

Screening for tetrahydrobiopterin deficiencies using dried blood spots on filter paper

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Abstract

Tetrahydrobiopterin (BH₄) deficiency among newborns with hyperphenylalaninemia must be rapidly diagnosed and distinguished from classical phenylketonuria (PKU) to initiate immediately specific treatment and to prevent irreversible neurological damage. The characteristic pattern of urinary pterins makes it possible to differentiate between PKU and BH₄ deficiencies, and to identify different variants of BH₄ deficiency. However, collection, storage, and shipment of urine samples for pterin analysis is cumbersome. A method for the measurement of different pterins (neopterin, biopterin, and pterin) in blood collected on filter paper was developed as a potential alternative to the screening for BH₄ deficiencies in urine and for the monitoring of BH₄ pharmacokinetics. Pterins pattern in blood spots was comparable with those in plasma and urine. We thus established reference values for pterins in blood spots in patients with hyperphenylalaninemia and identified new patients with GTP cyclohydrolase I deficiency, 6-pyruvoyl-tetrahydropterin synthase deficiency, and dihydropteridine reductase deficiency using dried blood spots on filter paper.

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Introduction

Tetrahydrobiopterin (BH₄) is the essential co-factor/co-substrate of phenylalanine hydroxylases (PAH) and several other monooxygenases [1]. Measurement of pterins in different biological fluids is the most common method for the screening and differential diagnosis of inborn errors of BH₄ metabolism. Five distinct genetic defects are known to cause hyperphenylalaninemia (HPA), including the classical form of Phenylketonuria (PKU) with a defect in the apo-enzyme PAH or a defect in four out of five BH₄ co-factor-synthesizing or regenerating enzymes [2]. Either of two defects in biosynthesis of BH₄, i.e., GTP cyclohydro-

lase I (GTPCH) or 6-pyruvoyl-tetrahydropterin synthase (PTPS) or defects in regeneration, i.e., pterin-4a-carbinolamine dehydratase (PCD) and dihydropteridine reductase (DHPR) may be responsible for BH₄ deficiency. BH₄ deficiency is a severe but treatable disease and early detection in newborns is essential to avoid irreversible brain damage.

According to the current protocol, the following investigations should be performed in all newborns with HPA (blood phenylalanine >120 μmol/L): 1. analysis of pterins (neopterin, biopterin, and primapterin) in urine; 2. measurement of DHPR activity in dried blood spots from Guthrie card; and 3. analysis of phenylalanine and tyrosine in plasma or blood before and after BH₄ loading with 20 mg/kg body weight. Tests 1 and 2 are essential for all newborns and identify primarily variants of BH₄ deficiency in older children due to characteristic pterin patterns: GTPCH deficiency with low neopterin and biopterin, PTPS deficiency

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with high neopterin and only traces of biopterin, PCD deficiency with high neopterin, moderately biopterin and high primapterin, and DHPR deficiency with normal or moderate elevated neopterin and high biopterin. Test 2 identifies patients with DHPR deficiency, which can sometimes be missed by test 1 if the urine is collected under low-protein diet. Test 3 is useful in all forms of BH₄ deficiency and can also detect patients with BH₄-responsive PAH deficiency [3].

With the introduction of tandem mass-spectrometry (TMS) in newborn screening, blood sample collection on filter paper became a routine procedure. The aim of this study was to test the use of filter paper blood spots (Guthrie cards) instead of urine for screening of BH₄ deficiencies and to study the pharmacokinetics of BH₄ by monitoring blood concentrations of pterins following oral loading test in patients with HPA. The new method should enable simultaneous measurement of pterins (neopterin, biopterin, isoxanthopterin, and pterin), DHPR activity, and amino acids from a single Guthrie card specimen. The main advantage of using Guthrie cards instead of urine is the easy handling and sample collection, and the less expensive shipping of the samples at room temperature.

Materials and methods

Pterins were purchased from Schircks Laboratories (Jona, Switzerland). All other chemicals were of the highest quality available.

