



## An enzymatic method to distinguish tetrahydrobiopterin from oxidized biopterins using UDP-glucose:tetrahydrobiopterin glucosyltransferase

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### ABSTRACT

The quantitative determination of tetrahydrobiopterin (BH4) and its oxidized forms (dihydrobiopterin and biopterin) is important in searching for possible markers of neuropsychiatric and cardiovascular disorders as well as in diagnosing BH4 deficiencies. Currently, two high-performance liquid chromatography (HPLC) methods are available, although both have some limitations. We developed an enzymatic method to distinguish BH4 from the oxidized forms by employing BH4:UDP-glucose  $\alpha$ -glucosyltransferase (BGLuT), which catalyzes glucosyl transfer from UDP-glucose to BH4. The recombinant BGLuT isolated from *Escherichia coli* converted essentially all of the BH4 in a mixture containing oxidized biopterins to the glucoside while leaving the oxidized forms intact. Therefore, acidic iodine oxidation of the reaction mixture followed by single fluorescence HPLC permitted the determination of biopterin and biopterin-glucoside, which represent oxidized biopterins and BH4, respectively. The validity of the method was evaluated using authentic biopterins and animal samples such as human urine, rat plasma, and rat liver. The BGLuT-catalyzed reaction not only would reduce the burden of chromatographic separation but also would promise non-HPLC analysis of BH4.

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As a cofactor for aromatic amino acid hydroxylases, tetrahydrobiopterin (BH4)<sup>2</sup> is essential for hepatic phenylalanine metabolism and neurotransmitter synthesis in humans [1]. It is well known that an inherent error in BH4 synthesis can cause hyperphenylalaninemia, neurotransmitter deficiencies, and DOPA-responsive dystonia [1,2]. BH4 has also been studied for a possible association with several neuropsychiatric disorders such as Parkinson's disease, Alzheimer's disease, depression, autism, and schizophrenia [1,3–6]. In addition, BH4 plays a crucial role in endothelial dysfunction as a cofactor and regulator of nitric oxide synthase (NOS) [7]. Although BH4 is functional in the fully reduced form, it can be partially oxidized to dihydrobiopterin (BH2) and fully oxidized to biopterin in vivo, especially under oxidative stress conditions. Insufficient availability of BH4 in endothelial cells results in NOS-uncoupled production of superoxide anions instead of nitric oxide [8]. The situation is further ameliorated by the competitive binding of BH2 to NOS [9].

The resulting endothelial dysfunction is a well-known prognostic marker of cardiovascular diseases. Therefore, both BH4 and its oxidized forms have been measured extensively in biological samples for clinical diagnostics and biomedical research.

Two high-performance liquid chromatography (HPLC) methods are currently available to quantify BH4 and its oxidized forms. A chemical method originally developed by Fukushima and Nixon [10] requires differential oxidation of specimens before HPLC. Total biopterin (BH4 + BH2 + biopterin) is simply determined by acidic iodine oxidation. Alkaline iodine oxidation converts BH2 to biopterin and converts BH4 to pterin. The subtracted amount of biopterin represents the amount of BH4. Another method depends on HPLC in anaerobic conditions to separate BH4 from its oxidized forms, which are measured directly by electrochemical detection or by fluorescence after postcolumn oxidation [11].

We previously isolated a novel enzyme named UDP-glucose:BH4 glucosyltransferase (BGLuT) from the cyanobacterium *Synechococcus* sp. PCC 7942 [12–14]. The enzyme catalyzes the glucosyl transfer from UDP-glucose to BH4, generating BH4- $\alpha$ -glucoside. While studying BGLuT, we hypothesized that the enzyme may be useful in a BH4 assay. If BGLuT catalysis is specific and irreversible, essentially all of the BH4 in a mixture containing oxidized biopterins would be converted to BH4-glucoside while the oxidized biopterins would remain intact. An acidic iodine oxidation

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<sup>2</sup> Abbreviations used: BH4, tetrahydrobiopterin; NOS, nitric oxide synthase; BH2, dihydrobiopterin; HPLC, high-performance liquid chromatography; BGLuT, UDP-glucose:BH4 glucosyltransferase; IPTG, isopropyl  $\beta$ -D-1-thiogalactopyranoside; Ni-NTA, nickel-nitrilotriacetic acid; DTT, dithiothreitol; %RSD, percentage relative standard deviation; ELISA, enzyme-linked immunosorbent assay.

of the mixture will generate biopterin–glucoside from BH4 and biopterin from the oxidized forms, which can then be separated by HPLC and quantified by fluorescence. The method, herein named the BGluT method, was validated using authentic biopterins and animal samples.

