Kinetic profile of overall elimination of 5-methyltetrahydropteroylglutamate in rats

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The physiological level of folates in the body is considered to be finely regulated, as is the case with other vitamins. Major components involved in folate homeostasis are enterohepatic circulation (45, 49, 50, 55) and renal conservation (30, 38, 40). Of the different folate derivatives, the physiologically transportable form, 5-methyltetrahydropteroylglutamate (5-CH3-H4PteGlu), is the major folate present in plasma (42), urine (2), and bile (48, 50). Liver is the main storage organ for folates (7, 35, 41, 53). 5-CH3-H4PteGlu taken up by hepatocytes is largely secreted in bile and subsequently translocated to the small intestine where it is reabsorbed (48). When such enterohepatic cycling is interrupted by a procedure such as bile cannulation, plasma folate levels fall rapidly (49). Thus enterohepatic recycling contributes to the maintenance of folate levels in the body (49). 5-CH3-H4PteGlu is freely filtered through the glomeruli and reabsorbed within the proximal tubes in the kidney (38, 56). This suggests that reabsorption in the kidney may also be involved in the homeostatic regulation of folates (42).

Folate deficiency can result from inadequate dietary intake, malabsorption, and altered hepatic metabolism. Increased urinary excretion of folate, which occurs in alcoholism (24, 55) and pregnancy (23), has also been shown to reduce plasma levels of folate in humans and animals. A deficiency of folate reabsorption in the small intestine could be a mechanism for malabsorption syndromes (42). The mechanism of membrane transport involved in such folate homeostasis in the body remains poorly understood despite its important physiological roles, although several studies have indicated that the hepatic uptake of folates is via carrier-mediated transport (15, 17, 19, 51), and canicular excretion is via a canicular multispecific organic anion transporter (cMOAT; see Ref. 21). The renal tubular reabsorption process involves binding of folate to folate-binding protein (FBP) localized on the brush-border membranes (6, 13, 39). In single-dose studies, Selhub et al. (38) found that the urinary folate clearance in rats was increased on increasing the dose injected. Muldoon and McMartin (31) reported that net reabsorption was governed by both a high-capacity-nonsaturable system and a low-capacity-saturable system in isolated perfused rat kidney. Until now, however, no kinetic analysis has been carried out to compare the nonlinear pharmacokinetics of 5-CH3-H4PteGlu with respect to biliary excretion and renal reabsorption.

Natural folates are present in tissues entirely in their polyglutamate forms. Folate, after assimilation into the cytoplasm in the monoglutamate form, is subsequently converted to the corresponding polyglutamate form by addition of several glutamic acid residues and is retained in cells (16). These polyglutamates serve to keep the folates within the cells (26). To understand the characteristics of folate excretion from liver and kidney, transport of the monoglutamate form should be investigated since only this form of folates is believed to be transported across biological membranes (42). However, no report of excretion kinetics based on tissue folate concentrations has been published because of difficulties in performing a quantitative analysis in tissue samples. A number of chromatographic methods have been used to determine folates after conversion to their corresponding monoglutamates (9, 10, 12, 14, 33, 52, 57). No assay for the determination of only the monoglutamate form, which is actually present in tissues, has been reported. In this paper, we established a method for determining 5-CH3-H4PteGlu
in the monoglutamate form in liver and kidney to examine the biliary and urinary excretion kinetics of the 5-CH$_3$-H$_4$PteGlu form, evaluated by changing plasma and tissue concentrations by use of various continuous infusion rates to achieve steady-state folate levels in plasma, liver, and kidney.

**METHODS**

Chemicals and reagents. The calcium salt of (R,S)-5-CH$_3$-H$_4$PteGlu (purity, 99.6%) was obtained from Dr. B. Schircks Laboratories (Jona, Switzerland). Sodium salt of p-hydromercurobenzoate (pHMB), zinc oxide, benzamidine, leupeptin, pepstatin A, and phenylmethylsulfonyl fluoride were obtained from Sigma Chemical (St. Louis, MO). Sodium ascorbate, sodium acetate (trihydrate), acetic acid, disodium salt of tannin, and paraformaldehyde were obtained from Wako Pure Chemical Industries (Osaka, Japan). All chemicals and reagents were of analytical grade.