Sample preparation

Pterins were eluted from dried blood spots on filter paper (Guthrie cards). For every single measurement four blood spots (Ø 6 mm) were cut out and pterins were extracted with 250 µL of 20 mmol/L HCl and placed in an ultrasonic bath (Sonorex RK31, Bandelin) for 30 s. Extraction was continued for 10 min by mixing the filter spots solution five times for 5 s at room temperature. The extract was centrifuged at 1800g for 5 min at room temperature. Sixty microliters of the clear supernatant were used for analysis of hemoglobin on the hematology analyzer Sysmex KX-21N (Sysmex, Japan). The remaining supernatant was ultra-filtrated on Ultrafree (NMWL 10000; Millipore) at 5000g for 5 min. Pterins were analyzed in clear filtrate by HPLC and fluorescence detection without prior oxidation.

HPLC of pterins

HPLC of pterins (neopterin, biopterin, isoxanthopterin, and pterin) was performed as described previously [4b] with some modifications. Separation was performed on a C8 Spherisorb, 5 µm pre-column (40 × 4.6 mm) and ODS-1 Spherisorb, 5 µm analytical column (250 × 4.6 mm) (both from Stagroma, Rheinach, Switzerland), using 1.5 mmol/L potassium hydrogen phosphate buffer, pH 4.6, with 8% (v/v) methanol at a flow rate of 1.2 mL/min. Pterins were detected by their native fluorescence at λ_{EX} : 350 nm, λ_{EM} :

450 nm using a fluorescence Detector FP-920 (Jasco, Tokyo, Japan).

Patients

Seventy patients with HPA (age 1 week to 15 years) were either tested for BH₄ responsiveness (BH₄ loading test with 20 mg/kg) or on treatment with BH₄ (Schircks Laboratories, Jona, Switzerland). Blood sampling was a part of the routine screening for BH₄ deficiency and was approved by corresponding boards.

Controls

Healthy adult controls (authors of this work) were administered BH₄ (2 mg/kg, orally or sublingually) as described previously [4a].

Statistical analyses

WinSTAT for Excel (v. 2003.1) was used for descriptive statistics and for regression analysis. Correlation between blood spots, plasma, and urine was evaluated by debiased regression analysis according to the method of Passing and Bablok.

Results

BH₄ is extremely unstable in collected blood and about 30–40% is readily decomposed to pterin. Thus, in this study total biopterin was calculated as the sum of biopterin and pterin (biopterin + pterin).

Extraction of pterins from filter paper

Neopterin, biopterin, and pterin were eluted from filter paper with 20 mmol/L HCl. Addition of 10 or 20% (v/v) methanol did not improve the efficiency (Fig. 1A). Extending the sonification time from 30 s to 5 min and extraction time from 10 to 30 min, and increasing the volume of solvent from 250 to 500 µL also did not change the elution profile significantly (Figs. 1B and C).

Recovery

Recovery of pterins from Guthrie cards, calculated by spiking blood samples with neopterin (20 nmol/L), biopterin (20 nmol/L), and pterin (5 nmol/L) standards, yielded 63–69%.

Stability

The stability of pterins in dried blood spots was tested by storing Guthrie cards at room temperature in the dark for a period of up to 16 days. This time was estimated to be sufficient to send samples by ordinary mail to the laboratory. Pterins were analyzed on 5 different days, and as shown in Fig. 2, the profile did not change significantly during the first 16