## Materials and methods

### Materials

Pteridines were purchased from Schircks Laboratories (Switzerland). Biopterin–glucoside was isolated from *Synechococcus* sp. PCC 7942 [14]. BH4 was quantified using the molar extinction coefficient ( $\epsilon$ ) of  $16,000\text{ M}^{-1}\text{ cm}^{-1}$  at 267 nm in 0.1 N HCl [10].

The BGluT recombinant protein was isolated from *Escherichia coli* strain BL21(DE3), which was described in a previous study [13]. Overexpression and purification procedures were modified slightly. Briefly, the transformed *E. coli* was induced to overexpress the protein with 0.3 mM isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG). After being incubated at 37 °C for 4 h, the harvested cells were washed with lysis buffer (50 mM sodium phosphate [pH 8.0], 300 mM NaCl, and 10 mM imidazole), resuspended in the same buffer, and disrupted by sonication. The crude extract obtained after centrifugation was applied to a column of nickel–nitrilotriacetic acid (Ni–NTA) gel (Qiagen, USA) and purified according to the product manual. The recombinant BGluT protein was eluted with 250 mM imidazole in lysis buffer. The purified protein was dialyzed against 20 mM Tris–HCl (pH 7.5), mixed with glycerol to a concentration of 30% (v/v), and stored in aliquots at  $-70\text{ }^{\circ}\text{C}$  until use. The protein was stable for up to 4 months.

Human urine was collected in 1 mM dithiothreitol (DTT) solution, and aliquots were stored at  $-70\text{ }^{\circ}\text{C}$  until use. An 8-week-old male Sprague–Dawley rat was sacrificed to collect the plasma and liver. The liver was homogenized with a glass homogenizer in 50 mM Tris–HCl (pH 7.5) and 1 mM DTT and was centrifuged at 13,000 rpm for 10 min to save the supernatant. The supernatant was used immediately for the analysis of biopterins. Protein concentration was determined by the Bradford method using bovine serum albumin as a standard.

### Standard reaction conditions of BGluT

Unless otherwise specified, the following standard reaction conditions were used for the determination of BH4 and its oxidized forms in this study. Reactions were carried out in a final volume of 100  $\mu\text{L}$ , which consisted of 50 mM Tris–HCl (pH 7.5), 10 mM  $\text{MgCl}_2$ , 0.05% ascorbic acid, 500  $\mu\text{M}$  UDP–glucose, 2  $\mu\text{g}$  of BGluT, and an aliquot of the pteridine/animal samples (10  $\mu\text{L}$  for urine, 20  $\mu\text{L}$  for rat plasma, and 10  $\mu\text{L}$  for rat liver extract). The reaction mixture was incubated for 10 min at 37 °C and then mixed with 30  $\mu\text{L}$  of acidic iodine solution (2% KI/1%  $\text{I}_2$  in 1 N HCl). After 1 h in the dark at room temperature, the oxidized mixture was centrifuged to remove protein precipitate. The supernatant (125  $\mu\text{L}$ ) was mixed with 10  $\mu\text{L}$  of 5% ascorbic acid and 25  $\mu\text{L}$  of water. Nonenzymatic reactions (–BGluT) were conducted using the same procedures but without enzyme incubation. Finally, 5–50  $\mu\text{L}$  of the reaction mixture was injected for HPLC.

