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# Identification of 5,10-methylenetetrahydrofolate in rat bile

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**Abstract**

5,10-Methylenetetrahydrofolate (5,10-$CH_2$-$H_4$PteGlu) was identified as a major active reduced folate in rat bile using high-performance liquid chromatography with electrochemical detection (HPLC–ED). The identification of the folate derivative was based on the similarities in the retention-time profiles, electrochemical properties, UV absorption characteristics and demethylenation profiles of the bile folate and the synthetic standard. An HPLC–ED method was developed for the simultaneous determination of reduced folates including 5,10-$CH_2$-$H_4$PteGlu, tetrahydrofolate ($H_4$PteGlu), 10-formyltetrahydrofolate (10-HCO-$H_4$PteGlu) and 5-methyltetrahydrofolate (5-$CH_3$-$H_4$PteGlu) in rat bile. All peaks of the reduced folates in bile were separated using this method with a total retention time of less than 15 min. The detection limit was 0.01 ng/injection for $H_4$PteGlu, 10-HCO-$H_4$PteGlu and 5-$CH_3$-$H_4$PteGlu, and 0.02 ng/injection for 5,10-$CH_2$-$H_4$PteGlu at a signal-to-noise ratio of 3 and an injection volume of 100 $\mu$l. Recoveries of synthetic folates from rat bile were higher than 90%. The distribution percentages of 5,10-$CH_2$-$H_4$PteGlu, $H_4$PteGlu, 10-HCO-$H_4$PteGlu and 5-$CH_3$-$H_4$PteGlu in rat bile were $29.6 \pm 7.2$, $17.7 \pm 3.5$, $24.4 \pm 6.5$ and $28.2 \pm 7.1$%, respectively, and total secretion rate of the bile reduced folates was $1514 \pm 663$ ng/h (mean ± S.D., $n = 9$).

## 1. Introduction

5,10-Methylenetetrahydrofolate (5,10-$CH_2$-$H_4$PteGlu) is the source of the methyl one-carbon unit for thymidylate synthesis [1–3], and is also required as substrate for several folate-dependent enzymes involved in purine synthesis as well as in amino acid synthesis [1,4,5]. 5,10-$CH_2$-$H_4$PteGlu has been an attractive target not only to physiologists but also to cancer investigators, because of its critical role as the donor of the methyl one-carbon unit to deoxyuridylate [6–10]. Spears [11] demonstrated that 5,10-$CH_2$-$H_4$PteGlu potentiated the anticancer effect of 5-fluorouracil, a notable anticancer drug. It has been well demonstrated that 5,10-$CH_2$-$H_4$PteGlu occurs in the intracellular fraction of rapidly proliferating cells such as tumor cell lines [6–8,12]. However, the occurrence of endogenous 5,10-$CH_2$-$H_4$PteGlu in extracellular fluid has never been demonstrated.

Recently, we identified tetrahydrofolate ($H_4$PteGlu) and 10-formyltetrahydrofolate (10-HCO-$H_4$PteGlu) together with 5-methyltetrahydrofolate (5-$CH_3$-$H_4$PteGlu) in rat bile, and developed a high-performance liquid chromatographic method with electrochemical detection (HPLC–ED) [13,14]. With this analytical meth-

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od, we examined bile folate secretion in rabbits. In the course of our study, we found that $H_4$PteGlu concentrations increased significantly when a bile sample containing ascorbate was kept at room temperature. The $H_4$PteGlu concentrations also increased by heat treatment (unpublished results). These phenomena were also observed in rat bile, although the increase was smaller than in rabbits. We speculated that these phenomena may result from demethylenation of 5,10-$CH_2$-$H_4$PteGlu. Demethylenation of 5,10-$CH_2$-$H_4$PteGlu to $H_4$PteGlu by heat treatment has been described by Wilson and Horne [15], Houghton et al. [16], and Kashani and Cooper [17].

The present study was undertaken to identify the endogenous 5,10-$CH_2$-$H_4$PteGlu in rat bile, and to develop a method for the simultaneous determination of bile folates including, 5,10-$CH_2$-$H_4$PteGlu, $H_4$PteGlu, 10-HCO-$H_4$PteGlu and 5-$CH_3$-$H_4$PteGlu using a HPLC–ED system.