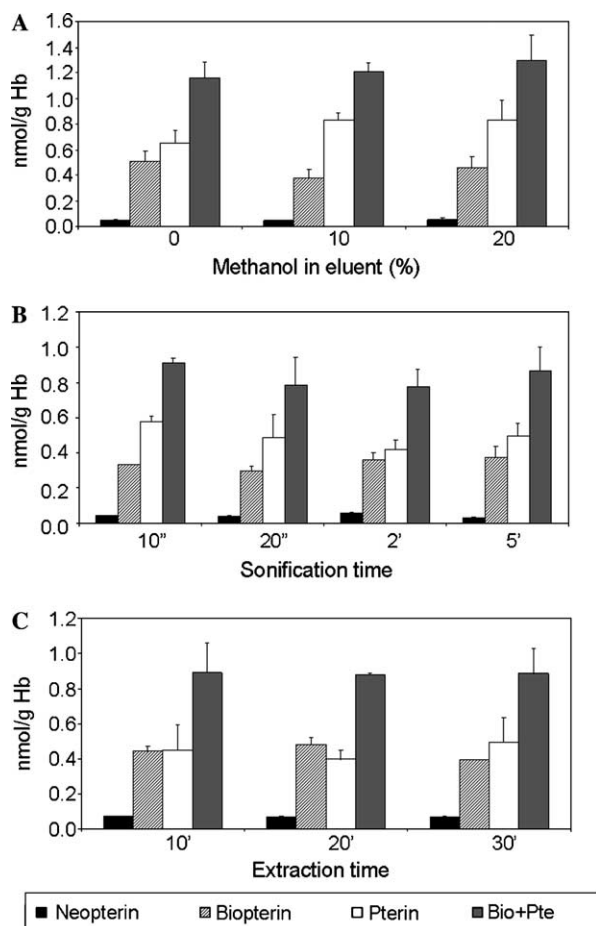


Fig. 1. Concentrations of neopterin, biopterin, and pterin in blood spots on filter paper investigated at: (A) different content of methanol in eluent; (B) different sonification times; and (C) different extraction times $n = 3$.

days. For long-time storage, Guthrie cards were kept at -20°C and retested after 6 and 18 months. Compared with initial values, neopterin was 102%, biopterin 105%, pterin 67%, and biopterin + pterin 80% (data not shown).

Reproducibility

The run-to-run imprecision was determined with 20 identical blood samples from a patient with HPA (high

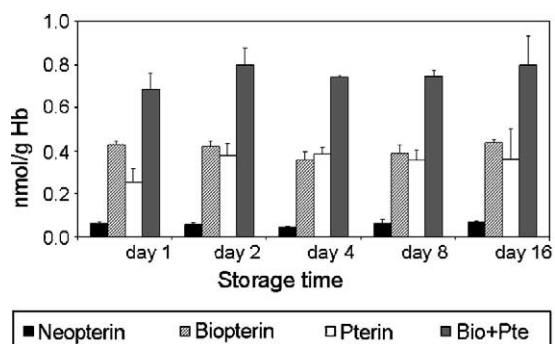


Fig. 2. Stability of neopterin, biopterin, and pterin in blood spots on filter paper after storage at room temperature $n = 3$.

Table 1

Run-to-run and within-run imprecision of identical 20 samples with low-value pterins (adult control) and high-value pterins (patient with HPA)

| $n = 20$ | Low value | | | | High value | | | |
|-------------------|-----------|------|------|-----------|------------|-------|-------|-----------|
| | Neo | Bio | Pte | Bio + Pte | Neo | Bio | Pte | Bio + Pte |
| <i>Run-to-run</i> | | | | | | | | |
| x | 0.08 | 0.26 | 0.22 | 0.33 | 2.81 | 12.44 | 14.86 | 27.29 |
| SD | 0.02 | 0.08 | 0.08 | 0.17 | 0.40 | 2.12 | 2.52 | 4.58 |
| CV(%) | 24.0 | 28.4 | 36.0 | 52.9 | 14.3 | 17.0 | 17.0 | 16.8 |
| <i>Within-run</i> | | | | | | | | |
| x | 0.05 | 0.21 | 0.41 | 0.62 | 0.32 | 54.23 | 84.69 | 138.92 |
| SD | 0.004 | 0.01 | 0.05 | 0.06 | 0.041 | 1.812 | 3.619 | 4.652 |
| CV(%) | 9.1 | 5.5 | 12.2 | 8.8 | 13.1 | 3.34 | 4.27 | 3.35 |

For details, see Materials and methods. Values are expressed in nmol/g Hb (not corrected for the recovery). Neo, neopterin; Bio, biopterin; Pte, pterin.

value) and of an adult healthy control (low value). The rather high coefficient of variation (CV) in the control sample (low value) may be explained by the fact that concentrations of pterins were close to detection limit of the HPLC system (around 0.02 nmol/g Hb). The within-run imprecision was measured in the same fashion but samples were measured in the same series (Table 1).

Comparison with plasma and urine

Concentrations of pterins in dried blood spots were compared with those in plasma obtained at same time points from four healthy persons loaded with 2 mg BH_4/kg and regression was calculated using the method of Passing–Bablok (Table 2). A relative good correlation was found between two methods for biopterin + pterin and neopterin (Figs. 3A and D) and less good but still acceptable for biopterin and pterin alone (Figs. 3B and C). Analysis of pterins in dried blood spots and plasma of healthy persons loaded with BH_4 (2 mg/kg) showed identical profiles for biopterin + pterin and neopterin (data not shown).