### Chromatographic conditions

The HPLC system consisted of a Gilson 321 pump, a Gilson 234 autoinjector, and a fluorescence detector (Shimadzu RF-10AXL). Chromatography was performed on a guard column (10  $\mu\text{m}$ , 4.3 mm  $\times$  1 cm) and an Inertsil ODS-3 (5  $\mu\text{m}$ , 150  $\times$  2.3 mm) (GL Science, Japan) at room temperature. Pteridines were eluted iso-

cratically with 10 mM potassium phosphate buffer (pH 6.0) at a flow rate of 1.2 ml/min and were monitored at 350/450 nm (excitation/emission). The fluorescence detector was set at sens 1 and gain 3, which are the highest levels for that machine. The chromatograms were recorded using a Gilson 506C interface and integrated with the system software (Gilson Unipoint 5.11).

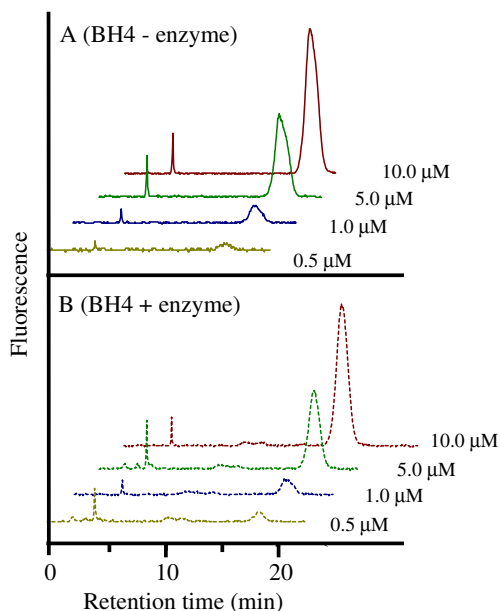
## Results

### Complete conversion of BH4 to BH4–glucoside by BGluT

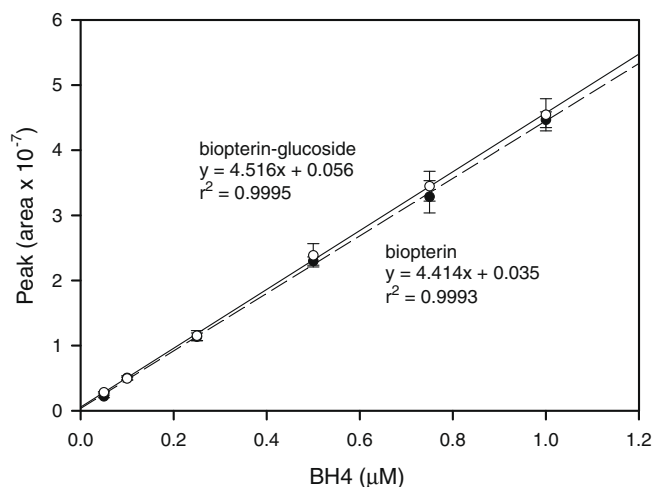
To demonstrate the ability of BGluT to convert essentially all of the BH4 in the reaction mixture to the glucoside, we incubated the enzyme with increasing amounts of authentic BH4 (Fig. 1). All of the reaction mixtures exhibited biopterin–glucoside peaks eluting at approximately 18 min, leaving apparently no biopterin, as is shown in the chromatograms of the nonenzymatic reaction mixtures. Both biopterin–glucoside and biopterin peaks similarly increased in height with increasing BH4 concentrations, indicating a stoichiometric yield of BH4–glucoside from BH4. The calibration curves for the two peaks were constructed by plotting the peak area versus BH4 concentration, which was obtained in an interday assay of six replicates. The curves were linear over the concentration range of 0.05–1.0  $\mu\text{M}$  BH4 (Fig. 2). The amounts actually injected into the column ranged from 0.325 to 6.25 pmol (Table 1). The precision values of the interday assay for biopterin and biopterin–glucoside, expressed as percentage relative standard deviation (%RSD), were 2.7–9.7% and 3.9–7.5%, respectively, similar to those of an intraday assay (Table 1). A slight difference between the slopes of the regression lines of biopterin (4.414) and biopterin–glucoside (4.516) generated a ratio of 1:1.02 (Fig. 2, inset), establishing that the peak areas of biopterin and biopterin–glucoside obtained from an equal amount of BH4 were nearly identical.