## 2. Experimental

### 2.1. Materials and reagents

The magnesium salts of [6*R*,*S*]-5,10-$CH_2$-$H_4$PteGlu (purity, 96.5%), [6*R*,*S*]-$H_4$PteGlu (purity, >98%) and 10-formylfolate (10-HCO-PteGlu; purity, 98.2%) were obtained from Dr. B. Schircks Laboratories (Jona, Switzerland), and the disodium salt of [6*R*,*S*]-5-$CH_3$-$H_4$PteGlu (purity, 90%) from Sigma (St. Louis, MO, USA). The calcium salt of 5-formyltetrahydrofolate (5-HCO-$H_4$PteGlu; purity >90%) was a gift from Lederle Japan (Tokyo, Japan). 10-HCO-$H_4$PteGlu was prepared using the method described by Scott [18]. Sodium ascorbate, 2-mercaptoethanol, sodium acetate (trihydrate), acetic acid, the disodium salt of ethylenediaminetetraacetic acid (EDTA) and *p*-formaldehyde were obtained from Wako Pure Chemical Industries (Osaka, Japan). All folate derivatives were dissolved in 0.2% sodium ascorbate solution except for 5,10-$CH_2$-$H_4$PteGlu which was dissolved in 0.2% sodium ascorbate solution containing $3 \cdot 10^{-3}$% of *p*-formaldehyde. These solutions were stored at −80°C.

### 2.2. Animals and sampling

Female Sprague–Dawley rats (200–230 g of body weight, 9–11 week old; Clea Japan, Tokyo, Japan) were used. The animals were given a pelleted diet (CE-2, Clea Japan) and water ad libitum, and were acclimatized for ca. 2 weeks at our laboratory. The diet contained 2 mg of folate per kg of diet. After anesthesia with urethan (0.8–1 g/kg) intraperitoneal injection, the bile duct was isolated and cannulated with a polyethylene catheter (0.28 mm I.D.). This procedure was carried out at 32°C and 70% relative humidity. Bile samples were collected into tubes placed on ice 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 analysis.

### 2.3. Chromatography

Chromatographic analysis was performed using HPLC systems with three types of detectors. Analytical detectors included an electrochemical detector (ED, LC-4C, Bioanalytical Systems, IN, USA), a photodiode-array detector (PAD, Multi-340, Jasco, Tokyo, Japan), and an ultraviolet detector (UV; SPD-6A, Shimadzu, Kyoto, Japan). The HPLC system had the following setup: analytical column of phenyl-bonded phase (PN, Nova-Pak phenyl, 4 $\mu$m, $100 \times 8$ mm I.D., Waters Assoc., Milford, MA, USA) or $C_8$ (RP-8, 5 $\mu$m, $250 \times 4$ mm I.D., Merck, Darmstadt, Germany), a pump (LC-9A, Shimadzu), a 100-$\mu$l fixed-loop injector (Model 7125, Rheodyne, Cotati, CA, USA) and a data processor (C-R4A, Shimadzu). The mobile phase of the HPLC–ED system was a mixture of 20 m*M* acetate buffer (pH 5) containing 0.1 m*M* EDTA and acetonitrile (95:5, v/v), and that of the HPLC–PAD system was 50 m*M* phosphate buffer (pH 5.0)–acetonitrile (95:5, v/v). The flow-rate was 0.8 ml/min.

### 2.4. Identification of endogenous 5,10-$CH_2$-$H_4$PteGlu in rat bile

Identification was carried by comparison of the following four profiles between bile folate and the synthetic standard of 5,10-$CH_2$-$H_4$PteGlu; retention time profile, hydrodynamic voltammogram, demethylenation profile and UV absorption spectrum. The former three profiles were obtained using the HPLC–ED system, and the last profile was obtained using the HPLC–PAD. Retention time profiles were examined using two different analytical columns, PN and $C_8$. The retention times were determined for various pH values (4.0, 5.0 and 5.5 for PN and 4.0, 4.5 and 5.0 for $C_8$) of the acetate buffer in the mobile phase, and for various fractions of acetonitrile (3, 5, and 7% for PN and 4, 5 and 6% for $C_8$) in the mobile phase. The hydrodynamic voltammogram was obtained from the responses of the ED at various potentials ranging between 50 and 450 mV. The demethylenation profile was obtained after heat treatment (80°C, 2 min). The UV spectrum was traced between the wavelengths of 200 and 400 nm of the PAD.