Comparison of pterins from Guthrie cards with those from urine specimen from both control persons and patients with HPA revealed a similar correlation as between blood spots and plasma (Table 2 and Figs. 4A–D). Furthermore,

Table 2

Regression analysis of pterins in blood spots, plasma, and urine according to Passing–Bablok

| | n | r | Slope (95% range) | Intercept (95% range) |
|-------------------------------|-----|-------|---------------------|---------------------------|
| <i>Blood spots vs. plasma</i> | | | | |
| Bio + Pte | 72 | 0.883 | 0.006 (0.005–0.007) | −0.026 (−0.056 to 0.012) |
| Bio | 72 | 0.773 | 0.004 (0.003–0.005) | −0.003 (−0.038 to 0.020) |
| Pte | 72 | 0.728 | 0.010 (0.008–0.012) | 0.000 (−0.019 to 0.037) |
| Neo | 72 | 0.842 | 0.010 (0.009–0.011) | −0.025 (−0.042 to −0.012) |
| <i>Blood spots vs. urine</i> | | | | |
| Bio + Pte | 84 | 0.852 | 0.261 (0.217–0.350) | −0.062 (−0.171 to −0.021) |
| Bio | 84 | 0.788 | 0.182 (0.152–0.242) | −0.019 (−0.084 to 0.016) |
| Pte | 84 | 0.502 | 0.520 (0.341–0.703) | −0.005 (−0.035 to 0.000) |
| Neo | 84 | 0.874 | 0.163 (0.151–0.195) | 0.026 (0.009–0.036) |

See also Figs. 3 and 4.

