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Identification of 10-formyltetrahydrofolate, tetrahydrofolate and 5-methyltetrahydrofolate as major reduced folate derivatives in rat bile

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ABSTRACT

Reduced folate derivatives in rat bile were examined using high-performance liquid chromatography with electrochemical detection (HPLC–ED). Three peaks of folate compounds were observed on the chromatograms. From the retention-time profiles and hydrodynamic voltammograms, and the profiles of ultraviolet (UV) absorbance spectra obtained by HPLC with photodiode array detection, these 3 peaks were identified as 10-formyltetrahydrofolate (10-HCO-H_4PteGlu), tetrahydrofolate (H_4PteGlu) and 5-methyltetrahydrofolate (5-CH_3-H_4PteGlu). The rates of bile secretion of 10-HCO-H_4PteGlu, H_4PteGlu and 5-CH_3H_4PteGlu were 314 ± 181, 321 ± 179 and 449 ± 198 ng/h (mean ± S.D.), respectively. 10-HCO-H_4PteGlu and H_4PteGlu together with 5-CH_3-H_4PteGlu are found to be the major folate derivatives in rat bile. The nonmethylated folates, 10-HCO-H_4PteGlu and H_4PteGlu, may also play an important role in folate homeostasis.

INTRODUCTION

Bile folates are secreted into the small intestine and reabsorbed [1]. The enterohepatic circulation of folates plays an important role in folate homeostasis. This is demonstrated for example by the drastic decrease in serum folate levels after interruption of the circulation by bile drainage [2,3]. Steinberg *et al.* [3] described that the secretion rate of bile folates is *ca.* 1 μg per hour, which is equivalent to 20 times the daily folate requirement.

The principal folate in bile has been found to be 5-methyltetrahydrofolate (5-CH_3-H_4PteGlu) [1,4]. Pratt *et al.* [5], however, described that the amount of 5-CH_3-H_4PteGlu in bile is only 20–60% of the total bile folates. Moreover, several investigators reported that some other forms of folates appear in rat bile after intravenous injection of [^{3}H]pteroylglutamic acid (PteGlu) [3,6,7]. Hillman *et al.* [6] demonstrated the secretion of radioactive 5-CH_3-H_4PteGlu, 10-formyltetrahydrofolate (10-HCO-H_4PteGlu) and 5-formyltetrahydrofolate (5-HCO-H_4PteGlu) after injection of [^{3}H]PteGlu in rats. Pheasant *et al.* [7] demonstrated bile secretion of radioactive 5-CH_3-H_4PteGlu, 10-HCO-H_4PteGlu and 10-HCO-PteGlu after injection of [^{3}H]PteGlu.

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Thus, it is quite possible that folates other than $5\text{-}CH_3\text{-}H_4PteGlu$ are secreted into bile. Nevertheless, to our knowledge, there have been no reports on folates other than $5\text{-}CH_3\text{-}H_4PteGlu$ in rat bile. Recently, we have developed a sensitive method to determine tetrahydrofolate ($H_4PteGlu$) and $5\text{-}CH_3\text{-}H_4PteGlu$ in pig plasma using high-performance liquid chromatography with electrochemical detection (HPLC–ED) [8,9]. In the present study, the identification and secretion rate of bile folates were examined in rats using the HPLC–ED.