### 2.5. Other folates in rat bile

The possible occurrence of 5-HCO-$H_4$PteGlu and 10-HCO-PteGlu in rat bile was examined. 5-HCO-$H_4$PteGlu was detected using HPLC–ED at a potential of the ED 800 mV, and 10-HCO-PteGlu was detected using HPLC–UV at a wavelength of 360 nm. The mobile phase was a mixture of 20 m*M* acetate buffer (pH 5) containing 0.1 m*M* EDTA and acetonitrile (95:5, v/v). The detection limit was 0.2 ng/injection for 5-HCO-$H_4$PteGlu and 0.8 ng/injection for 10-HCO-PteGlu at a signal-to-noise ratio of 3.

### 2.6. Simultaneous determinations of 5.10-$CH_2$-$H_4$PteGlu, $H_4$PteGlu, 10-HCO-$H_4$PteGlu and 5-$CH_3$-$H_4$PteGlu in rat bile

The bile folates were simultaneously determined using the HPLC–ED method. Bile samples were diluted with 0.2% sodium ascorbate solution containing $1.8 \cdot 10^{-5}$% of *p*-formaldehyde (1:10, v/v), and were centrifuged at 5000 *g* for 2 min. After filtration (0.45-$\mu$m microfilter), the filtrates (100 $\mu$l) were injected onto the HPLC PN column, and an ED potential of 350 mV was selected to optimize sensitivity and selectivity. The linearity of the peak area was examined over the range 1–100 ng/ml, and the linear-regression equation and the correlation coefficient were calculated by the least-squares method. The recoveries were determined by addition of standards (1 $\mu$g) to 1-ml bile samples.

The stability of endogenous 5,10-$CH_2$-$H_4$PteGlu, $H_4$PteGlu, 10-HCO-$H_4$PteGlu and 5-$CH_3$-$H_4$PteGlu in the bile samples was examined over a 4-h period at 4°C under the following conditions: without sodium ascorbate and *p*-formaldehyde, with 0.2% sodium ascorbate, and with 0.2% sodium ascorbate and $1.8 \cdot 10^{-5}$% *p*-formaldehyde. In addition, the stability of these bile folates was also examined at 32°C without the presence of sodium ascorbate and *p*-formaldehyde.

### 2.7. Secretion rates of the bile folates

The secretion rates of the bile folates were determined in 9 rats under urethan anesthesia. Bile samples were collected for 90 min with 30 min intervals.

## 3. Results

### 3.1. Identification of endogenous 5,10-$CH_2$-$H_4$PteGlu in rat bile

An ED chromatogram of rat bile shows that peak A had the same retention time as that of synthetic 5,10-$CH_2$-$H_4$PteGlu (Fig. 1). The retention times of the bile folate eluting as peak A were similar to those of the synthetic standard at different pHs of the acetate buffer and at different acetonitrile concentrations in the mobile phase (Table 1). The hydrodynamic voltammogram and UV absorption spectrum of peak A were also similar to those of the synthetic stan-

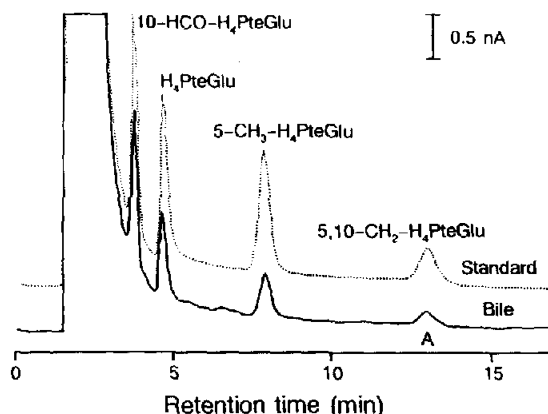


Fig. 1. HPLC–ED chromatogram of rat bile sample (——) and the synthetic folate standards (----).

dard (Fig. 2). Peak A and the synthetic 5,10-$CH_2$-$H_4$PteGlu decreased in a similar way by heat treatment at 80°C for 2 min, while the peaks of $H_4$PteGlu both in the bile sample and the synthetic standard increased similarly (Fig. 3).