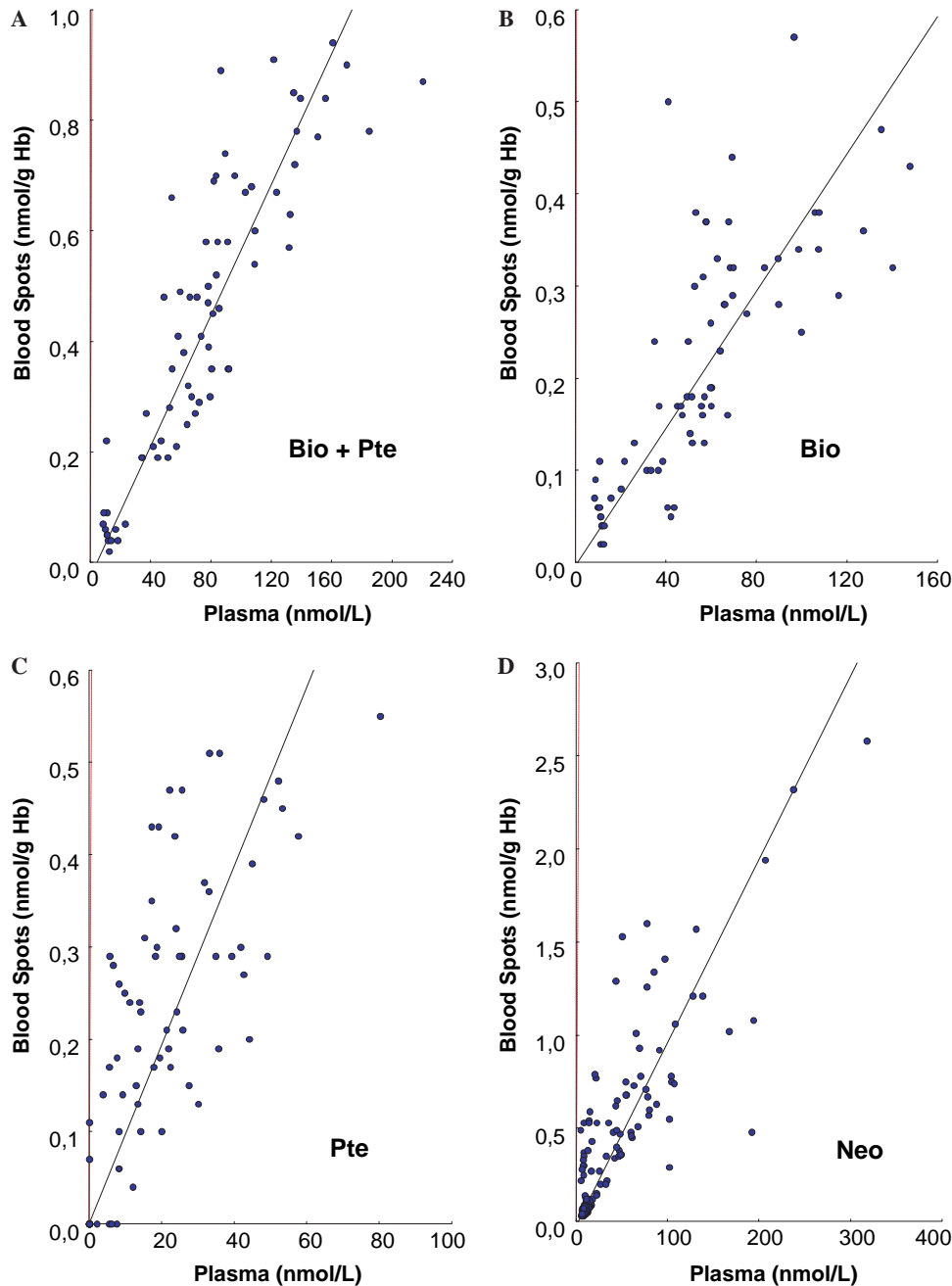


Fig. 3. Regression analysis between blood spot and plasma: (A) biopterin plus pterin (Bio + Pte), (B) biopterin (Bio), (C) pterin (Pte), and (D) Neopterin (Neo).

the percentage of biopterin + pterin (of the sum of all pterins) in two patients with HPA were found to be comparable for all three methods (Fig. 5). The first patient underwent an extended loading test with BH₄ (2 × 20 mg/kg BH₄ at T₀ and T₂₄) (Fig. 5A) and in the second patient a combined loading test (100 mg/kg phenylalanine at T₋₃ and 20 mg/kg BH₄ at T₀) was performed (Fig. 5B). The pterins profile showed that biopterin + pterin levels peaked 3–4 h after BH₄ administration in both cases (Figs. 6A and B), while neopterin concentrations remained unchanged (data not shown). The maximal BH₄ concentrations in blood spots were found to be extremely variable (3.6–96.4 nmol/g Hb) in a group of patients loaded with the same amount of BH₄ (20 mg/kg). In

one patient who was, based on the genotype, suggested to be BH₄-responsive, pterins were measured in blood spots on two occasions (Fig. 7). In the first loading test, he was found to be a non-responder and at that time the blood spot biopterin + pterin concentration was rather low (9.7 nmol/g Hb). In the second test, patient was found to be a BH₄-responder and the maximal biopterin + pterin concentration was much higher (33.2 nmol/g Hb).

Pterins profile in dried blood spots

To be able to screen for BH₄ deficiency in patients with HPA, reference values for neopterin and biopterin + pterin