EXPERIMENTAL

Materials and reagents

[6*R*,*S*]-$H_4PteGlu$, the disodium salt of [6*R*,*S*]-$5\text{-}CH_3\text{-}H_4PteGlu$, pterine, neopterin, biopterin, dimethylpterin, hydroxymethylpterin, dimethyltetrahydropterin, pteroic acid and dihydrofolate were obtained from Sigma (St. Louis, MO, USA). *p*-Aminobenzoyl glutamate (*p*ABG) was obtained from Fluka (Buchs, Switzerland) and [3′,5′,7,9-^{3}H]PteGlu from Amersham International (Buckinghamshire, UK). PteGlu was obtained from Wako Pure Chemical Industries (Osaka, Japan), pterin carboxylic acid from Aldrich (MI, USA) and tetrahydrobiopterin from Dr. B. Schircks Laboratory (Jona, Switzerland). The calcium salt of $5\text{-}HCO\text{-}H_4PteGlu$ was a gift from Lederle Japan Co.(Tokyo, Japan). Sodium ascorbate, sodium acetate (trihydrate), acetic acid and the disodium salt of ethylenediaminetetraacetic acid (EDTA) were obtained from Wako Pure Chemical Industries. $10\text{-}HCO\text{-}H_4PteGlu$ was prepared from [6*R*,*S*]-$5\text{-}HCO\text{-}H_4PteGlu$ using the method described by Scott [10]. $10\text{-}HCO\text{-}H_4PteGlu$ and $H_4PteGlu$ were purified by anion-exchange chromatography using QAE-Sephadex A-25 (Pharmacia LKB, Uppsala, Sweden) [11]. The concentrations of these folates in the eluent were determined by spectrophotometry. All folate-related compounds except for [^{3}H]PteGlu were dissolved in 0.2% sodium ascorbate solution and stored at $-80°C$.

Sampling

Female Sprague–Dawley rats weighing 250–300 g (*ca.* 13 weeks old; Clea Japan, Tokyo, Japan) were used. The animals were fed a pelleted feed (CE-2, Clea Japan) and allowed to drink water *ad libitum*. The animals were anesthetized with an intraperitoneal injection of urethan (0.8–1.0 g/kg). After the abdominal wall had been incised, the bile duct was isolated and cannulated with a polyethylene catheter (0.28 mm I.D., SP-10, Natsume Co., Tokyo, Japan). The bile samples were collected into ice-cold tubes containing 0.4% sodium ascorbate solution (bile:sodium ascorbate solution = *ca.* 1:1) at intervals of 30 min. After measuring the volume, the bile samples were stored at $-80°C$ until HPLC analysis.

The bile samples were centrifuged at 5000 *g* for 2 min immediately after thawing. The supernatants were diluted with 0.2% sodium ascorbate solution (1:10), and filtered with a 0.45-μm microfilter (Chromatodisk, Biofield, Osaka, Japan). Aliquots (100 μl) were injected into the HPLC system.

$10\text{-}HCO\text{-}H_4PteGlu$, $H_4PteGlu$ and $5\text{-}CH_3\text{-}H_4PteGlu$ were stable in the bile sample containing 0.2% sodium ascorbate. Degradation of these folates was negligible up to 1 h at 4°C. The stability of these folates was confirmed in the samples which were stored at $-80°C$ for at least several weeks.

Chromatography

Chromatographic analysis was performed using HPLC systems with different detectors. Analytical detectors included an electrochemical detector (L-ECD-6A, Shimadzu, Kyoto, Japan), a photodiode array detector (Multi-340, Jasco, Tokyo, Japan), a liquid scintillation counter (Trace II 7150, Hewlett-Packard, Penna, USA) and an ultraviolet detector (UV; SPD-6A, Shimadzu). The HPLC system had the following setup; a pump (LC-9A, Shimadzu), a 100-μl fixed-loop injector (Model 7125, Rheodyne, Cotati, CA, USA), a guard column (5 μm RP-8, 4 mm $\times$ 4 mm I.D., Merck, Darmstadt, Germany), an analytical column (C_8 column, RP-8 (5 μm), 250 mm

× 4 mm I.D., Merck) and a data processor (C-R4A, Shimadzu). The flow-rate was 0.8 ml/min.

Identification of bile folates

The retention-time profiles. Retention times of the bile folates and standards were examined using the HPLC–ED method. The profiles were determined for various pH values (3.5, 4.0, 4.5 and 5.0) of acetate buffer in the mobile phase, and for various fractions of acetonitrile (3, 4, 6 and 8%) in the mobile phase.

Electrochemical properties. The hydrodynamic voltammograms were obtained from the responses *vs.* the potentials (50, 100, 150, 200, 250, 300, 350, 400 and 450 mV) applied in the electrochemical detector. The mobile phase was a mixture of 20 m*M* acetate buffer containing 0.1 m*M* EDTA and acetonitrile (94:6, v/v).

