Determination of 5-Methyltetrahydrofolate in Citrus Juice by Reversed-Phase High-Performance Liquid Chromatography with Electrochemical Detection†

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The principal forms of folate in citrus are 5-methyltetrahydrofolic acid (5-MeTHF) and its polyglutamate derivatives. A method for the rapid determination of 5-MeTHF in citrus juice has been developed and involves solid-phase extraction using a phenyl-type bonded phase with the aid of an ion-pairing reagent, followed by reversed-phase HPLC and amperometric detection on a glassy carbon electrode (+200 mV vs Ag;AgCl, 3 M NaCl). No preconcentration was necessary; the detection limit was in the low nanogram range. Treatment of orange and grapefruit juices with a folate polyglutamate hydrolase (conjugase) enzyme prior to analysis increased the apparent level of 5-MeTHF from 30% to 70%, depending on the sample, and yielded results in reasonable agreement with those reported by microbiological assay using *Lactobacillus casei*. The method shows promise for the routine monitoring of the folate content of citrus juices.

INTRODUCTION

Since the establishment of a Recommended Daily Allowance (RDA) for dietary folate (FNB, 1968), there has been widespread demand for information regarding the levels of this vitamin in foods. The use of nutritional labeling of certain foods has prompted a desire on the part of food industries for reliable data on the folate content of many products.

Folate compounds play an important role in human nutrition, acting as essential coenzymes in the biosynthesis of proteins and nucleic acids (Blakley, 1969). The many naturally occurring forms differ in the oxidation state of the pteridine ring, substitution at the N-5 and N-10 positions, and the number of glutamic acid residues in γ-peptide linkage to the parent folic acid molecule.

The principal forms of folacin in plant tissues are reduced 5-methyltetrahydrofolic acid (5-MeTHF) and its polyglutamate derivatives (Gregory et al., 1984; Tamura et al., 1976). Citrus juice, in particular orange juice, has been reported to be a good source of dietary folate (Hill and Attaway, 1971; Streiff, 1971), yet inhibition of uptake and consequently a low bioavailability of folate polyglutamates in orange juice has been reported (Tamura and Stokstad, 1973; Tamura et al., 1976). Results from gel filtration combined with anion-exchange chromatography suggest that the 5-MeTHF monoglutamate comprises 30–40% of the total folate in orange juice, the rest being di- through tetraglutamate (15–30%), and pentaglutamate derivatives (40–50%) (Stokstad et al., 1977).

Although reduced folates are labile when subjected to heat, light, and air, the presence of mild reducing agents such as ascorbate inhibits the oxidation of 5-MeTHF to the corresponding 5-methyl-5,8-dihydrofolic acid (Donaldson and Keresztesy, 1962). Thus, 5-MeTHF is relatively stable in an ascorbate-rich medium such as citrus juice.

Several schemes for separation of folate monoglutamates utilizing reversed-phase "ion-pair" or anion-exchange high-performance liquid chromatography (HPLC) are reported in the literature (Reed and Archer, 1976; Chapman et al., 1978; Horne et al., 1981; McMartin et al., 1981; Reingold and Picciano, 1982; Duch et al., 1983; Kohashi and Inoue, 1986; Wegner et al., 1986; Holt et al., 1988). Optical detection methods, typically UV absorption in the 280-nm range, are useful for fortified or preconcentrated samples containing relatively high levels of folate (>10 ng/injection). Microbiological assay of HPLC fractions (McMartin et al., 1981; Wilson and Horne, 1984) can provide for lower detection limits (picogram range); however, it is a lengthy and cumbersome procedure. Fluorescence monitoring has been shown to be a satisfactory means of detection (Gregory et al., 1984), yet the fluorescence of reduced folates is pH dependent, requiring either an acidic mobile phase (pH <3) or post-column acidification (Holt et al., 1988).