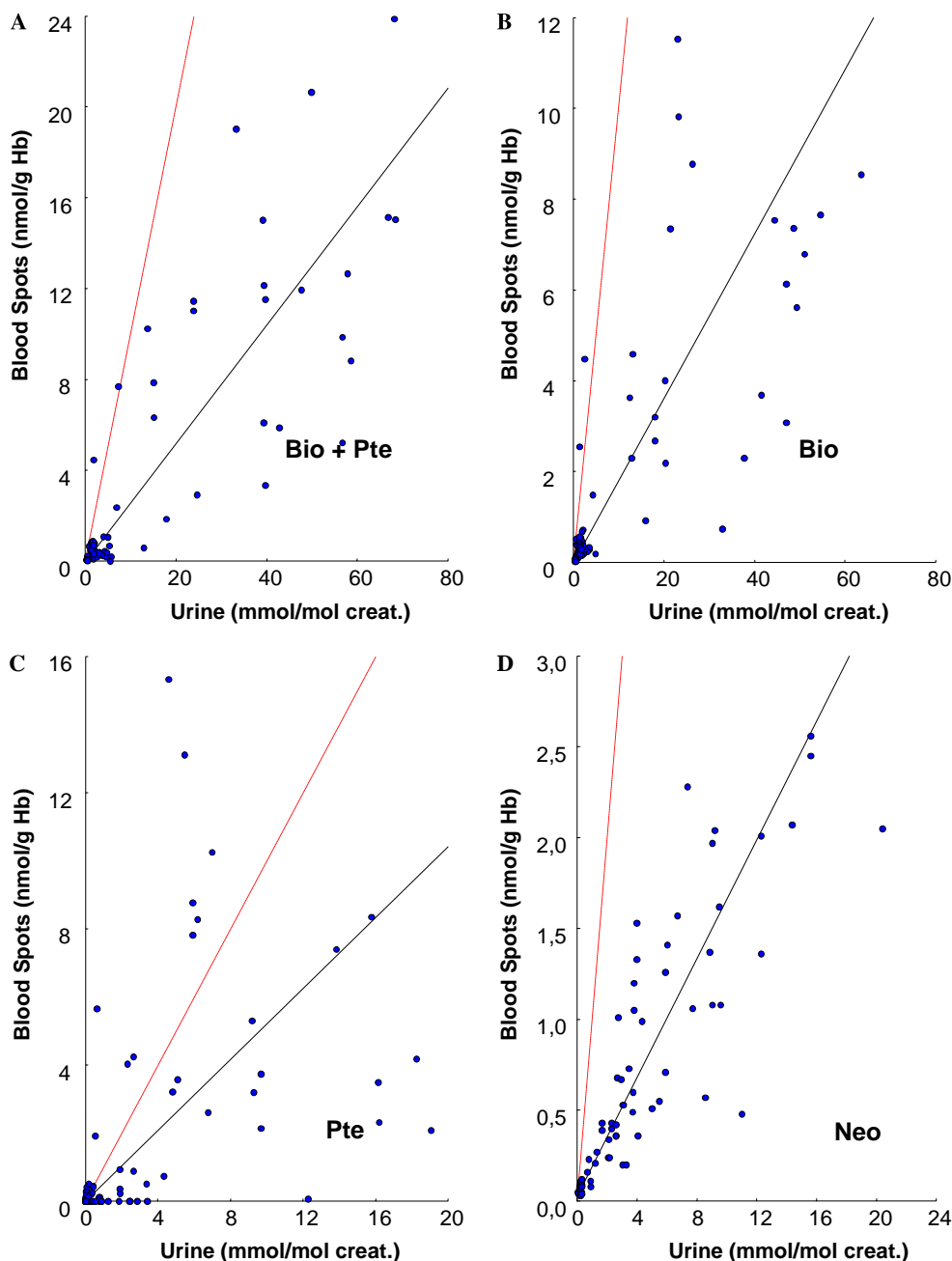


Fig. 4. Regression analysis between blood spots and urine. (A) Biopterin plus pterin (Bio + Pte), (B) biopterin (Bio), (C) pterin (Pte), and (D) neopterin (Neo).

were established (Table 3). For this purpose, blood spots from 70 patients with various degrees of HPA in whom BH_4 deficiency was excluded by standard tests were analyzed. Value were corrected for the reported recovery of $\sim 65\%$.

In a heterogeneous group of patients with HPA, two patients with PTPS deficiency, one with DHPR deficiency, and one with GTPCH deficiency were identified by analysis of the pterins profile in blood spots. Fig. 8A shows a patient suffering from classical PKU with a PAH deficiency. Neopterin and biopterin levels are slightly increased, with the profiles fitting well those in urine. A patient with a PTPS deficiency shows increased neopterin and no biopterin (Fig. 8B) and a patient suffering from DHPR deficiency has

normal to increased neopterin and increased biopterin levels in blood spots (Fig. 8C). The patient with GTPCH deficiency is characterized by reduced neopterin and biopterin levels (Fig. 8D). We also investigated blood spots and urine samples from an older patient previously diagnosed with PCD deficiency, but primapterin was very low in urine and was not detected in blood spots (data not shown).

