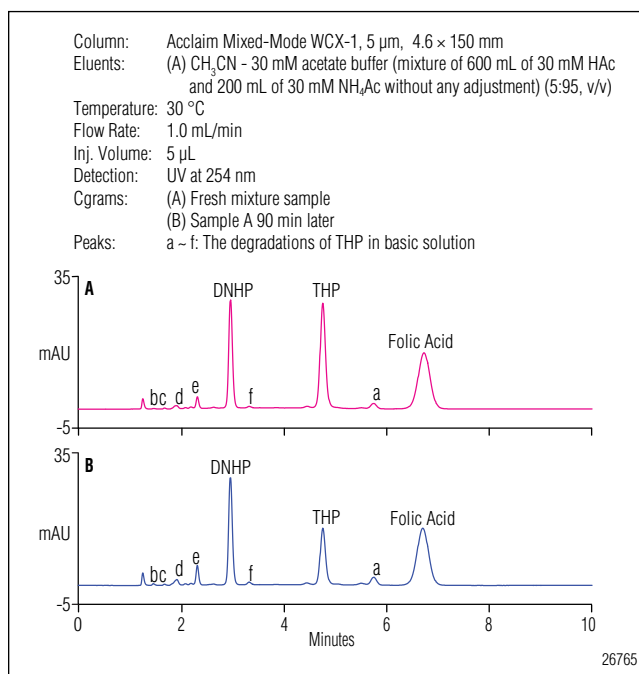


Figure 4. Chromatograms of (A) a mixture sample (a stock solution diluted with water) and (B) the same one obtained 90 min later, using 30 mM NH₄Ac-HAc buffer (~pH 4.0) in the mobile phase.

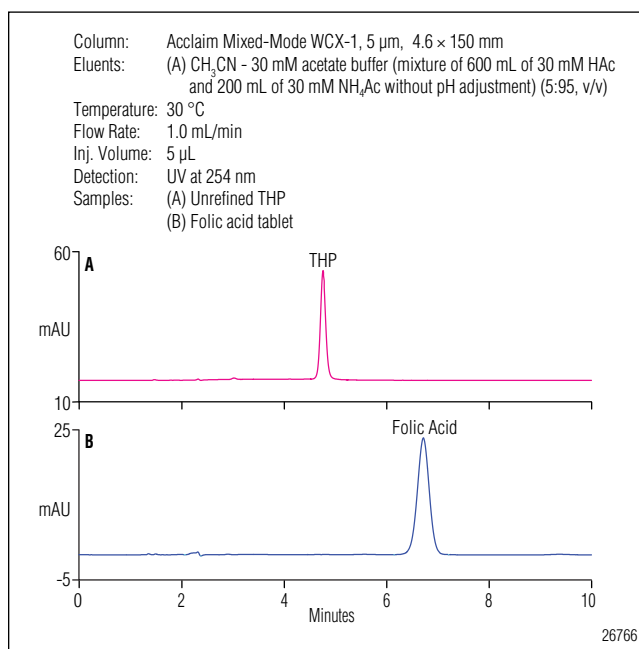


Figure 5. Chromatograms of (A) unrefined THP product and (B) folic acid tablet sample.

CONCLUSION

This application update describes an efficient HPLC method for the simultaneous separation of 2,4,5-Triamino-6-hydroxypyrimidine sulfate, 2,4-Diamino-5-isonitroso-6-hydroxypyrimidine, and folic acid on the Acclaim Mixed-Mode WCX-1 column and UltiMate 3000 HPLC system. The Acclaim Mixed-Mode WCX-1 column incorporates both hydrophobic and weak cation-exchange properties, and exhibits good selectivity for a mixture of ionic and neutral compounds, thereby facilitating separation of folic acid and its synthetic precursors.

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