Table 1
Retention times of peak A and 5,10-$CH_2$-$H_4$PteGlu under various pHs of the acetate buffer and various concentrations of acetonitrile (AN) in the mobile phase

| pH | AN (%) | Retention time (min) | |
|---|---|---|---|
| | | Peak A | 5,10-$CH_2$-$H_4$PteGlu |
| *Phenyl column* | | | |
| 4.0 | 5 | 37.3 ± 1.1 | 38.1 ± 2.0 |
| 5.0 | 5 | 13.1 ± 0.1 | 13.2 ± 0.2 |
| 5.5 | 5 | 8.6 ± 0.0 | 8.6 ± 0.0 |
| 5.0 | 3 | 35.8 ± 0.7 | 36.2 ± 1.5 |
| 5.0 | 5 | 13.1 ± 0.1 | 13.2 ± 0.2 |
| 5.0 | 7 | 9.8 ± 0.0 | 9.8 ± 0.1 |
| *$C_8$ column* | | | |
| 4.0 | 6 | 45.0 ± 0.0 | 45.6 ± 0.6 |
| 4.5 | 6 | 27.5 ± 0.6 | 28.2 ± 0.6 |
| 5.0 | 6 | 18.0 ± 0.0 | 18.2 ± 0.2 |
| 5.0 | 4 | 43.0 ± 0.7 | 43.1 ± 0.7 |
| 5.0 | 5 | 25.6 ± 0.3 | 26.3 ± 0.7 |
| 5.0 | 6 | 18.0 ± 0.0 | 18.2 ± 0.2 |

Values are mean ± S.D. ($n$ = 3).

### 3.2. Other folates in rat bile

We examined whether 5-HCO-$H_4$PteGlu and 10-HCO-PteGlu were secreted in rat bile. However, 5-HCO-$H_4$PteGlu and 10-HCO-PteGlu were not detected (Fig. 4).

### 3.3. Simultaneous determination of 5,10-$CH_2$-$H_4$PteGlu, $H_4$PteGlu, 10-HCO-$H_4$PteGlu and 5-$CH_3$-$H_4$PteGlu in rat bile

Analytical parameters including retention time, linear-regression, detection limit and recovery are presented in Table 2. All bile folates were eluted within 15 min after injection using ED chromatography, and no interferences from other components in the bile were observed. Peak areas correlated well with the concentrations of the folates over the range 1–100 ng/ml. The detection limits were 0.01–0.02 ng/injection, and the recoveries were higher than 90%. Results of the stability test of endogenous 5,10-$CH_2$-$H_4$PteGlu, $H_4$PteGlu, 10-HCO-$H_4$PteGlu and 5-$CH_3$-$H_4$PteGlu are shown in Fig. 5. Without the presence of sodium ascorbate and $p$-formaldehyde the bile folates were rapidly degraded at 4°C, and most of the $H_4$PteGlu and 10-HCO-$H_4$PteGlu had disappeared after 1 h. With 0.2% sodium ascorbate, approximately 30% of the 5,10-$CH_2$-$H_4$PteGlu was converted to $H_4$PteGlu after 4-h, while 10-HCO-$H_4$PteGlu and 5-$CH_3$-$H_4$PteGlu were relatively stable during this 4-h period. However, in the presence of 0.2% sodium ascorbate and $1.8 \cdot 10^{-5}$% $p$-formaldehyde, all bile folates were rather stable for at least a 4-h period. At 32°C, the bile folates were rapidly degraded without the presence of sodium ascorbate and $p$-formaldehyde.