UV absorption characteristics. The spectral curves of the UV absorbance of the bile folates and standards were obtained using HPLC with a photodiode array detection system (HPLC–PAD). The mobile phase used in this analysis was a mixture of 50 m*M* phosphate buffer (pH 5.0)–acetonitrile (95:5, v/v). As an index for the similarity of the UV spectra between bile folates and standards, a correlation coefficient was calculated from the following equation.

$$r = \frac{N\sum(X_i\, Y_i) - (\sum X_i)(\sum Y_i)}{\sqrt{\{N\sum X_i^2 - (\sum X_i)^2\}\{N\sum Y_i^2 - (\sum Y_i)^2\}}} \quad (1)$$
$$(i = 0, 1, 2, 3, ..., N - 1)$$

where X_i and Y_i are the absorbances of bile folate and standard at $200 + 4\,i$ nm, respectively.

Retention profile of radioactive metabolites of [^{3}H]PteGlu in bile

The [^{3}H]PteGlu (specific activity, 20–50 Ci/mmol) was diluted with PteGlu solution and intravenously injected in the rats at a dose of 240×10^6 dpm/kg of body weight (PteGlu, 1.1 mg/kg of body weight). After collecting bile, an aliquot of the sample (100 μl) was subjected to HPLC with a liquid scintillation counting system (HPLC–LSC) combined with a UV detector. The remainder of the bile sample was infused into the duodenum at a constant rate (0.6 ml/hr) for enterohepatic recirculation. The mobile phase was a mixture of 20 m*M* acetate buffer (pH 3.5) containing 0.1 m*M* EDTA and acetonitrile (95:5, v/v).

Five radioactive peaks were detected on the HPLC–LSC chromatogram. The retention times of each radioactive peak were compared with those of the following 16 folate-related compounds using HPLC with ED and UV detection system. The folate-related compounds were 10-HCO-H_4PteGlu, H_4PteGlu, 5-CH_3-H_4PteGlu, pterine, neopterin, biopterin, dimethylpterin, hydroxymethylpterin, dimethyltetrahydropterin, pteroic acid, tetrahydrobiopterin, pterin carboxylic acid, *p*ABG, dihydrofolate, PteGlu and 5-HCO-H_4PteGlu.

Secretion rate of bile folates

The rate of bile-folate secretion was examined in 11 rats. The secretion rate per animal was calculated as the mean value of 3 bile samples collected up to 1.5 h after cannulation. The time course of bile-folate secretion was examined after intravenous injection of PteGlu (1 mg/kg) *via* the jugular vein in 4 rats. The concentrations of 10-HCO-H_4PteGlu, H_4PteGlu and 5-CH_3-H_4PteGlu including PteGlu in bile were determined simultaneously using an HPLC–ED–UV system. The mobile phase was a mixture of 20 m*M* acetate buffer (pH 4.5) containing 0.1 m*M* EDTA–acetonitrile (94:6, v/v). The applied potential of the ED was 350 mV for the determination of the reduced folates and the UV wavelength for detection of PteGlu was 310 nm. Detection limits were *ca.* 0.5 ng/ml for the reduced folates and 10 ng/ml for PteGlu.

RESULTS

Identification of bile folates

The chromatogram of rat bile obtained from HPLC–ED analysis is shown in Fig. 1. Peaks A, B and C had the same retention time as those of standard 10-HCO-H_4PteGlu, H_4PteGlu and 5-CH_3-H_4PteGlu, respectively. Consistency of the retention times between these compounds in bile and in the standard solution was obtained for different pH values of the acetate buffer (Table I) and for different concentrations of acetonitrile in