Animal preparation. Male Sprague-Dawley rats (Nissei'ai, Tokyo, Japan) weighing 250–300 g were used throughout the study. All animals were provided with water and food ad libitum. Those animals had free access to water and food. This study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. Under light ether anesthesia, both the femoral artery and vein were cannulated with PE-50 tubing (ID 0.58 mm; OD 0.965 mm; Becton-Dickinson) for blood sampling and for infusion of 5-CH$_3$-H$_4$PteGlu, respectively, and the bile duct was cannulated with PE-10 tubing (ID 0.28 mm; OD 0.61 mm; Becton-Dickinson) for bile collection. The bladder was catheterized with polyethylene tubing (no. 8; Hibiki, Tokyo, Japan). Rats were divided into five groups, and each received an intravenous bolus injection, followed by an infusion, to achieve steady-state plasma concentrations, biliary excretion, and urinary excretion of 5-CH$_3$-H$_4$PteGlu. After being dissolved in saline, 5-CH$_3$-H$_4$PteGlu was infused in each group at a rate of 48.3, 121, 241, 483, or 965 nmol·h$^{-1}$·kg$^{-1}$ after a bolus administration of 40.2, 101, 201, 402, or 804 nmol/kg. These infusion rates were selected in our preliminary experiment to attain an ~1.5 times higher plasma 5-CH$_3$-H$_4$PteGlu concentration than the endogenous level (100 nM) at the lowest dose and to investigate any nonlinearity in the urinary and biliary excretion kinetics at the higher doses. The rats were given a pelleted diet (CE-2; Clea, Tokyo, Japan), the folic acid content of which was 2 mg/kg diet, ad libitum. If we assume that each male rat consumes 20 g diet/day (20, 54), the rate of intake of folate is 40 µg/day. The infused folate in the present study was 7.2–144 µg/h, i.e., ~3.6–72 times higher than the daily dietary folate. The rate of infused saline was adjusted to 7 ml/4-h infusion period to minimize the loss of biological fluids during the experiment.

At fixed times after initiation of the infusion, 0.4 ml of arterial blood was collected in 1.5-ml Eppendorf microcuvette tubes containing 8 µl of 25% sodium ascorbate solution, and plasma was obtained by centrifugation. Bile was collected in preweighed 1.5-ml Eppendorf microcuvette tubes placed on ice containing 0.4% sodium ascorbate solution (bile-sodium ascorbate, ~1.1 vol/vol) at 30-min intervals for 4 h. Urine was collected in preweighed 1.5-ml Eppendorf microcuvette tubes placed on ice containing 0.2 ml of 1.0% sodium ascorbate solution at 1-h intervals. At the time of urine sampling, the remaining urine in the bladder was flushed out with 1% sodium ascorbate solution. At the end of the infusion, the liver and kidneys were removed and immediately frozen in liquid nitrogen. Plasma, bile, urine, liver, and kidney samples were kept at −80°C until the determination of folates.

