

Simultaneous Determination of Tetrahydrofolate and N⁵-Methyltetrahydrofolate in Pig Plasma by High-Performance Liquid Chromatography with Electrochemical Detection

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ABSTRACT. An analytical method for measurement of tetrahydrofolate (THF) and N⁵-methyltetrahydrofolate (5MF) in pig plasma by the high performance liquid chromatography and electrochemical detection is described. The plasma sample was deproteinized by perchloric acid and the supernatant was analyzed using the following conditions; (a) phenyl bonded phase column as an analytical column; (b) mobile phase consisting of 20 mM acetate buffer (pH 3.6) containing 0.1 mM EDTA and acetonitrile (96.5:3.5, v/v); (c) an applied potential of +300 mV. Under the above condition, both peaks of THF and 5MF in the plasma were well separated. The detection limits of THF and 5MF were 0.15 and 0.13 ng/ml, respectively, at S/N=3. The recoveries of THF and 5MF from the plasma spiked with standard THF and 5MF were 77.6±2.1% and 83.0±1.7% (mean±S.D.), respectively.—**KEY WORDS:** HPLC-ECD, N⁵-methyltetrahydrofolate, pig, plasma tetrahydrofolate.

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In general, N⁵-methyltetrahydrofolate (5MF) is the principal folate congener in plasma [2, 4, 8, 11, 15]. Plasma folate levels in pigs has been reported as 70–125 ng/ml using radioligand assay [6, 7, 13]. We determined 5MF level in pig plasma by high-performance liquid chromatography with electrochemical detection (HPLC-ECD) and obtained levels of 2–5 ng/ml [10]. The 70–125 ng/ml and 2–5 ng/ml ranges are not contradictory provided that another folate exists in pig plasma. We examined other folate congeners than 5MF in pig plasma. As a result, tetrahydrofolate (THF) as well as 5MF was detected in the plasma. This report describes a simultaneous analytical method of THF and 5MF in pig plasma by HPLC-ECD. ECD was useful for substances having a low redox potential, such as THF and 5MF.

MATERIALS AND METHODS

HPLC system: The HPLC system consisted of a pump (Model 576, Gaskurokogyo Inc., Tokyo, Japan), a fixed loop injector (Model 7125, Rheodine Inc., California, U.S.A.), an analytical column (phenyl bonded phase; 4.6×150 mm, IRICA PHENYL, Irikakogyo Inc., Kyoto, Japan), an amperometric detector with a grassy carbon (E-502, Iricakogyo Inc.) and a data processor (C-R4A, Shimadzu Corporation, Kyoto, Japan). A mobile phase was a mixture of 20 mM acetate buffer (pH 3.6) containing 0.1 mM EDTA and acetonitrile

(96.5:3.5, v/v).

Preparation of standard solution: Stock solutions of 100 µg/ml of THF and 5MF were prepared with 0.2% ascorbic acid solution. Ascorbic acid was used as antioxidant. The stock solutions were divided into 1 ml each and stored at –20°C until the HPLC analysis. They were used within 4 weeks.

The frozen stock solutions were thawed in running water for 2 min. A standard solution containing THF and/or 5MF was prepared by diluting with 0.2% ascorbic acid solution. Since THF and 5MF are sensitive to UV light, all used test tubes and flasks were covered with aluminium foil.

Sample treatment: Blood samples were collected from 7 Goettingen miniature pigs (15–40 kg). The blood was put into an EDTA treated test tube and centrifuged at 2,000 g for 5 min to separate plasma. The plasma was put into a test tube containing ascorbic acid (1 mg/ml plasma) and stored at –20°C until the HPLC analysis.

After thawing the plasma in running water for 2 min, 0.2 ml of plasma was deproteinized by adding an equal volume of 0.5 M perchloric acid and centrifuged at 5,000 g for 2 min at 4°C. The supernatant was filtered through a 0.45 µm filter (Chromatodisk 4A, Biofield Inc., Tokyo, Japan). A 100 µl of the filtrate was injected into the HPLC-ECD system.

Voltammogram: Hydrodynamic voltammograms of THF and 5MF in plasma and standard solution were made by using the above HPLC-ECD system.

Weibull's equation was regressed to the plots of the obtained response against volt plots by the nonlinear least squares method using program MULTI [16]. Weibull's equation is as follows,

$$y = 1 - \exp(-\alpha(x - \gamma)^\beta)$$

Linear range: The stock solutions of THF and 5MF were diluted into 0.5–100 ng/ml with 0.2% ascorbic acid. The linearity was examined in the following three ranges; 0.5–2.5 ng/ml, 2–10 ng/ml and 20–100 ng/ml. Each range had 5 concentrations at same intervals. Peak areas of the prepared solutions were plotted against the corresponding concentrations, and the slope, intercept and regression coefficient were calculated by the least squares method.