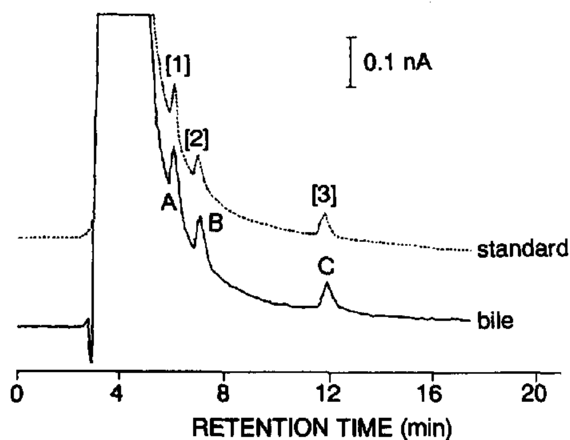

Fig. 1. HPLC–ED chromatogram of rat bile folates and standards. Standards [1], [2] and [3] represent 10-formyltetrahydrofolate (10-HCO-H_4PteGlu), tetrahydrofolate (H_4PteGlu) and 5-methyltetrahydrofolate (5-CH_3-H_4PteGlu), respectively. Three peaks, A, B and C were identified in bile samples.

the mobile phase (Table II). The hydrodynamic voltammograms of the bile compounds indicating peaks A, B and C were almost identical to those of standard solutions, including 10-HCO-H_4PteGlu, H_4PteGlu and 5-CH_3-H_4PteGlu, respectively (Fig. 2).

Furthermore, the UV absorbance curves of the bile substances obtained from peaks A, B and C were very similar to those of standard solutions of 10-HCO-H_4PteGlu, H_4PteGlu and 5-CH_3-H_4PteGlu, respectively (Fig. 3). The correlation coefficients were calculated by eqn. (1) as an index of similarity of the UV spectra for bile folates and standards. The values were 0.9828 for 10-HCO-H_4PteGlu, 0.9938 for H_4PteGlu and 0.9969 for 5-CH_3-H_4PteGlu.

Retention profile of radioactive metabolites of [^{3}H]PteGlu

The retention times of radioactive metabolites after an intravenous injection of [^{3}H]PteGlu were analyzed with an HPLC–LSC system. Fig. 4 shows the chromatogram of a bile sample collected between 1.5 and 2 h after an injection with [^{3}H]PteGlu. Five predominant radioactive peaks (I–V) were observed. Peaks I–V had the same retention times as those of *p*ABG, H_4PteGlu, 10-HCO-H_4PteGlu, 5-CH_3-H_4PteGlu and PteGlu, respectively.

Secretion rate of bile folates

Table III shows the bile secretion rates of 10-HCO-H_4PteGlu, H_4PteGlu and 5-CH_3-H_4PteGlu. The secretion rates of 10-HCO-H_4PteGlu

TABLE I

RETENTION TIMES OBTAINED FROM HPLC–ED ANALYSES UNDER THE VARIOUS pH VALUES OF ACETATE BUFFER IN THE MOBILE PHASE

pH	Retention time (min)[a]					
	Peak A	10-HCO-H_4PteGlu	Peak B	H_4PteGlu	Peak C	5-CH_3-H_4PteGlu
3.5	10.63 ± 0.03	10.73 ± 0.13	9.22 ± 0.05	9.19 ± 0.08	12.26 ± 0.10	12.25 ± 0.10
4.0	8.67 ± 0.05	8.63 ± 0.12	9.28 ± 0.06	9.18 ± 0.07	13.70 ± 0.07	13.60 ± 0.16
4.5	6.82 ± 0.11	6.82 ± 0.07	7.94 ± 0.08	7.87 ± 0.11	13.33 ± 0.21	13.30 ± 0.17
5.0	5.43 ± 0.13	5.31 ± 0.02	6.56 ± 0.10	6.42 ± 0.03	12.03 ± 0.05	11.99 ± 0.05

[a] Values are means ± S.D. (n = 3). Peaks A, B and C were found in rat bile, and the peaks of 10-formyltetrahydrofolate (10-HCO-H_4PteGlu), tetrahydrofolate (H_4PteGlu) and 5-methyltetrahydrofolate (5-CH_3-H_4PteGlu) were obtained from standard solution. The fraction of acetonitrile in the mobile phase was 6%.