Determination of plasma, urine, and bile 5-CH$_3$-H$_4$PteGlu. 5-CH$_3$-H$_4$PteGlu was measured in plasma, bile, and urine using high-performance liquid chromatography (HPLC) with an electrochemical detector (ECD) as previously described (43, 44). An HPLC-ECD system included the following items: a pump (LC-10AD; Shimadzu, Tokyo, Japan), a fixed-loop injector (model 7125; Rheodine, Cotani, CA), an analytical column (4 µm phenyl-bonded phase, 8 × 100 mm; Radical-Pak cartridge, 5NVPH4u; Waters), an amperometric detector (Nanospace SI-1; Shisuido, Tokyo, Japan), and a data processor (Chromatopack C-R4A; Shimadzu, Kyoto, Japan). For the determination of 5-CH$_3$-H$_4$PteGlu in plasma and urine samples, the mobile phase was a mixture of 20 mM acetic acid buffer (pH 3.6) containing 0.1 mM EDTA and acetoni-trile (97.6:2.4), and the ECD applied potential was +300 mV (43). For the determination of 5-CH$_3$-H$_4$PteGlu in bile, a mixture of 20 mM acetic acid buffer (pH 5) containing 0.1 mM EDTA and acetoni-trile (97.6:2.4) was used as the mobile phase, and the ECD applied potential was +350 mV (44). These ECD potentials were selected to minimize the noise peaks in each sample. The flow rate was 0.8 ml/min. For the analysis of plasma samples, 100 µl of 0.5 M perchloric acid were added to 100 µl plasma and were vortexed for 10 s and then centrifuged at 5,000 g and 4°C for 2 min. The supernatant was filtered through a 0.45-µm filter (Chromatodisk 4A; Biofield, Tokyo, Japan), and 50 µl of filtrate were injected in HPLC. For detection of folate derivatives in bile and urine, samples were diluted with 6 and 10 volumes of 0.2% sodium ascorbate, respectively. Diluted samples were filtered through a 0.45-µm filter (Chromatodisk 4A; Biofield), and 50 µl of filtrate were injected in the HPLC.

Determination of liver and kidney concentrations of 5-CH$_3$-H$_4$PteGlu. Additives such as 1 mM ZnSO$_4$, protease inhibitors mixture (0.5 mg/ml aprotinin, 0.1 µg/ml benzamidine, 0.5 mg/ml leupeptin, 0.5 mmol sodium ascorbate, and 0.3 mmol phenylmethylsulfonyl fluoride), 0.5 mM pHMB, or 100 mM EDTA were added to extraction buffer (50 mM HEPES containing 2% sodium ascorbate) that had a pH of 9.0. Frozen livers or kidneys were homogenized in 4 volumes of ice-cold extraction buffer using a Polytron homogenizer for 10 s at 15,000 rpm. Homogenates prepared in this way were stored on ice for the specified time, and 500 µl of homogenate were added to 500 µl of 0.5 M perchloric acid. After vortexing for 10 s, the mixture was centrifuged at 2,000 g for 20 s. The supernatant was filtered through a 0.45-µm filter (Millex-HA; Millipore), and 100 µl of filtrate were injected in the HPLC. The time taken from homogenization to the injection of each sample in the HPLC did not exceed 2 min. The concentration of 5-CH$_3$-H$_4$PteGlu in liver or kidney was assayed by the method described above for plasma and urine samples. The recovery of 5-CH$_3$-H$_4$PteGlu in the tissue homogenates was 93.6 ± 7.9%. The coefficient of variation in the triplicate determinations of 5-CH$_3$-H$_4$PteGlu under the HPLC conditions was 8.4%.

Determination of liver concentrations of 5-methyltetrahydrofolic acid. Rat plasma was used as the source of conjugase to hydrolyze folate polyglutamates. It was prepared from heparinized blood by centrifugation and dialysis against phosphate buffer (pH 7.0) containing activated charcoal using published procedures (56). The 5-CH$_3$-H$_4$PteGlu content of this preparation was 54.3 nM. Minced liver was transferred to a test tube, and 10 volumes of extraction buffer (pH 7.85) containing 0.2 M 2-mercaptoethanol were added. The test tube was heated for 10 min at 100°C in the dark and was cooled to 0°C in an ice bath. The liver was homogenized at...
0°C and was centrifuged at 30,000 g for 20 min to remove insoluble materials. Next, 500 µl of supernatant were mixed with 500 µl of plasma conjugase and were incubated in the dark for 4 h; 500 µl of 0.5 M perchloric acid were added to 500 µl of incubated solution, and the concentration of 5-CH₃-H₄PteGlu was assayed by the method described for plasma and urine samples. To confirm whether all of the 5-methyltetrahydropteroylglutamate (5-CH₃-H₄PteGlun) had degraded, 500 µl of plasma conjugase were added to the remaining incubation medium after 4 h of incubation, and the concentration of 5-CH₃-H₄PteGlu was reassayed. Further incubation failed to show any additional increase in 5-CH₃-H₄PteGlu concentration.