Electrochemical detection of 5-MeTHF in plasma and spinal fluid after HPLC separation was first reported in 1980 (Lankelma and Van der Kleijn, 1980) with a detection limit of about 1 ng, a 10-fold improvement over the UV method. More recently, Lunte and Kissinger (1983) have reported detection limits as low as 0.2 ng for 17 known pterin compounds. Kohashi and Inoue (1986) separated and detected five folate monoglutamates with an estimated detection limit of 1–150 pg. The objective of the current work was to develop a rapid method for the determination of 5-MeTHF in citrus juice. Reversed-phase HPLC coupled with electrochemical detection is capable of determining this compound in the low nanogram range, permitting its analysis in citrus juice with little or no preconcentration.

MATERIALS AND METHODS

Preparation of Standards. 5-Methyltetrahydrofolic acid (barium salt, 90% pure) was obtained from Sigma Chemical Co. (St. Louis, MO) and used without further purification. L-Ascorbic acid was obtained from Aldrich Chemical Co. Inc. (Milwaukee, WI). The integrity of the 5-MeTHF standard was qualitatively assessed by reversed-phase ion-pair HPLC using an isocratic modification of the method of Rebello (1987). Photo-

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diode array detection permitted confirmation of 5-MeTHF. By use of a molar absorptivity value of 2.9×10^4 (Larabee et al., 1961), the absorbance (λ = 290 nm) of a freshly prepared standard solution led to a calculated value of 87% purity, in reasonably good agreement with the nominal purity (90%) supplied by the manufacturer. The nominal purity was used in all calculations. A primary stock solution of 5-MeTHF was prepared by dissolving approximately 10 mg of the salt in 100 mL of 0.1 M phosphate buffer (pH 6.8) containing 0.1% (w/v) ascorbic acid. This was kept frozen at −20 °C until needed. Secondary stock solutions of approximately 100 μg/100 mL were prepared as needed by dilution of the primary stock with the HPLC mobile phase.

Preparation of Samples. Commercial orange juice from concentrate (OJFC) and canned and bottled grapefruit juice from concentrate (GJFC) were obtained from local grocery store shelves. Samples were centrifuged at 10 000 rpm and 2 °C for 15 min. The pH of the supernatant liquid was adjusted to 5.0 by using concentrated NaOH. Five-milliliter portions were then either directly subjected to the solid-phase extraction procedure outlined below or treated with conjugase enzyme prior to SPE. Enzyme treatment was performed essentially according to the procedure of Gregory et al. (1984). Hog kidney, for extraction of the folacin polyglutamate hydrolase (conjugase), was obtained "fresh frozen" from a local market. The resulting enzyme suspension was added to samples as 0.5 mL of enzyme/5 mL of sample. This was roughly twice the amount required to give maximum yield of the monoglutamate as determined from preliminary HPLC studies of treated samples. After incubation at 37 °C for 1 h, samples were put on ice and then centrifuged at 15 000 rpm for 20 min at 2 °C. The supernatant liquid was then applied to SPE cartridges.

Solid-phase extraction was carried out by using disposable BondElut (Analytichem International, Harbor City, CA) phenyl solid-phase extraction cartridges of 500-mg capacity. The cartridges were conditioned by wetting with 5 mL of methanol followed by 10 mL of water and then equilibrated with 3 mL of a 0.05 M phosphate/acetate buffer (pH 5) containing 0.005 M tetrabutylammonium phosphate (TBAP, Waters PIC A ion-pairing reagent, Milford MA). Five-milliliter portions of the clarified juice samples were passed through the cartridges at a rate of about 2.0 mL/min. The cartridges were then washed with 3 mL of the equilibration buffer followed by 3 mL of the buffer containing 10% methanol. Finally, retained folate was eluted with 5 mL of buffer containing 30% methanol without TBAP. All samples and standards were kept on ice in amber vials throughout the procedure. Eluted samples were chromatographed as soon as possible to minimize oxidative loss; there was no noticeable decrease in 5-MeTHF content observed in either the standard (prepared fresh daily) or the eluted samples within the time frame of the experiments. Recovery of 5-MeTHF from a citrus juice matrix was determined by spiking orange juice samples at levels at least 10 times that of endogenous folate and applying the extraction method described. The extracts were chromatographed as usual except that the detector sensitivity was reduced 10-fold to 50 nAFS.