Recovery: The stock solutions of THF and 5MF were diluted into 1 µg/ml with 0.2% ascorbic acid. The solution of THF or 5MF was added into 0.95 ml of plasma containing 0.1% ascorbic acid and diluted into 50 ng/ml. The added and not added plasma samples were analyzed by the HPLC-ECD. The recovery was calculated by dividing the difference in the peak area between added and not added plasma by the peak area of 50 ng/ml standard solution.

Stability of THF and 5MF: Stabilities of THF and 5MF in standard solutions were examined after an adding of deproteinizing agents including perchloric acid, trichloroacetic acid, acetone and acetonitrile. 0.2 ml of the deproteinizing agent was added into 0.2 ml of standard solution of THF or 5MF (50 ng/ml). The mixture was placed at 4°C for 2 min and then injected into the HPLC-ECD system.

Stabilities of THF and 5MF in plasma were also examined after the deproteinization. 0.05 ml of a standard solution containing THF and 5MF (1 µg/ml, each) was added into 0.95 ml of plasma containing 0.1% ascorbic acid. The mixture was deproteinized, and the supernatant was kept at a room temperature and injected into the HPLC system 0, 40, 80 and 120 min after deproteinization.

Chemicals: Trihydrate of sodium acetate, acetic acid, ascorbic acid, sodium ascorbate, disodium salt of ethylenediaminetetraacetic acid (EDTA), acetonitrile, methanol, acetone and perchloric acid were obtained from Wako Pure Chemical (Osaka, Japan). They were of analytical grade. THF (70% purity) and disodium salt of 5MF (90% purity) were obtained from Sigma Chemical Company (St. Louis, U. S. A.).

RESULTS

Separation on chromatogram: Figure 1 shows chromatograms of plasma sample and the standard solution under the following analytical condition; phenyl column, mobile phase consisting of 20 mM acetate buffer (pH 3.6) and acetonitrile (96.5:3.5, v/v), an applied potential of +300 mV. Both peaks of THF and 5MF in pig plasma were well separated on the chromatogram.

In order to examine whether the peak of THF or 5MF contains any other substance than THF or 5MF, hydrodynamic voltammogram of THF and 5MF in the plasma sample were made and compared with those of THF and 5MF in the standard solution. As is shown in Fig. 2, the voltammograms of each folate in the plasma were well consistent with those in the standard solution. Weibull's equation was regressed to each voltammogram. The obtained parameter are shown in Table 1. Each parameter for THF and 5MF was almost same between plasma and standard solution.

Retention characteristics: The effects of mobile phase on the retention times of THF and 5MF were examined by changing concentration of organic solvent or pH value of the buffer in mobile phase. As is shown in Fig. 3, the retention times of both folates decreased as increasing concentration of acetonitrile or methanol. In Fig. 4 the peak areas of both folates were plotted against retention time. The peak area of THF decreased as increasing retention time, or decreasing concentration of acetonitrile or methanol. The decrease in the peak area was smaller in acetonitrile case than in methanol case. On the other hand, the peak area of 5MF was almost constant.

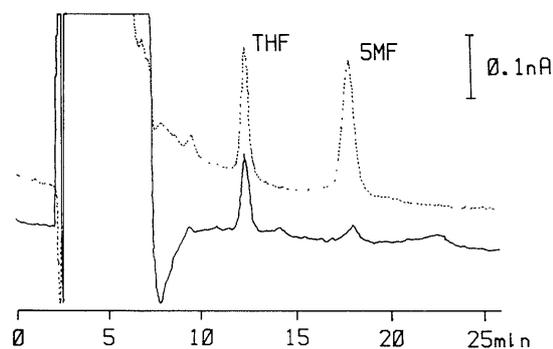


Fig. 1. HPLC-ECD chromatogram of pig plasma and standard solution containing THF and 5MF. Solid line represents the chromatogram of plasma and dotted line represents that of standard solution.

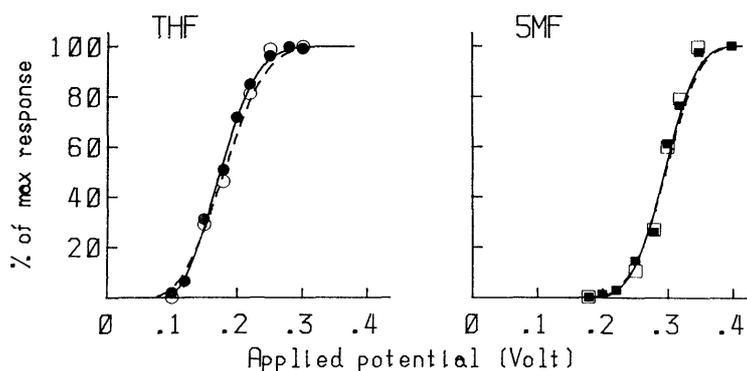


Fig. 2. Hydrodynamic voltammograms of THF and 5MF in pig plasma (open symbols) and standard solution (closed symbols). Solid or dotted line was obtained from the nonlinear least squares regression of Weibull's equation to the plots for standard solution or plasma.