### Selective conversion of BH4 to BH4–glucoside by BGluT

Animal tissues contain not only BH4 but also several kinds of pteridines, including oxidized forms of BH4, which might interfere



**Fig. 1.** Representative HPLC chromatograms of biopterin (A) and biopterin–glucoside (B) produced from authentic BH4. The indicated amounts of BH4 were incubated in a mixture of 100  $\mu\text{L}$  either with or without 0.15  $\mu\text{g}$  of BGluT for 1 h at 37 °C and were subjected to HPLC after acidic iodine oxidation. Nonenzymatic reaction mixtures were subjected to iodine oxidation without the incubation step.

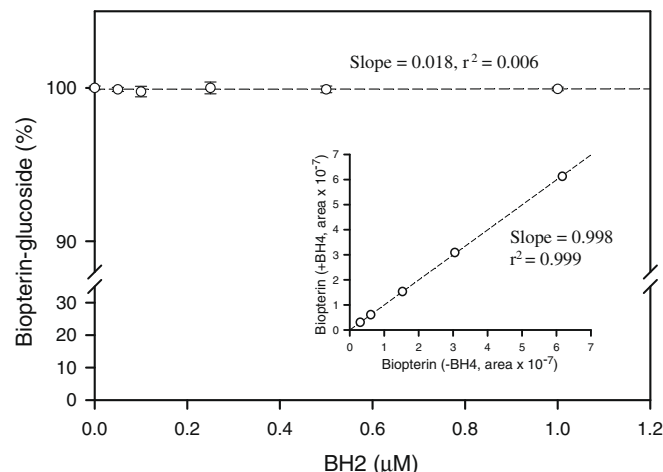


**Fig. 2.** Calibration curve of peak areas of biopterin (closed circles) or biopterin-glucoside (open circles) versus the BH4 concentration. Data are the means  $\pm$  standard deviations of intraday assays ( $n = 6$ ) in 0.05–1.0  $\mu\text{M}$  BH4 solutions.  $r^2$  values and linear regression equations are provided.

with the BGluT reaction as a nonspecific substrate or inhibitor. Among several pteridines tested (BH2, biopterin, dihydroneopterin, tetrahydroneopterin, L-threo-BH4, and 6S-BH4), BH2 showed a small amount of glucosylated product ( $\sim 1.8\%$  of the total) when the concentration was increased to 5  $\mu\text{M}$  (data not shown), which is much higher than the amount actually encountered in animal tissues. To examine whether BH2 interferes with the BGluT reaction, BH2 was coincubated with BH4. The yield of biopterin-glucoside was nearly constant, even in the presence of a fourfold higher amount of BH2 (Fig. 3), proving that there is no obvious interference by BH2. Supporting this, the biopterin peak areas measured in the coincubation assay were identical to those obtained from the nonenzymatic reaction of BH2 (Fig. 3, inset). Biopterin also did not inhibit the BGluT reaction (data not shown). The results strongly support the idea that the simultaneous determination of BH4 and its oxidized forms in animal samples may be possible via an assay using BGluT.

#### Optimal conditions for the BGluT-catalyzed reaction

Because BGluT was already studied for optimal reaction conditions [12], we determined, in the current study, the minimal



**Fig. 3.** Effect of BH2 on the production of BH4-glucoside. A fixed amount of BH4 (0.25  $\mu\text{M}$ ) was coincubated with increasing concentrations (0–1  $\mu\text{M}$ ) of BH2 using the standard reaction conditions. The peak areas of biopterin-glucoside were plotted against the BH2 concentration. The peak areas of biopterin were also compared with those determined from the replicate without both BH4 and the enzyme (see inset). Data are the means  $\pm$  standard deviations of triplicate assays.

amount of protein required to convert physiological concentrations of BH4 within a short time, which is critical for overcoming possible problems of BH4 oxidation during the enzyme reaction as well as for saving time. BH4 analysis is usually performed in a 100- $\mu\text{l}$  reaction volume that contains the proper amount of tissue homogenates. Animal samples, except urine, were known to contain biopterins at concentrations of less than 1  $\mu\text{M}$ . Therefore, the BGluT reaction was conducted in 100  $\mu\text{l}$  of a reaction mixture containing 1  $\mu\text{M}$  BH4. When incubated for 10 min with increasing amounts of BGluT, BH4 was completely converted if more than 1  $\mu\text{g}$  of enzyme was used (Fig. 4). The amount of product was nearly constant even when the incubation time was extended up to 1 h (data not shown). Therefore, we routinely used 2  $\mu\text{g}$  of BGluT in a reaction volume of 100  $\mu\text{l}$  to ensure a complete reaction in 10 min at 37  $^{\circ}\text{C}$ .