TABLE II

RETENTION TIMES OBTAINED FROM HPLC–ED ANALYSES UNDER THE VARIOUS FRACTIONS OF ACETONITRILE IN THE MOBILE PHASE

Acetonitrile (%)	Retention time (min)[a]					
	Peak A	10-HCO-H_4PteGlu	Peak B	H_4PteGlu	Peak C	5-CH_3-H_4PteGlu
3	15.01 ± 0.01	15.03 ± 0.68	23.63 ± 1.02	23.73 ± 1.40	41.97 ± 1.87	42.10 ± 2.60
4	11.58 ± 0.64	11.31 ± 0.24	16.35 ± 0.19	16.27 ± 0.15	28.03 ± 0.36	27.93 ± 0.23
6	6.82 ± 0.11	6.82 ± 0.07	7.94 ± 0.08	7.87 ± 0.11	13.33 ± 0.21	13.30 ± 0.17
8	5.42 ± 0.08	5.42 ± 0.02	5.85 ± 0.00	5.82 ± 0.04	7.86 ± 0.01	7.85 ± 0.03

[a] Values are means ± S.D. (n = 3). Peaks A, B and C were found in rat bile, and the peaks of 10-HCO-H_4PteGlu, H_4PteGlu and 5-CH_3-H_4PteGlu were obtained from standard solution. The pH of the acetate buffer in the mobile phase was 4.5.

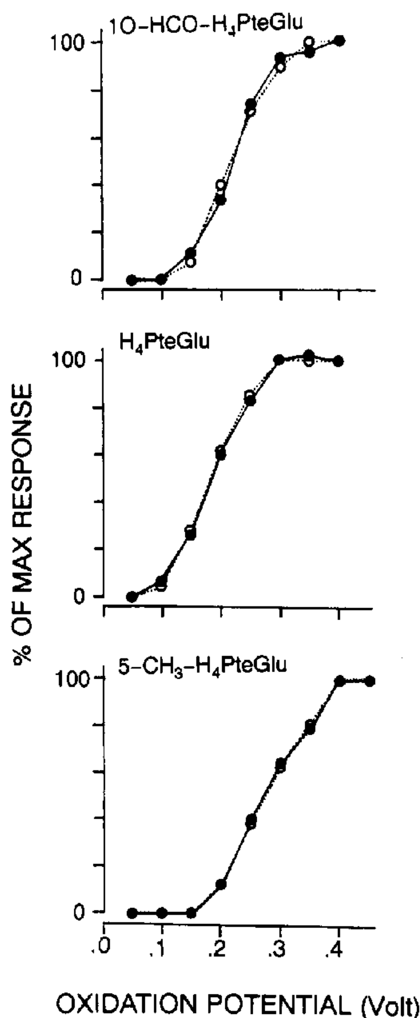

Fig. 2. HPLC–ED hydrodynamic voltammograms of the compounds from peaks A, B and C in rat bile (●) and standards (○). Peak A was compared with 10-HCO-H_4PteGlu, peak B with H_4PteGlu and peak C with 5-CH_3-H_4PteGlu.