### 3.4. Secretion rates of bile reduced folates in rats

The secretion rates of 5,10-$CH_2$-$H_4$PteGlu, $H_4$PteGlu, 10-HCO-$H_4$PteGlu and 5-$CH_3$-$H_4$PteGlu were 427 ± 131, 267 ± 112, 383 ± 233 and 436 ± 254 ng/h (mean ± S.D., $n$ = 9), respectively, and the distribution percentages in

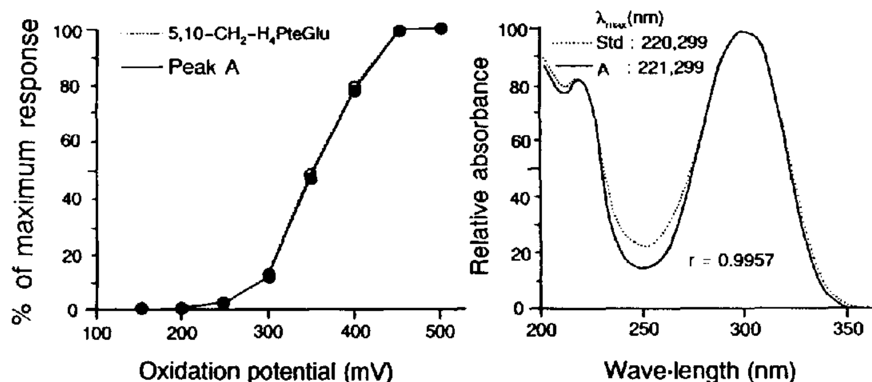


Fig. 2. Hydrodynamic voltammogram (left) and ultraviolet absorption spectrum (right) for peak A (——) and the synthetic standard 5,10-$CH_2$-$H_4$PteGlu (----). The $r$ value represents the correlation coefficient.

total folates were 29.6 ± 7.2, 17.7 ± 3.5, 24.4 ± 6.5 and 28.2 ± 7.1%, respectively.

## 4. Discussion

We studied the secretion of 5,10-$CH_2$-$H_4$PteGlu in rat bile. The identification of 5,10-$CH_2$-$H_4$PteGlu was based on the similarity of the following profiles between the bile folate and the synthetic standard of 5,10-$CH_2$-$H_4$PteGlu: (a) retention times at the different pHs of acetate buffer or the different concentrations of acetonitrile in the mobile phase (Table 1), (b) hydrodynamic voltammogram (Fig. 2), and (c) UV spectrum (Fig. 2). The similarities of the re-

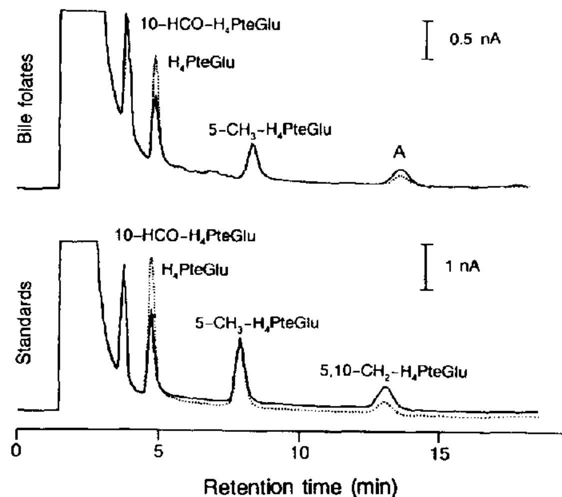


Fig. 3. HPLC–ED chromatograms of rat bile folate and the synthetic standard solution before (——) and after heat treatment at 80°C for 2 min (----).