#### Validation of BGluT method using animal samples

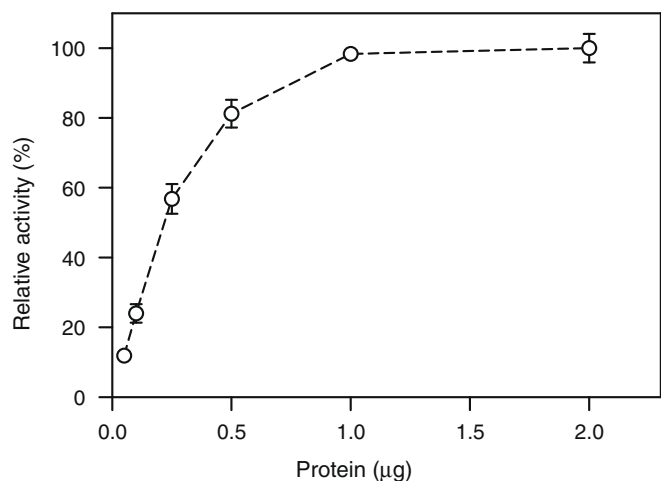
The validity of the BGluT method was evaluated in human urine, rat plasma, and rat liver extract, all of which are easily available and known to contain BH4. Each sample was assayed with

**Table 1**  
Precision of the BGluT method.

Specimen <sup>a</sup>		Precision (%RSD) <sup>b</sup>					
		Instrumental		Intraday		Interday	
		–BGluT	+BGluT	–BGluT	+BGluT	–BGluT	+BGluT
BH4	1.00 $\mu\text{M}$			2.3	3.4	2.7	5.4
	0.50 $\mu\text{M}$	0.9	1.1	3.4	4.1	2.7	7.5
	0.10 $\mu\text{M}$	3.0	1.3	4.8	3.7	6.5	4.3
	0.05 $\mu\text{M}$	3.9	4.6	9.6	3.9	9.7	3.9
	0.01 $\mu\text{M}$	10.2	6.7	8.7	12.9		
Human urine, >2.0 $\mu\text{M}$				4.1	0.4		
Rat plasma, >0.02 $\mu\text{M}$				3.5	1.0		
Rat liver, >0.2 $\mu\text{M}$				10.9	4.6		

<sup>a</sup> The concentrations of biopterins in the reaction mixtures are shown. Because the mixture volume increases due to the acidic iodine oxidation, the concentrations should be multiplied by the dilution factor of 0.65 to calculate the actual ones in the final samples for HPLC analysis. The injection volumes onto column were 10, 10, 50, and 20  $\mu\text{l}$  for BH4, human urine, rat plasma, and rat liver, respectively.

<sup>b</sup> Precision was calculated separately for biopterin and biopterin-glucoside, which were determined by the nonenzymatic and BGluT methods, respectively. The instrumental precision was obtained by measuring a reaction mixture five times in 1 day. The intraday precision was determined from triplicate assays, and the interday precision was determined from six separate assays.



**Fig. 4.** Enzyme-dependent production of BH4–glucoside. Increasing amounts of BGluT were incubated with 1 μM BH4 at 37 °C for 10 min using the standard reaction conditions. Data are the means ± standard deviations of triplicates.

or without BGluT in the presence or absence of authentic BH4. Representative chromatograms are shown in Fig. 5. Total biopterin and the ratio of BH4/total biopterin were calculated and are summarized in Table 2. In the nonenzymatic reaction mixtures (–BGluT), biopterin peaks were clearly seen just after the pterin peak without any interfering molecules close to the position of biopterin–glucoside. When incubated with BGluT (+BGluT), biopterin–glucoside appeared in the chromatograms, whereas a corresponding amount of biopterin disappeared. Accordingly, the sum of biopterin–glucoside (BH4) and residual biopterin (BH2 + biopterin) was similar to the total biopterin determined from the nonenzymatic reaction mixture (Table 2). There was less than a 5% difference between