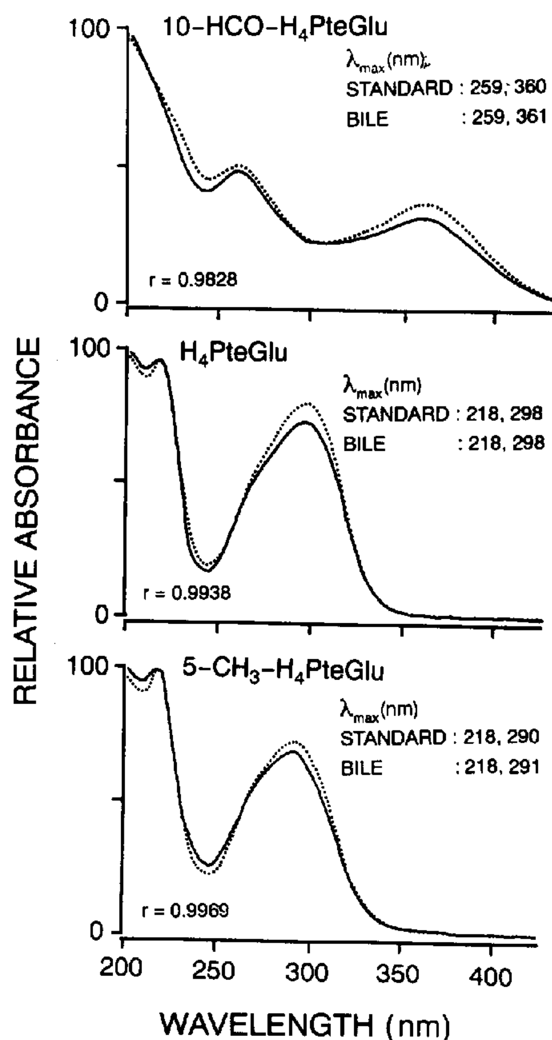

Fig. 3. UV-absorption spectra of the compounds from peaks A, B and C in rat bile and standards. The spectra were obtained at pH 5 with a HPLC–photodiode array detection system. Solid lines represent bile compounds and dotted lines represent standard folates; r-values (correlation coefficients) were calculated by eqn. (1).

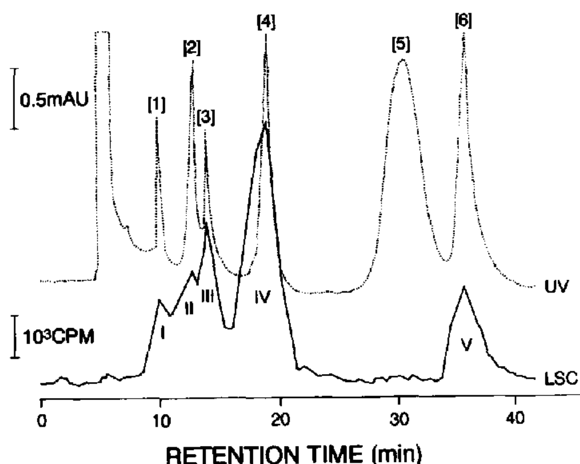

Fig. 4. Chromatograms of rat bile, collected 1.5–2 h after an intravenous injection of [^{3}H]PteGlu, and standards ([1] *p*ABG; [2] H_4PteGlu; [3] 10-HCO-H_4PteGlu; [4] 5-CH_3-H_4PteGlu; [5] 5-HCO-H_4PteGlu; [6] PteGlu). The applied amounts of the standards were 80–100 ng. The chromatogram of bile (solid line) was obtained with HPLC–liquid scintillation counting and the chromatogram of the standards (dotted line) with HPLC–UV detection at 300 nm.

TABLE III

SECRETION RATE OF BILE FOLATES IN RATS

No. of animals = 11 rats. Distribution = percentage of total. The secretion rate of each animal is the mean value of 3 intervals up to 1.5 h after cannulation.

Folate	Secretion rate (mean ± S.D.) (ng/h)	Distribution (mean ± S.D.) (%)
10-HCO-H_4PteGlu	314 ± 181	28.3 ± 5.2
H_4PteGlu	321 ± 179	29.7 ± 9.3
5-CH_3-H_4PteGlu	449 ± 198	42.0 ± 8.5

and H_4PteGlu were comparable to that of 5-CH_3-H_4PteGlu.

Bile-folate secretion during a 5-h period after an intravenous injection with PteGlu (1 mg/kg of body weight) is shown in Fig. 5. 10-HCO-H_4PteGlu, H_4PteGlu and 5-CH_3-H_4PteGlu were significantly increased. The total amounts of 10-HCO-H_4PteGlu (16.3 ± 6.3 μg) and H_4PteGlu (19.4 ± 5.2 μg) secreted in bile during the 5-h