HPLC Methods. Separation and identification of 5-MeTHF in juice samples were accomplished by reversed-phase HPLC with electrochemical detection. A Waters HPLC system consisting of a Model 510 LC pump, a Model 721 system controller, and a Model 710B WISP autosampler (injection loop 1–200 μL) was used. A ZORBAX ODS column (4.6 mm × 25 cm) at ambient temperature was used for the separation. Data were acquired with a 20-bit A/D converter (CSI Model 160S, Autochrom Inc., Milford, MA) at a rate of no less than 2 Hz. Integration and peak height calculation were performed by using APEX Chromatography Software (Autochrom) with the aid of a 286 AT-style computer (CompuAdd Corp., Austin, TX). The mobile phase was 25% methanol in phosphate/acetate buffer (pH 5.5), prepared by mixing 0.05 M phosphate/acetate buffer (adjusted to pH 5.0 with concentrated NaOH) with methanol in a ratio of 75:25 by volume, filtered through a 0.45-μm filter, and degassed by sparging with helium. The injection volume was usually 10 μL, and the flow rate 1.0 mL/min. Electrochemical detection was accomplished by using an EG&G Princeton Applied Research Model 400 (Princeton, NJ) amperometric detector equipped with a glassy carbon electrode and operated at +200 mV (vs Ag;AgCl, 3 M NaCl) with sensitivity set at 5 nAFS. A Hewlett-Packard HP 1040A (Corvallis, OR) diode array detector was used for UV spectral data acquisition.

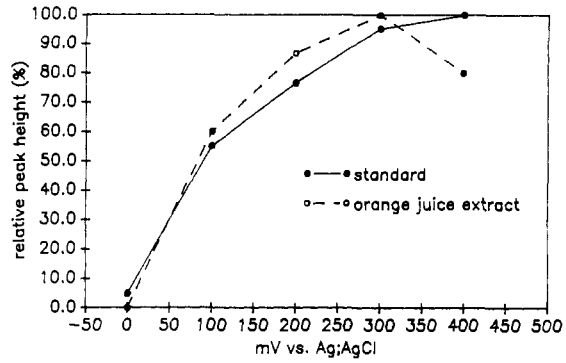

Figure 1. Hydrodynamic voltammogram of 5-MeTHF in a standard solution (1.0 μg mL^{-1}) and in an orange juice extract. Chromatographic conditions are described in the text.

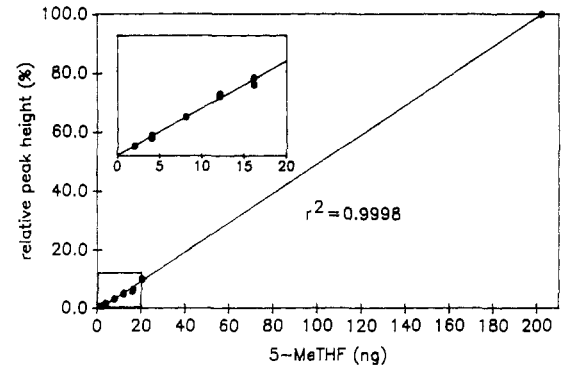

Figure 2. Detector response (+200 mV vs Ag;AgCl) vs nanograms of 5-MeTHF injected.