Table 1. Parameters obtained by the regression of Weibull's equation to voltammograms of THF and 5MF in plasma and standard solution

Parameter	THF		5MF	
	plasma	standard	plasma	standard
α (volt ⁻¹)	289	226	1306	1254
β^a	2.94	2.43	3.89	3.67
γ (volt)	0.0518	0.0824	0.154	0.167

a) No dimension.

The pH value of the buffer also affected the retention times of THF and 5MF. Longer retention time was observed at lower pH value of the buffer in the examined pH range of 3–5.

Linear range, detection limit and recovery: In three concentration ranges examined for linear range, the peak areas of THF and 5MF were well correlated with their concentrations. The slopes of regression lines obtained from the three concentration ranges of THF or 5MF were almost same (Table

2). Detection limits of THF and 5MF were 0.15 ng/ml (0.034 pM/injection) and 0.13 ng/ml (0.027 pM/injection), respectively, at S/N = 3. The recoveries of THF and 5MF from the plasma were $77.6 \pm 2.1\%$ and $83.0 \pm 1.7\%$ (mean \pm S.D., n=5), respectively.

Stability of THF and 5MF: Table 3 shows the peak areas of THF and 5MF in the standard solution after an addition of perchloric acid, trichloroacetic acid, acetone or acetonitrile. The peak area of THF decreased by the addition of those agents. The decrease was the least in the case of perchloric acid. The peak area of 5MF did not change by the addition of perchloric acid or trichloroacetic acid.

Table 4 shows the peak areas of THF and 5MF after the deproteinization of plasma spiked with THF and 5MF. The peak area of THF exponentially decreased with the passage of time after the deproteinization. A degradation half life of THF was about 40 min. The peak area of 5MF was nearly

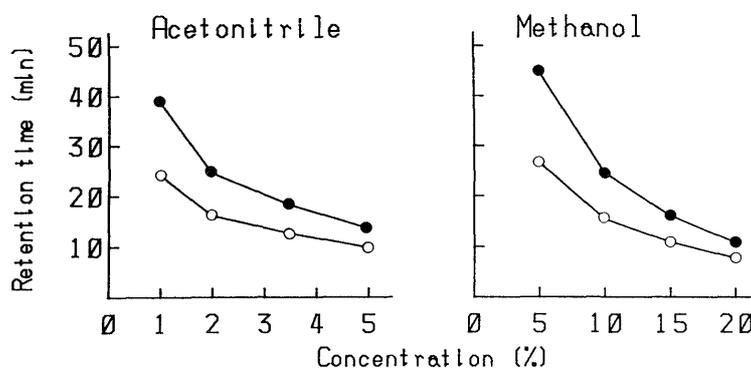


Fig. 3. Effect of the organic solvent concentration in mobile phase on retention times of THF (○) and 5MF (●). The buffer was 20 mM acetate buffer of which pH value was 3.6.

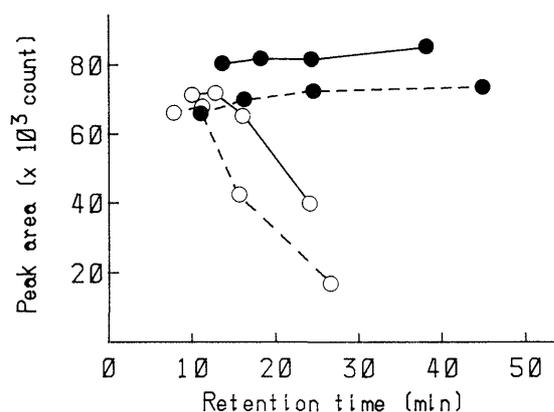


Fig. 4. Retention time-dependent decrease in peak area of THF (○) and 5MF (●). Solid line represents the case where acetonitrile was used as organic solvent of the mobile phase. Dotted line represents the case where methanol was used.

Table 2. Linear range of THF and 5MF by the HPLC-ECD analysis

range (ng/ml)	THF			5MF		
	a	b	r	a	b	r
0.5–2.5	3308	-2	.9995	3943	-44	.9993
2–10	3256	200	.9995	3564	725	.9966
20–100	3128	10102	.9980	3536	14373	.9989

$Y=ax+b$ was regressed to 5 points with same interval. r is correlation coefficient.