Determination of kinetic parameters. The plasma concentration (Cₚ) of 5-CH₃-H₄PteGlu (µM) was fitted to the following equation to estimate the total body clearance (CL_total) of 5-CH₃-H₄PteGlu (ml·h⁻¹·kg⁻¹)

\[ C_p = (1/CL_{total})I - (1 - (CL_{total}D)/(V_d)) \exp(-CL_{total}t/V_d) \]  

where I is the infusion rate of 5-CH₃-H₄PteGlu (nmol·h⁻¹·kg⁻¹), D is the loading dose of 5-CH₃-H₄PteGlu (nmol/kg), V_d is the distribution volume of 5-CH₃-H₄PteGlu (ml/kg), and t is time. Pharmacokinetic parameters were calculated using the following equations

\[ CL_{bile,p} = (V_{bile}/I) \times CL_{total} \]  
\[ CL_{urine,p} = (V_{urine}/I) \times CL_{total} \]  
\[ CL_{bile,h} = V_{bile}/C_{kss} \]  
\[ CL_{urine,k} = V_{urine}/C_{kss} \]

where C_{kss}, V_{bile}, and V_{urine} are the concentration of 5-CH₃-H₄PteGlu (µM) in the liver 4 h after infusion, the concentration of 5-CH₃-H₄PteGlu (µM) in the kidney 4 h after infusion, the biliary excretion rate of 5-CH₃-H₄PteGlu from 3.5 to 4 h (nmol·h⁻¹·kg⁻¹), and the urinary excretion rate of 5-CH₃-H₄PteGlu from 3 to 4 h (nmol·h⁻¹·kg⁻¹), respectively. CL_bile,h (ml·h⁻¹·kg⁻¹) and CL_bile is the biliary excretion clearance with regard to the plasma concentration and hepatic concentration of 5-CH₃-H₄PteGlu, respectively. CL_{urine,p} (ml·h⁻¹·kg⁻¹) and CL_{urine,k} (ml·h⁻¹·kg⁻¹) are the urinary excretion clearance with regard to the plasma concentration and kidney concentration of 5-CH₃-H₄PteGlu, respectively. The plasma concentration of 5-CH₃-H₄PteGlu (µM) at steady state (C_{pss}) was calculated by

\[ C_{pss} = I/CL_{total} \]

Statistical analysis. For the analysis of the difference between two data sets (liver concentration of both 5-CH₃-H₄PteGlu and 5-CH₃-H₄PteGlu and time profiles for 5-CH₃-H₄PteGlu formation in kidney), the test for equal variance (F-test) and a subsequent Student’s t-test were performed on the two means of the unpaired data. For the analysis of the other multiple data, a test for homogeneity of variance (Bartlett’s test) and a subsequent Tukey test were performed on the means for unpaired data. A P value of <0.05 was considered to be statistically significant.