RESULTS AND DISCUSSION

Detector Response and Peak Identification. The hydrodynamic voltammograms (HV) illustrated in Figure 1 are the result of repeat injections of either standard 5-MeTHF or sample extract containing the analyte at various working electrode potential settings (0–400 mV vs Ag;AgCl) on the detector. The fact that the detector signal is a function of the applied potential at the working electrode was used to advantage for peak confirmation. The potential at which oxidation begins and the shape of the HV are unique features of a given compound and, as such, offer reliable criteria for peak confirmation when combined with a known chromatographic retention time. Comparison of the two curves in Figure 1 illustrates this point. It should be emphasized that the potentials reported here are uniquely dependent on the absolute potential of the reference Ag;AgCl electrode. A change in the reference electrode potential manifests itself as a shift of the HV along the potential axis, which leads to an apparent change in detector response at any one potential setting. This has no effect on the shape of the HV curve, however, and the relative responses retain their significance. Nevertheless, comparisons of this type are best made by using a standard–sample–standard injection sequence to ensure that any changes in the potential of the reference electrode, and hence the detector response, can be compensated.

Detector response was essentially linear over the concentration range expected in the juice samples (0.2–0.4 μg mL^{-1}). A plot of relative peak height vs nanograms of 5-MeTHF is shown in Figure 2.

Retention Behavior and Recovery. Rebello (1987) illustrated the retention behavior of several folate

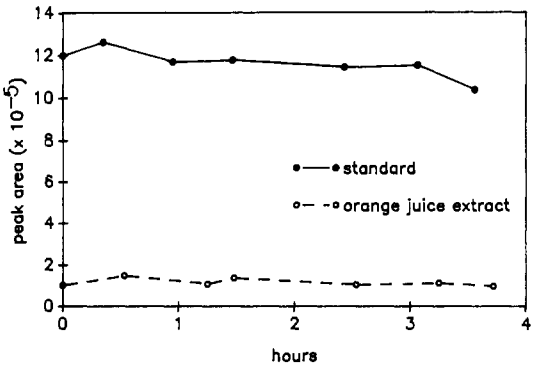

Figure 3. Stability of 5-MeTHF in a standard solution (1.0 μg mL^{-1}) containing 10 μg mL^{-1} ascorbic acid and in orange juice extract.

Table I. 5-Methyltetrahydrofolate Content of Four Commercial Citrus Juices

sample	5-MeTHF, μg mL^{-1}	enzyme treated, μg mL^{-1}
OJFC (1)	0.191 ± 0.018	0.243 ± 0.023
OJFC (2)	0.132 ± 0.012	0.229 ± 0.021
GJFC (canned)	0.067 ± 0.006	0.091 ± 0.008
FJFC (bottled)	0.064 ± 0.006	0.085 ± 0.008

compounds separated by using reversed-phase ion-pair chromatography. At a fixed concentration of ion-pairing reagent, an inverse relation between pH and retention time was noted above pH 6, presumably due to competition for exchange sites by hydroxide ion, similar to retention behavior in anion-exchange HPLC. As expected, retention time increased with the concentration of ion-pairing reagent for a given pH. Similar results were obtained in this laboratory. The strong binding affinity for 5-MeTHF of a phenyl-bonded phase conditioned with TBAP was used to advantage for solid-phase extraction of the citrus juice samples. This permitted the separation of the analyte from less tightly bound compounds likely to interfere with the analysis. The folate was then easily liberated from the cartridge with an elution buffer containing methanol without TBAP. Other reversed-phase SPE cartridges were tried (C2, C8, and C18); however, the phenyl-bonded cartridges appeared to be more selective and yielded higher recoveries. Results of 12 analyses following extraction of spiked orange juice using the phenyl-bonded cartridge averaged 89.9% recovery with a standard deviation of 6.7%.