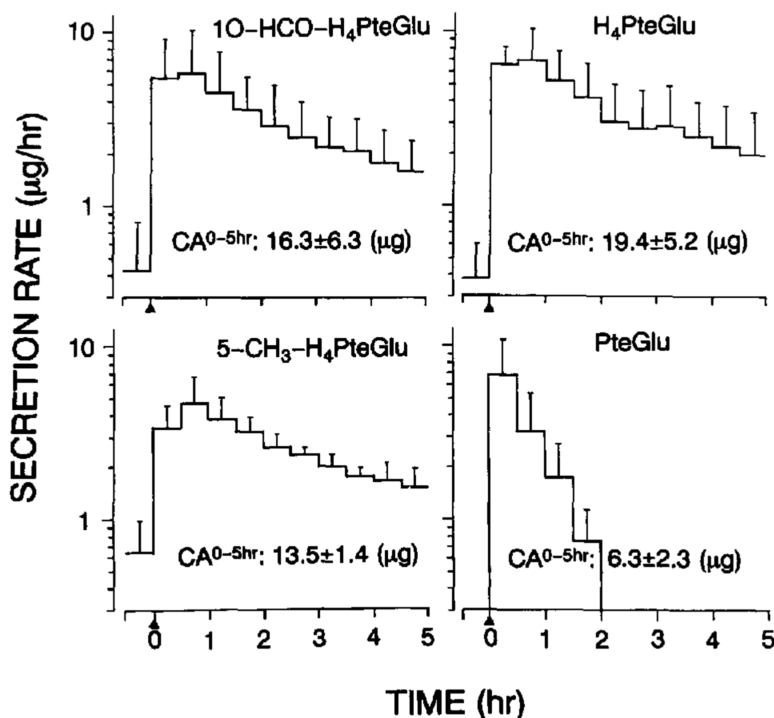

Fig. 5. Time courses of bile-folate secretion during a 5-h period after an intravenous injection of PteGlu (1 mg/kg). CA^{0-5hr} represents the cumulative amount of each folate during the 5-h period after injection. (▲) Represents the injection time of PteGlu. Values are mean ± S.D. (n = 4).

period after the injection of PteGlu were considerably higher than that of 5-CH_3-H_4PteGlu (13.5 ± 1.4 μg). The parent compound (PteGlu) disappeared from bile within 2 h after injection.

DISCUSSION

It has been suggested that 5-CH_3-H_4PteGlu is the principal folate in rat bile [1,4]. However, this study demonstrated that 10-HCO-H_4PteGlu and H_4PteGlu were also secreted as major folates into bile. The identification of 10-HCO-H_4PteGlu and H_4PteGlu in rat bile was based on the following verification: (a) the similarity of the retention times of the compounds from peaks A and B in the bile samples and the standards of 10-HCO-H_4PteGlu and H_4PteGlu for the different pH values of the acetate buffer in the mobile phase, or the different concentrations of acetonitrile in the mobile phase (Fig. 1; Tables I and II); (b) the similarity of the hydrodynamic voltammograms of the bile substances and the standards (Fig. 2); and (c) the similarity of the UV absorbance spectra of the bile substances and the standards (Fig. 3). The first observation concerning the retention time indicates that bile substances from peaks A and B have the same values for the negative logarithm of the acid dissociation constant (pK_a) and the same lipid solubility as 10-HCO-H_4PteGlu and H_4PteGlu, respectively. The second finding, from the hydrodynamic voltammograms, indicates that the bile substances have the same electrochemical properties or redox potentials as the standard folates. The last observation, concerning the UV absorption spectra, indicates that the bile substances have the same UV absorption characteristics as the standard folates.

The predominant peaks of radioactive metabolites of [^{3}H]PteGlu in bile were eluted at the same position as the standards of 10-HCO-H_4PteGlu and H_4PteGlu on the HPLC–LSC chromatogram. This also supports their positive identification. While Hillman *et al.* [6] described the presence of 5-HCO-H_4PteGlu as a radioactive metabolite of [^{3}H]PteGlu in rat bile, no 5-HCO-H_4PteGlu peak was detected on the LSC chromatogram in the present study. This may suggest that the amount of 5-HCO-H_4PteGlu in rat bile is negligible in the physiological state or that the identification of 5-HCO-H_4PteGlu by Hillman *et al.* [6] was due to an artifact. Pheasant *et al.* [7] did not find 5-HCO-H_4PteGlu in rat bile after injection with [^{3}H]PteGlu and their results are consistent with ours.