RESULTS

Effect of various additives on the increase in 5-CH₃-H₄PteGlu concentrations in the liver. To set up the conditions for homogenate preparation for the quantification of 5-CH₃-H₄PteGlu in the liver, each blank liver homogenate was prepared in extraction buffer containing a variety of additives and was stored on ice, and the concentration change over 1 h for 5-CH₃-H₄PteGlu, which was normalized with respect to the monoglutamate form at time 0, was monitored (Fig. 1). Liver homogenate prepared in extraction buffer of pH 7.85 showed that the 5-CH₃-H₄PteGlu content considerably increased with time, being 12 times higher after 1 h (Fig. 1). When the pH of the extraction buffer was changed from 7.85 to 9.0, the increase in 5-CH₃-H₄PteGlu concentration was about eightfold after 1 h; this value was relatively low compared with the result at pH of 7.85 (Fig. 1). When the pH of the extraction buffer was changed from 7.85 to 9.0, the increase in 5-CH₃-H₄PteGlu content considerably increased with time, being 12 times higher after 1 h (Fig. 1). When the pH of the extraction buffer was changed from 7.85 to 9.0, the increase in 5-CH₃-H₄PteGlu concentration was about eightfold after 1 h; this value was relatively low compared with the result at pH of 7.85 (Fig. 1). When protease inhibitors were also added to the extraction buffer (pH 9.0), there was still an increase in 5-CH₃-H₄PteGlu (Fig. 1). EDTA and pHMB in this extraction buffer inhibited the increase in 5-CH₃-H₄PteGlu in homogenate (Fig. 1), but the inhibitory effect was not complete; the concentration was ~4.5-fold higher after 1 h compared with the initial
value (Fig. 1). ZnSO₄ inhibited the concentration change in 5-CH₃-H₄PteGlu among tested additives; the concentration increased, reaching a value at most 50% higher than the initial concentration (Fig. 1). When both ZnSO₄ and pHMB were added to extraction buffer, the increase in 5-CH₃-H₄PteGlu was only 15% up to 30 min and 30% at 1 h of the monoglutamate form at time 0 (Fig. 1). The statistical analysis was performed by the Tukey test on the seven means of the data shown in Fig. 1A. For the data at both 30 and 60 min, a statistically significant difference was observed 1) between the data at pH 7.85 and all the other data, 2) between pH 9.0 and pH 9.0 + pHMB + ZnSO₄, 3) between pH 9.0 and pH 9.0 + ZnSO₄, 4) between pH 9.0 + protease inhibitors and pH 9.0 + pHMB + ZnSO₄, and 5) between pH 9.0 + protease inhibitors and pH 9.0 + ZnSO₄. Similarly, kidney homogenate prepared in pH 7.85 extraction buffer showed an increase in 5-CH₃-H₄PteGlu content (Fig. 1B). When both ZnSO₄ and pHMB were added to the extraction buffer, the increase was only 5% up to 30 min and 11% at 1 h (Fig. 1B). On applying Student’s t-test, the data at both 30 and 60 min in the presence of pHMB + ZnSO₄ (pH 9.0) were found to be significantly different from those at pH 7.85. On the basis of these results, buffer (pH 9.0) containing 1 mM ZnSO₄ and pHMB was selected as the most reliable extraction buffer for the determination of hepatic 5-CH₃-H₄PteGlu.

Biliary and urinary excretion of 5-CH₃-H₄PteGlu. To determine the hepatobiliary and urinary transport of 5-CH₃-H₄PteGlu, plasma concentration, renal excretion, and biliary excretion of 5-CH₃-H₄PteGlu were determined at various infusion rates ranging from 48 to 965 nmol·h⁻¹·kg⁻¹ (Fig. 2A). The plasma concentration had almost reached steady state 4 h after initiation of the infusion. The Cₚss ranged from 0.154 to 1.74 μM and increased as the infusion rate increased (Fig. 2A). Figure 2, B and C, shows the biliary and urinary excretion rate of 5-CH₃-H₄PteGlu, respectively. Both excretion rates reached steady state 4 h after infusion. The statistical analysis was performed using Tukey’s test after the data shown in Fig. 2 were normalized by the corresponding infusion rate. A statistically significant difference was observed in the plasma concentrations at 0.5, 1, 2, 3, and 4 h between all the infusion rates examined, whereas no significant difference was found in the biliary and urinary excretion rates at these time points.