Ascorbic acid was the major interferent; its concentration in natural orange juice is roughly 1000 times that of 5-MeTHF. Ascorbate is easily oxidized at the potentials employed here to detect 5-MeTHF, and the detector response was found to be about equal for the two compounds. Although ascorbate was not retained on the chromatographic column under the described conditions, significant tailing was observed, which swamped the detector signal and obscured the 5-MeTHF peak. Solid-phase extraction was capable of reducing ascorbate to a residual concentration of only about 10 times that of 5-MeTHF, facilitating selective detection of the analyte. Figure 3 is a plot of peak area vs time for a 5-MeTHF standard solution (1.0 μg mL^{-1}) and an extracted juice sample. The standard was prepared to contain ascorbate at 10 times the concentration of 5-MeTHF. Repeat injections were made over a 4-h period and illustrate the stability of 5-MeTHF in the two solutions under the experimental conditions.

5-Methyltetrahydrofolate in Citrus Juice. The plots of relative peak height vs potential (millivolts) (Figure 1) reveal a maximum detector signal between +300 and +400 mV (vs Ag;AgCl) under the chromatographic conditions employed. Although these results might suggest +400 mV as the optimal detector setting for 5-MeTHF, other compounds present in the extracted OJ sample, and which elute the HPLC column close to the analyte, are also oxidized at this potential, making accurate quantitation difficult. After a few runs at different potentials, +200 mV was found to be the optimal trade-off potential in that it offered near maximum response without detecting interfering compounds. Figure 4 illustrates typical chromatograms obtained for OJFC samples at +350 and +200 mV, superimposed on that of standard 5-MeTHF.

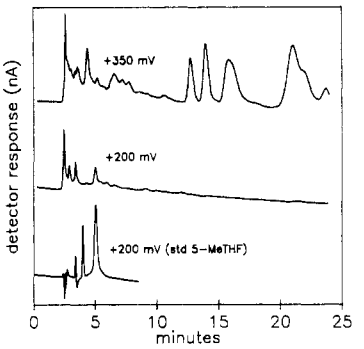

Figure 4. Typical chromatograms of orange juice extract and standard 5-MeTHF with detector settings at +350 and +200 mV vs Ag;AgCl. Sensitivity: 5 nAFS.

Repeat injections of a standard solution of 5-MeTHF were carried out to estimate the reproducibility of the HPLC instrumentation, namely, the injection repeatability, column performance, detection, and quantitation. Results of five injections of a 1.02 μg mL^{-1} standard with detection at +200 mV (vs Ag;AgCl) averaged 1.017 μg mL^{-1} with a standard deviation of 0.056 μg mL^{-1}. Detection at higher applied potentials improved the signal-to-noise ratio (sd = 0.019 μg mL^{-1} at +300 mV); however, as already mentioned, interference from other compounds in real samples, electroactive at potentials more positive than +200 mV, reduced the reliability of peak quantitation methods (see Figure 4).

Two samples of commercial OJFC and one sample each of canned and bottled GJFC were analyzed by using the above described method. The results are shown in Table I and take into account percent recovery and the nominal purity of the 5-MeTHF standard (90%). The error estimate was obtained by summing the relative variances for the HPLC and the sample handling procedures. The results obtained are in reasonably good agreement with previously reported data for orange and grapefruit juices as determined by microbiological assay (Hill and Attaway, 1971; Fellers et al., 1990), where the typical range is from 0.20 to 0.40 μg mL^{-1} and from 0.06 to 0.21 μg mL^{-1} for OJFC and GJFC, respectively. The increase in 5-MeTHF as a result of sample treatment with a folacin polyglutamate hydrolase enzyme (conjugase) supports earlier evidence that the 5-MeTHF monoglutamate only partially accounts for the total folate in citrus juices.

In conclusion, a rapid HPLC method for the determination of 5-MeTHF has been developed that shows promise as a technique for routine folate analysis in citrus juices. Our laboratory is currently using the method to investigate the relationship between 5-MeTHF concentration and total folate in a variety of fresh and processed juices, the effect of processing on folate distribution and content, and the correlation between electrochemical detection and microbiological assay for total folate content in processed citrus juices.

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