Table 3. Stability of THF and 5MF in the standard solution after an adding of deproteinizing agents

Agents	Peak area (% of control)	
	THF	5MF
Perchloric acid	79.1±1.1	102.0±3.3
Trichloro acetate	66.1±5.0	99.0±2.0
Acetone	21.3±1.4	30.6±0.8
Acetonitrile	30.1±2.0	33.7±0.8

Each value represents mean±S.D. (n=3)

Table 4. Time-dependent decrease in peak areas of THF and 5MF after deproteinization

Time (min)	THF (% of 0 time response)	5MF (% of 0 time response)
40	55.9±10.5	97.1±3.1
80	25.3±5.7	94.0±2.7
120	13.5±0.5	90.9±12.4

Each value represents mean±S.D. (n=5)

constant after the deproteinization.

Plasma levels in pig: Plasma levels of THF and 5MF in 7 Goettingen miniature pigs were determined by the HPLC-ECD analysis. The sample was taken at least 3 times from each animals during 3 months. The levels of THF and 5MF were 11.4 ± 4.3 and 2.74 ± 2.17 ng/ml (mean±S.D., n=24), respectively.

DISCUSSION

This study developed a simultaneous analytical method of THF and 5MF in pig plasma by using HPLC-ECD. As is shown in Fig. 1 both peaks of THF and 5MF were well separated. This was also confirmed by the consistencies in voltammogram of THF or 5MF between plasma sample and the standard solution. The detection limits for THF and 5MF of our method were more than 20 times lower than their basal levels in pig plasma. Those limits were 50–100 times lower than those by UV detection [1, 5, 12, 14] and 20 times lower than those by fluorometric detection [3].

As is shown in Fig. 2, the saturated response of THF was observed at 250 mV of the applied potential. On the other hand, that of 5MF was observed at 350 mV. This implies that 350 mV is most effective for the simultaneous determination of THF and 5MF. However, we adopted 300 mV in our method. This is because an unknown peak appeared just before the 5MF peak at more than 300 mV.

Our results on the retention characteristics indicated that the retention time of THF must be less than 15 min because of its degradation during the HPLC-ECD analysis. As is shown in Fig. 4, the degradation was not clear when the retention time of THF was less than 15 min. However it was evident when the retention time was more than 15 min. Our results also indicated that acetonitrile may be a better component of mobile phase than methanol, because acetonitrile caused a less degradation of THF than methanol (Fig. 4).

Ascorbic acid and 2-mercaptoethanol were compared as an antioxidant in this study. We adopted ascorbic acid because 2-mercaptoethanol had a similar retention time to THF on the chromatogram of HPLC-ECD. Ascorbic acid as antioxidant is generally used at 1–4% of the concentration for the determination of folate including THF by HPLC [1, 3, 5, 14]. At those levels, however, the peak of ascorbic acid was so large on the HPLC-ECD

chromatogram that both peaks of THF and 5MF were interfered by that of ascorbic acid. For the standard solution, therefore, ascorbic acid was used at the concentration of 0.2% in our method. When the THF standard solution containing 0.2% ascorbic acid was stored at 4°C, 50% degradation was observed after one day. When the THF standard solution was stored at -20°C for one month, the degradation was negligible. For plasma sample, ascorbic acid was used at the concentration of 0.1%. 0.1% ascorbic acid in plasma showed better efficacy of antioxidation than 0.2% in the standard solution. This fact may suggest that some endogenous substance in plasma is involved in the stability of THF.

After deproteinization, time-dependent degradation of THF was evident with the half life of 40 min. Therefore, we made the time from deproteinization to the injection of the supernatant into the HPLC-ECD system as short as possible. That time was 3 min in this study. As a result, a relatively high recovery was obtained with a low CV value.

For the determination of folate levels in pigs, radioligand assay has been used [6, 7, 13]. Since this assay is sensitive to many active folates, the obtained level represents a sum of active folate levels. Recently, Natsuhori *et al.* [9] demonstrated that the radioligand assay overestimated THF level about 4 times higher than real level but exactly estimated 5MF. Furthermore, they also demonstrated that the differences in folate levels between the HPLC-ECD analysis (THF+5MF) and the radioligand assay were resulted from that overestimation rather than from an existence of another folate in pig plasma. This indicates that the radioligand assay is not suitable for the determination of folate level in pig plasma. Alternatively, folate level in pig plasma must be determined by a method which can separately determine THF and 5MF, such as HPLC analysis.

It has been well established that plasma 5MF plays important roles in the homeostasis of folate metabolism [4, 10]. In contrast, there is no report that describes the physiological roles of plasma THF. Plasma THF, however, may have certain roles in the homeostasis of folate metabolism in pigs, since THF is an active folate which acts as a cofactor in intracellular folate metabolism. We are now studying physiological roles of plasma THF in pigs.

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