The CL_total calculated using Eq. 1 is shown in Fig. 3A. The CL_total of 5-CH₃-H₄PteGlu obtained ranged from 329 to 588 ml·h⁻¹·kg⁻¹ and increased slightly with an increase in Cₚss (Fig. 3A). The Tukey test was performed on all the data shown in Fig. 3A. A significant difference was found in CL_total of 5-CH₃-H₄PteGlu between 48.3 and 483 nmol·h⁻¹·kg⁻¹ and all the other infusion rates, 2) between 121 and 483 nmol·h⁻¹·kg⁻¹, and 3) between 121 and 965 nmol·h⁻¹·kg⁻¹. CL_bilep and CL_urenp were calculated on the basis of Eqs. 2 and 3, respectively. The CL_bilep ranged from 110 to 133 ml·h⁻¹·kg⁻¹ and remained constant, independent of the Cₚss (Fig. 3B). On the other hand, CL_urenp exhibited a Cₚss-dependent increase (Fig. 3C). The sum of CL_bilep and CL_urenp accounted for 70–80% of CL_total at any infusion rate examined. The CL_urenp was almost comparable with CL_bilep at the lowest infusion rate, whereas it was 3.3 times higher than CL_bilep at the highest infusion rate. No significant difference was found in CL_bilep between any of the infusion rates, although a significant difference was found in CL_urenp, 1) between 48.3 and 483 nmol·h⁻¹·kg⁻¹ and 2) between 48.3 and 965 nmol·h⁻¹·kg⁻¹.

Based on the liver and kidney concentrations of 5-CH₃-H₄PteGlu, CL_bilep and CL_urenp were calculated from Eqs. 4 and 5, respectively. As shown in Fig. 4A, CL_bilep ranges from 150 to 193 ml·h⁻¹·kg⁻¹ and showed minimal change when Cₚss increased from 0.123 to 1.06
µM. CLurine,k of 5-CH3-H4PteGlu at steady state ranged from 16.8 to 106 ml·h\(^{-1}\)·kg\(^{-1}\), showing an increase as C\(_{\text{KSS}}\) increased (Fig. 4B). No significant difference was found in CL bile,h between any of the infusion rates, although a significant difference was found in CL urine,k between 48.3 and 483 nmol·h\(^{-1}\)·kg\(^{-1}\), 2) between 48.3 and 965 nmol·h\(^{-1}\)·kg\(^{-1}\), 3) between 121 and 483 nmol·h\(^{-1}\)·kg\(^{-1}\), and 4) between 121 and 965 nmol·h\(^{-1}\)·kg\(^{-1}\).

Determination of 5-CH\(_3\)-H4PteGlu in liver. After 5-CH\(_3\)-H4PteGlu was infused at 965 nmol·h\(^{-1}\)·kg\(^{-1}\) for 4 h, the liver concentration of 5-CH\(_3\)-H4PteGlu was 1.06 µM, ~10 times the concentration in blank (noninfused) rat liver, 0.099 µM (Table 1). The 5-CH\(_3\)-H4PteGlu concentration in blank kidney was 531 ± 86 nM, which was five times higher than that in liver. 5-CH\(_3\)-H4PteGlu, in both blank and infused liver was ~30 µM, and the two values were almost comparable (Table 1).

**DISCUSSION**

One of the objectives of the present study was to characterize the transport of 5-CH\(_3\)-H4PteGlu across the bile canalicular membrane in the liver and brush-border membrane in the kidney by estimating biliary and urinary excretion clearance, based on tissue concentrations. To achieve this, it is essential to determine the liver and kidney concentrations of 5-CH\(_3\)-H4PteGlu. However, even when blank liver homogenate was stored on ice, a rapid increase in 5-CH\(_3\)-H4PteGlu occurred; this would lead to overestimation of the liver concentration of 5-CH\(_3\)-H4PteGlu (Fig. 1). Degradation of 5-CH\(_3\)-H4PteGlu by liver conjugase can be considered as a major reason for such an increase in 5-CH\(_3\)-H4PteGlu because the liver has a substantial quantity of this enzyme (22, 32, 47). By increasing the pH of the extraction buffer, 5-CH\(_3\)-H4PteGlu formation could be partially arrested (Fig. 1). This was thought to be due to the low conjugase activity at alkaline pH (18). Various compounds such as EDTA, ZnSO\(_4\), and pHMB, which have been reported to be conjugase inhibitors (11, 32, 34), reduced 5-CH\(_3\)-H4PteGlu formation, whereas protease inhibitors did not produce any significant reduction in 5-CH\(_3\)-H4PteGlu formation (Fig. 1). These results suggest that 5-CH\(_3\)-H4PteGlu formation may be due to degradation of 5-CH\(_3\)-H4PteGlu by conjugase. Although formation of 5-CH\(_3\)-H4PteGlu was still as much