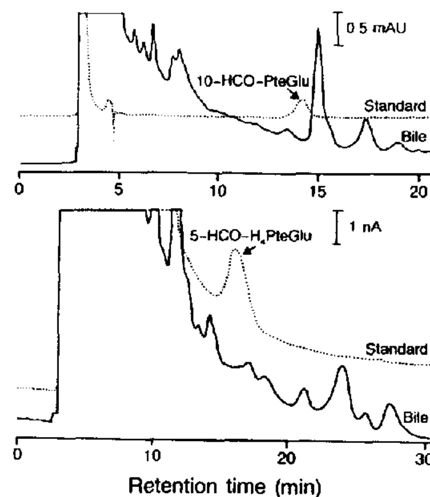


Fig. 4. HPLC–UV chromatogram (upper) and HPLC–ED chromatogram (lower) of rat bile sample and the synthetic standard 10-HCO-PteGlu or 5-HCO-$H_4$PteGlu. (——) bile samples; (----) standards. The concentrations of the standards were 100 ng/ml.

Table 2
Analytical parameters for the HPLC–ED analysis

| Folates | Retention time (min) | Linear-regression equation ($r$) | Detection limit[a] (ng/injection) | Recovery[b] (%) |
|---|---|---|---|---|
| 10-HCO-$H_4$PteGlu | 3.76 | $y = 4687x - 1672$ (0.999) | 0.01 | 99.5 ± 4.1 |
| $H_4$PteGlu | 4.74 | $y = 5336x - 1932$ (0.999) | 0.01 | 95.2 ± 7.7 |
| 5-$CH_3$-$H_4$PteGlu | 7.96 | $y = 4761x + 620$ (0.999) | 0.01 | 101.0 ± 6.0 |
| 5,10-$CH_2$-$H_4$PteGlu | 13.17 | $y = 1781x + 240$ (0.999) | 0.02 | 91.2 ± 5.8 |

Linear regression curves were determined by the least-squares regression analyses of the peak area over the range 1–100 ng/ml. The $r$-value is the correlation coefficient.
[a] The value was obtained at a signal-to-noise ratio of 3.
[b] The values are mean ± S.D. ($n = 3$).

tention times, voltammogram and UV spectrum indicate that bile folate has the same value for the negative logarithm of the acid dissociation constant ($pK_a$) and lipid solubility, the same electrochemical properties or redox potential, and the same UV absorption characteristics as the synthetic 5,10-$CH_2$-$H_4$PteGlu. The demethylenation of the bile folate into $H_4$PteGlu by heat treatment (Fig. 3) supports the identification of bile folate as 5,10-$CH_2$-$H_4$PteGlu. The demethylenation reaction of 5,10-$CH_2$-$H_4$PteGlu to $H_4$PteGlu was well confirmed by Wilson and Horne [15], Houghton et al. [16], and Kashani and Cooper [17].

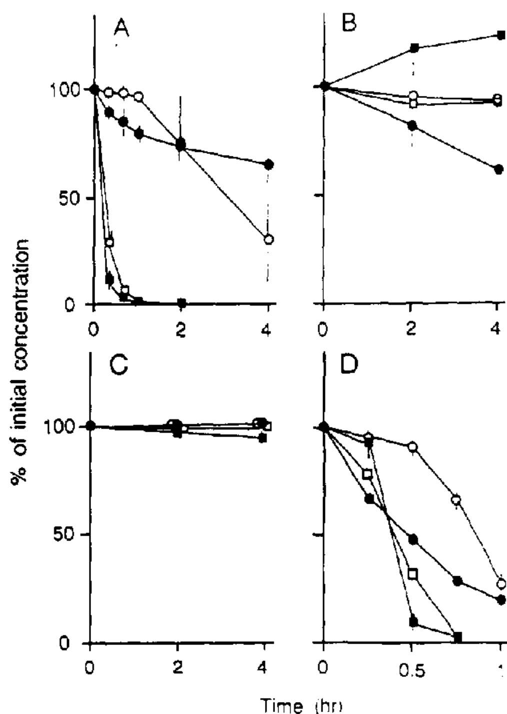


Fig. 5. Stabilities of bile folates. The stabilities were examined under four conditions: (A) without sodium ascorbate and $p$-formaldehyde at 4°C; (B) with 0.2% sodium ascorbate at 4°C; (C) with 0.2% sodium ascorbate and $1.8 \cdot 10^{-5}$% $p$-formaldehyde at 4°C; and (D) without sodium ascorbate and $p$-formaldehyde at 32°C, (●) 5,10-$CH_2$-$H_4$PteGlu, (■) $H_4$PteGlu, (□) 10-HCO-$H_4$PteGlu, and (○) 5-$CH_3$-$H_4$PteGlu.