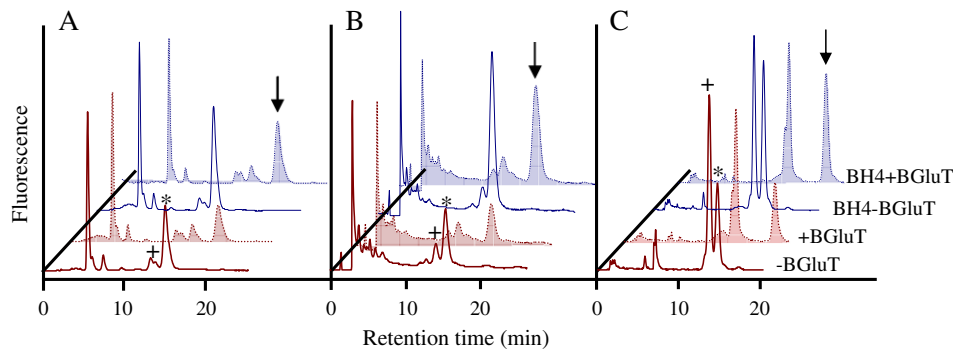
the mean values determined by the two methods. The precision values of the intraday nonenzymatic and BGluT methods were 3.5–10.9% and 0.4–4.6%, respectively (Tables 1 and 2), which was not much different from those determined in authentic BH4 solutions (2.3–9.6% and 3.4–4.1%, respectively) (Table 1). Instrumental precision was 0.9–10.2% RSD and became worse as the injected amounts decreased. The ratios of BH4/total biopterin determined via the BGluT method were remarkably high, ensuring complete glucosylation of the indigenous BH4 in the samples (Table 2). The ratios were also highly precise, showing a precision of 0.2–1.2% RSD. Therefore, the precision of the BGluT method in the animal samples was satisfactory.

Replicates of the reaction mixtures were spiked with known concentrations of authentic BH4 to evaluate the accuracy of the method (Table 2). As shown in the representative chromatograms (Fig. 5), recovery of spiked BH4 was clearly identifiable by the increased peak heights of biopterin in the nonenzymatic mixtures (BH4 – BGluT) and of biopterin–glucoside in the BGluT reaction mixtures (BH4 + BGluT). More than 89% of the spiked BH4 was recovered as biopterin–glucoside, similar to the levels for biopterin, except in the rat liver.

Discussion

For the simultaneous determination of reduced and oxidized biopterins in animal samples, we have developed a new method that consists of two steps: (i) BGluT-catalyzed glucosylation reaction of BH4 and (ii) acidic iodine oxidation followed by single fluorescence HPLC.

Above all, we made sure that BGluT converts all BH4 in the reaction mixture to the glucoside while leaving oxidized biopterins intact (Figs. 1 and 2). Although BGluT was also slightly active toward BH2 at high concentrations, there was no inhibition by BH2 and no false-positive production from BH2, at least in the physiological



**Fig. 5.** Representative chromatograms of the nonenzymatic reaction (–BGluT) and the BGluT reaction (+BGluT) in animal samples. Using the standard reaction conditions, 10 μl of human urine (A), 20 μl of rat plasma (B), and 10 μl of rat liver extract (C) were assayed either with or without BGluT. Replicates of the reaction mixtures were spiked with authentic BH4 and assayed via the nonenzymatic method (BH4 – BGluT) and the BGluT method (BH4 + BGluT). Spiked BH4 amounted to 1, 0.05, and 0.1 μM for human urine, rat plasma, and rat liver, respectively. The volumes injected into the column are described in Table 1. Pterin (+), biopterin (\*), and biopterin–glucoside (↓) are indicated.