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**Fig. 3.** Total body (CL\(_{\text{total}}\); A), biliary (CL\(_{\text{bile,h}}\); B), and urinary (CL\(_{\text{urine,k}}\); C) clearance of 5-CH\(_3\)-H4PteGlu. For the calculation of each 5-CH\(_3\)-H4PteGlu clearance, biliary, urinary, and infusion rates were divided by plasma concentration at steady state, respectively. Each point and bar represent the mean ± SE of 4 different rats.

**Fig. 4.** Biliary (CL\(_{\text{bile,h}}\); A) and urinary (CL\(_{\text{urine,k}}\); B) clearance of 5-CH\(_3\)-H4PteGlu with regard to tissue concentration. Liver and kidney were isolated 4 h after iv infusion and immediately frozen in liquid nitrogen to inhibit the degradation of 5-methyltetrahydropteroylpolyglutamate to 5-CH\(_3\)-H4PteGlu. Biliary and urinary clearances of 5-CH\(_3\)-H4PteGlu were calculated by dividing the excretion rate by the liver and kidney concentration at steady state. Each point and bar represent the mean ± SE of 4 different rats.
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Table 1. Liver concentration of 5-CH$_3$-H$_4$PteGlu$_n$ and 5-CH$_3$-H$_4$PteGlu

<table>
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<tr>
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<th>5-CH$_3$-H$_4$PteGlu$_n$</th>
<th>5-CH$_3$-H$_4$PteGlu</th>
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<tr>
<td>Blank liver</td>
<td>30.3 ± 3.0</td>
<td>0.099 ± 0.009</td>
</tr>
<tr>
<td>Infused liver</td>
<td>31.3 ± 2.9</td>
<td>1.06 ± 0.09*</td>
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Data are expressed as the mean ± SE of 3 different livers. 5-CH$_3$-H$_4$PteGlu$_n$, 5-methyltetrahydropteroypolyglutamate; 5-CH$_3$-H$_4$PteGlu, 5-methyltetrahydropteroylglutamate. Blank liver, without iv infusion of 5-CH$_3$-H$_4$PteGlu; infused liver, after infusion of 5-CH$_3$-H$_4$PteGlu at 965 nmol·h$^{-1}$·kg$^{-1}$ for 4 h. *Statistically significantly different from blank liver.

Abad and Gregory (1) also showed that the plasma folate concentration increased on increasing the level of folate in the diet. In their analysis, the plasma folate concentration ranged between 54.7 and 410 nM, whereas in the present study, the plasma 5-CH$_3$-H$_4$PteGlu concentration was found to be 154 to 1,740 nM (Fig. 2A). Thus, the plasma folate level found in the present study was near the range found in various dietary conditions. Within the range of C$_{pss}$ levels examined, CL$_{total}$ increased in parallel with the increase in C$_{pss}$, showing a nonlinear elimination profile (Fig. 3). The increase in CL$_{total}$ in parallel with the increase in C$_{pss}$ exhibited a nonlinear elimination profile (Fig. 3). When 5-CH$_3$-H$_4$PteGlu was infused at a low rate, CL$_{bile,p}$ and CL$_{urine,p}$ were about the same, and the sum of CL$_{bile,p}$ and CL$_{urine,p}$ was almost equal to CL$_{total}$ (Fig. 3), indicating that elimination is governed by both biliary and urinary excretion. On the other hand, at the highest infusion rate, CL$_{urine,p}$ was three times greater than CL$_{bile,p}$, which means that kidney is the major organ controlling folate homeostasis at such high plasma levels. These two clearances, the CL$_{urine,p}$ profile of 5-CH$_3$-H$_4$PteGlu was similar to the CL$_{total}$ profile when the C$_{pss}$ increased. This means that the nonlinear elimination of 5-CH$_3$-H$_4$PteGlu from the body is mainly due to a nonlinearity in urinary excretion, rather than biliary excretion.