In a previous report we identified $H_4$PteGlu, 10-HCO-$H_4$PteGlu and 5-$CH_3$-$H_4$PteGlu in rat bile [13]. However we could not detect 5,10-$CH_2$-$H_4$PteGlu in rat bile in that study. This was due to the following: (1) chromatograms of rat bile after [$^3$H]PteGlu injection showed only five radioactive major peaks indicating $p$-aminobenzoate, $H_4$PteGlu, 10-HCO-$H_4$PteGlu, 5-$CH_3$-$H_4$PteGlu and PteGlu, respectively, (2) the analytical conditions resulted in a detection limit much higher than the physiological levels of 5,10-$CH_2$-$H_4$PteGlu due to the long retention time (more than 40 min) during which this folate was possibly degraded.

In the present study, we further investigated the secretion of other folates besides the four identified in rat bile. Hillman et al. [19] and Pheasant et al. [20] reported the bile secretion of 5-HCO-$H_4$PteGlu or 10-HCO-PteGlu as a metabolite after dosing with [$^3$H]PteGlu in rats. This may suggest that 5-HCO-$H_4$PteGlu or 10-HCO-PteGlu endogenously exist in rat bile. However, endogenous 5-HCO-$H_4$PteGlu and 10-

HCO-PteGlu could not be detected under the analytical conditions used in the present study (Fig. 4). Pratt and Cooper [21] suggested that bile folates reflect the distribution of hepatic folate pools. Earlier studies [13,15,22] demonstrated that endogenous hepatic folates include 5,10-$CH_2$-$H_4$PteGlu, $H_4$PteGlu, 10-HCO-$H_4$PteGlu, 5-HCO-$H_4$PteGlu and 5-$CH_3$-$H_4$PteGlu. Eto and Krumdieck [23] described the occurrence of 5,10-methenyltetrahydrofolate (5,10-CH=$H_4$PteGlu) in hepatic cells of rats. 5,10-CH=$H_4$PteGlu readily converts to 10-HCO-$H_4$PteGlu in neutral and alkaline solution. Considering the pH (8.5) of rat bile, it may be that 5,10-CH=$H_4$PteGlu does not exist in rat bile.

For a long time, quantification of 5,10-$CH_2$-$H_4$PteGlu has been considered difficult due to instability [17,24–26]. Thus, in some cases, 5,10-$CH_2$-$H_4$PteGlu was not detected in tissue, and in other cases this folate was determined as $H_4$PteGlu after chemical conversion [15,24,25]. In previous reports [13,14], we identified and quantified $H_4$PteGlu, 10-HCO-$H_4$PteGlu and 5-$CH_3$-$H_4$PteGlu in rat bile using an HPLC–ED method. We further developed the HPLC–ED method that allows us to quantify not only $H_4$PteGlu, 10-HCO-$H_4$PteGlu and 5-$CH_3$-$H_4$PteGlu but also 5,10-$CH_2$-$H_4$PteGlu with reasonable sensitivity and recovery (Table 2). Moreover, all the bile folates are rapidly eluted within 15 min after injection. The problem of conversion of 5,10-$CH_2$-$H_4$PteGlu to $H_4$PteGlu was prevented by using a 0.2% sodium ascorbate solution containing $1.8 \cdot 10^{-5}$% *p*-formaldehyde (Fig. 5).

Most reduced folate derivatives are unstable under oxidative conditions. To estimate the stability of bile folate derivatives during canula passage of bile at sampling, we examined the oxidative degradation of these folates without the addition of an antioxidant at 32°C. The time of canula passage of bile is less than 30 s, during which degradation of bile folates was estimated to be less than 1%.

In summary, 5,10-$CH_2$-$H_4$PteGlu, $H_4$PteGlu, 10-HCO-$H_4$PteGlu and 5-$CH_3$-$H_4$PteGlu are major active folate derivatives identified in rat bile. The improved HPLC–ED method presented here is suitable to determine folates in rat bile.

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