Although we have not attempted to investigate the presence of polyglutamyl forms of folate in rat-bile samples, it may be that such forms do not exist due to the significant activity of folate conjugase in bile as demonstrated by Horne *et al.* [12].

With regard to the mechanism of bile secretion of 5-CH_3-H_4PteGlu in rats, Steinberg described that this derivative is secreted into bile and the amount is directly proportional to serum folate levels [1], suggesting that 5-CH_3-H_4PteGlu in bile is derived from plasma 5-CH_3-H_4PteGlu. The amounts of folates other than 5-CH_3-H_4PteGlu have been shown to be negligible in rat plasma [8,13,14]. Thus, the mechanism of bile secretion of 10-HCO-H_4PteGlu and H_4PteGlu may be different from that of 5-CH_3-H_4PteGlu. Possibly, 10-HCO-H_4PteGlu and H_4PteGlu in bile are derived from hepatic folate pools. It is interesting to note that the distribution pattern of bile folates in our study (Table III) is similar to that of liver folates reported in previous studies as shown in Table IV [15–24].

It has been well demonstrated that 5-CH_3-H_4PteGlu, through enterohepatic circulation, plays an important role in folate homeostasis [1–4]. However, there has been no report on the bile secretion and intestinal reabsorption of 10-HCO-H_4PteGlu and H_4PteGlu. In the present study, we demonstrated that high levels of 10-HO-H_4PteGlu, H_4PteGlu and 5-CH_3-H_4PteGlu exist in rat bile. Furthermore, after intravenous injection of PteGlu, a considerably larger increase in 10-HCO-H_4PteGlu and H_4PteGlu than in 5-CH_3-H_4PteGlu was observed in bile. These results indicate that both 10-HCO-H_4PteGlu and H_4PteGlu may play an important role in the homeostasis of folates through enterohepatic circulation.

Tani and Iwai [25] demonstrated that [^{3}H]Pte-

TABLE IV

DISTRIBUTION PATTERN OF HEPATIC FOLATES IN RATS IN THE LITERATURE

The values are percentages of total folates.

	H_4PteGlu (%)	HCO-H_4PteGlu[a] (%)	5-CH_3-HJ$_4$PteGlu (%)	Ref.
	22	17	61	15
	29	10	61	16
	44.2	23.3	23.5	17
	29	22	49	18
	45.1	18.2	36.8	19
	41	23.5	35	20
	36.4	15.4	48	21
	35.2	41.3	23.5	22
	39	14	48	23
	32.7	30.3	37.2	24
Range	22–45.1	10–41.3	23.5–61	

[a] HCO-H_4PteGlu represent both 10-HCO-H_4PteGlu and 5-HCO-H_4PteGlu.

Glu, an oxidized monoglutamate of folate, was converted into 5-CH_3-H_4PteGlu after reduction followed by methylation in intestinal mucosal cells of rats. Whitehead *et al.* [26] reported that most of the 5-HCO-H_4PteGlu was metabolized to 5-CH_3-H_4PteGlu during intestinal absorption after oral administration in man. Steinberg [1] pointed out that even though large amounts of folate are excreted into bile, little is found in the stool due to the very efficient absorption. Possibly, these nonmethylated folates, 10-HCO-H_4PteGlu and H_4PteGlu excreted into bile, are reabsorbed for further methylation.

In conclusion, 10-HCO-H_4PteGlu, H_4PteGlu and 5-CH_3-H_4PteGlu are the major reduced folate compounds in rat bile. The nonmethylated folates, 10-HCO-H_4PteGlu and H_4PteGlu, may also play an important role in the homeostasis of the folate metabolism through enterohepatic circulation. We are now studying the possible occurrence of other folate derivatives in rat bile and the kinetics of the reduced folates in enterohepatic circulation.

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