Selhub et al. (38) reported that the plasma protein binding of 5-CH$_3$-H$_4$PteGlu is constant (19.7%) over the concentration range 0.3 nM-30 µM, which covers the C$_{pss}$ obtained in the present study (0.154–1.74 µM). When account is taken of this plasma binding data and the CL$_{urine}$ shown in Fig. 3C, the CL$_{urine}$ defined in terms of the unbound form of 5-CH$_3$-H$_4$PteGlu was 161–454 ml·h$^{-1}$·kg$^{-1}$. This value at the lowest C$_{pss}$ (161 ml·h$^{-1}$·kg$^{-1}$) was lower than the glomerular filtration rate (GFR), calculated as the urinary excretion rate of $^{14}$C]inulin divided by its plasma concentration at steady state in our preliminary study (~240 ml·h$^{-1}$·kg$^{-1}$). This indicates that 5-CH$_3$-H$_4$PteGlu is reabsorbed in the renal proximal tubules. Therefore, one possible explanation for the increase in CL$_{urine}$ as the C$_{pss}$ increases is saturation of reabsorption in the kidney. This hypothesis is also supported by the present finding of the increase in CL$_{urine,n}$ as the C$_{pss}$ increases (Fig. 4B). This may reflect the saturation of membrane transport from the luminal space to proximal tubule cells based on the following information: FBP is abundantly expressed in the brush-border membranes of proximal tubule cells (7, 39, 38, 40) and actively internalizes folates by endocytosis (5, 25). Bhandari and co-workers (3, 4) have shown that folic acid is transported by isolated rat kidney brush-border membrane vesicles (BBMV) in a saturable and H$^+$-
H4PteGlu is a high-affinity one [Michaelis constant ($K_m$) $< 1 \mu M$], considering the fact that the $K_m$ of folic acid to BBMV is 0.67 $\mu M$ (4) and the binding affinity of 5-CH$_3$-H$_4$PteGlu to FBP is slightly lower than that of folic acid (38). Renal proximal tubule cells not only reabsorb folate from the glomerular filtrate but can also secrete it in the serosal-to-luminal direction (27–29). Such secretion may explain the present finding that the CL$_{urine}$, defined in terms of plasma unbound 5-CH$_3$-H$_4$PteGlu at the highest $C_{PSS}$ (454 m$\cdot$h$^{-1}$kg$^{-1}$) is higher than the GFR.

The CL$_{bile}$ and CL$_{bile}$ of 5-CH$_3$-H$_4$PteGlu did not change as the infusion rate increased (Figs. 3B and 4A). This constant biliary clearance is explained by a low-affinity system in its hepatobiliary transport. Strum change as the infusion rate increased (Figs. 3B and 4A). The finding that CL$_{bile}$ remains constant (Fig. 4A) also supports the hypothesis that cMOAT-mediated excretion in bile is not saturated even at the highest infusion rate. We reported that the $K_m$ of 5-CH$_3$-H$_4$PteGlu to cMOAT was $\sim 100 \mu M$ with use of rat canalicular membrane vesicles (21), which is much higher than the $C_{PSS}$ obtained in the present study (Fig. 4A). The synthesis of biologically active pteroyl-0L-glutamates (folic acid conjugates). Evaluation of [H]pteroylheptaglutamate for metabolic studies. J. Biol. Chem. 247: 226–2271, 1972.

In conclusion, regulation of plasma 5-CH$_3$-H$_4$PteGlu levels is governed by both biliary and urinary excretion. The hepatobiliary excretion process is a relatively low-affinity one compared with urinary excretion, and its efficiency is almost constant at high 5-CH$_3$-H$_4$PteGlu levels in the body. In contrast, the efficiency of renal excretion is sensitive to folate levels in the body and increases at high plasma folate concentrations. Therefore, at high 5-CH$_3$-H$_4$PteGlu levels in the body after a substantial intake of folate, the plasma 5-CH$_3$-H$_4$PteGlu level will be maintained at a relatively lower level by saturation of renal reabsorption.

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