**Table 2**  
Validation of the BGluT method in animal samples.

	Total biopterin <sup>a</sup>		% Ratio of BH4/total biopterin <sup>b</sup>	% Recovery of spiked BH4 <sup>c</sup>	
	+BGluT	–BGluT		+BGluT	–BGluT
Human urine	22.7 ± 0.1	22.1 ± 0.9	70.1 ± 1.2	97.5 ± 7.3	94.7 ± 6.8
Rat plasma	117.9 ± 1.2	112.2 ± 3.9	97.0 ± 0.8	89.6 ± 6.8	89.3 ± 3.0
Rat liver	63.2 ± 2.9	62.3 ± 6.8	91.8 ± 0.2	90.1 ± 6.0	98.7 ± 16.6

<sup>a</sup> The results obtained by the BGluT method (+BGluT) were compared with those obtained by the nonenzymatic method (–BGluT). Data are the means ± standard deviations of triplicate assays. The units were μM for human urine, nM for rat plasma, and pmol/mg protein for rat liver.  
<sup>b</sup> % Ratio of BH4/total biopterin was calculated from the data obtained by the BGluT method.  
<sup>c</sup> % Recovery of spiked BH4 was determined from biopterin (–BGluT) or biopterin–glucoside (+BGluT).



concentration range, as shown in the coinubation assay (Fig. 3). Although not disappointing, when BH4 was spiked in animal samples, it was not fully recovered as biopterin or biopterin-glucoside (Table 2). The recoveries as biopterin or biopterin-glucoside also varied for the different samples. It is presumed that the spiked BH4 might be rapidly oxidized when mixed with the samples due to contact with biological constituents or simply to its instability in solution [15]. Alternatively, there may be some sample-specific interference such as enzymatic degradation of biopterins during the incubation. Terminating the reaction after 1 min might be helpful in preventing any possible interference. We actually found that the BGluT reaction in 1  $\mu$ M BH4 solution was complete after 1 min with 5  $\mu$ g of protein (data not shown). In spite of the possible interference, the amount of total biopterins in the samples determined by the BGluT method agreed well with the amount determined by the nonenzymatic method (Table 2). Furthermore, the ratios of BH4/total biopterin (Table 1) were better than those determined previously using the differential oxidation method. According to the literature, the ratios of BH4/total biopterin in human urine, rat plasma, and rat liver were approximately 67% [10], approximately 80% [16,17], and 84.6% [18], respectively. Therefore, our results strongly support the hypothesis that the BGluT method is reliable and accurate, thereby advocating its application with any biological sample, including human plasma.

Although acidic iodine oxidation and subsequent fluorescence HPLC have been used extensively for pteridine analysis, we carried out some minimal works for the quantification of biopterin-glucoside. We established that biopterin and biopterin-glucoside produced from equimolar solutions of BH4 exhibit nearly identical peak areas of 1:1.02 (Fig. 2). However, the ratio changed to 1:1.12 when the reaction mixture was neutralized by adding an NaOH solution (data not shown), probably due to a pH-dependent change in fluorescence intensities. In this study, the final neutralization step in acidic iodine oxidation was excluded because nonneutralized samples did not exhibit a remarkable chromatographic change but rather had higher peak areas than the neutralized samples. In our HPLC conditions, no interfering peak was observed from the animal samples at the eluting position of biopterin-glucoside (Fig. 5). A minor problem was a partial overlap of the pterin and biopterin peaks (Fig. 5) such that if the column resolution worsened, it hampered the correct measurement of the peak areas. Finally, in our HPLC system, neither biopterin nor biopterin-glucoside could be determined in the reaction mixture of 5 nM BH4 solution, which corresponded to 32.5 fmol that was injected into the HPLC. At lower BH4 concentrations (10–50 nM), the precision of the intraday assay decreased approximately two-fold (Table 1).

In addition to its precision and accuracy, the BGluT method seems to guarantee that it will have a great advantage over the currently available methods. Owing to the simplified HPLC procedure, the BGluT method would save time and labor, thereby reducing the risk of experimental errors. Furthermore, the BGluT-catalyzed reaction may permit radioisotope assays and enzyme-linked immunosorbent assays (ELISAs). Using radiolabeled UDP-[ $^{14}$ C]glucose as a substrate, BH4-[ $^{14}$ C]glucoside can be quantified with a scintillation counter. Polyclonal or monoclonal antibodies against

biopterin [19] or biopterin-glucoside may be useful in an ELISA. Antibodies against UDP, which are commercially available, may also be useful.

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