Discussion

Dried blood spots on filter paper (Guthrie cards) were introduced in the early 1960s for the newborn screening of few common and treatable inherited metabolic diseases,

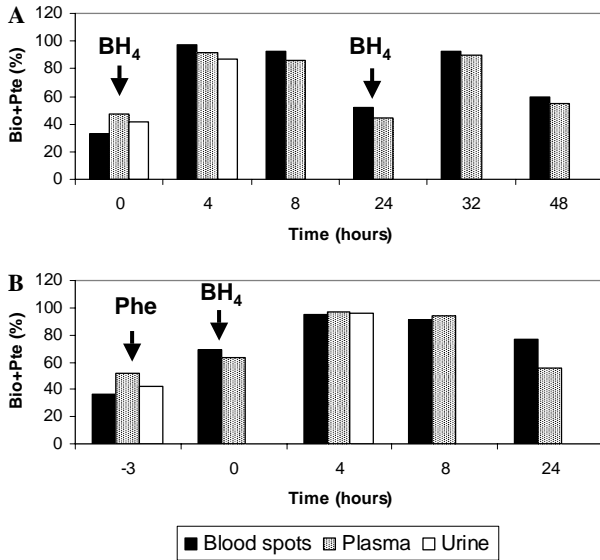


Fig. 5. Percentage of biopterin plus pterin ($100 \times (\text{Bio} + \text{Pte}) / (\text{Neo} + \text{Bio} + \text{Pte})$) in blood spots, plasma, and urine of two patients with HPA loaded with (A) $2 \times 20 \text{ mg/kg}$ of BH_4 at T_0 and T_{24} hours (extended loading test); and (B) 100 mg/kg phenylalanine at T_{-3} and 20 mg/kg BH_4 at T_0 (combined loading test).

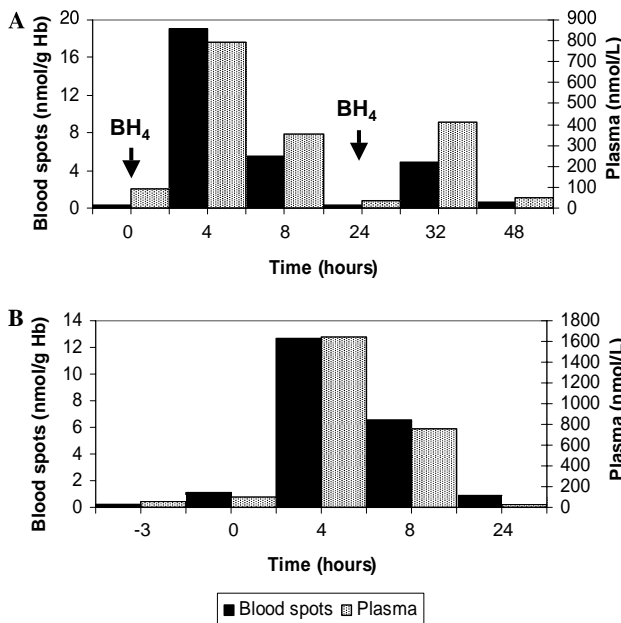


Fig. 6. Concentrations of biopterin plus pterin (Bio + Pte) in blood spots and plasma of two patients with HPA loaded with (A) $2 \times 20 \text{ mg/kg}$ of BH_4 at T_0 and T_{24} hours (extended loading test); and (B) 100 mg/kg phenylalanine at T_{-3} and 20 mg/kg BH_4 at T_0 (combined loading test).

including PKU [5]. With the introduction of TMS, a number of new tests were developed for blood spots and blood collection on filter paper became a practical alternative for measurement of metabolites such as amino acids and acylcarnitines in serum, plasma, or even urine. A minimal sample volume is required and samples can be transported at room temperature in an envelope. In addition to measurement of phenylalanine and tyrosine, blood spots are used

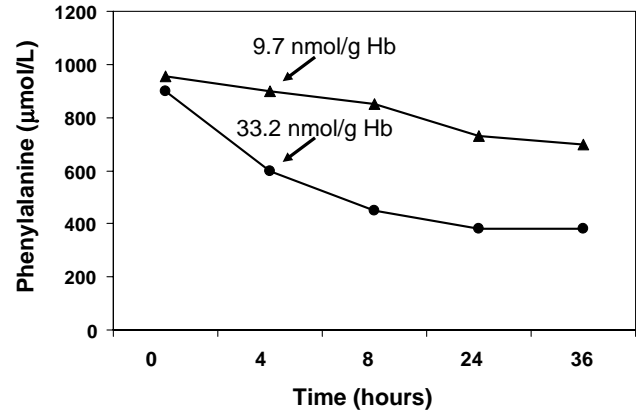


Fig. 7. Effect of oral administration of BH_4 (20 mg/kg) on blood phenylalanine levels in a patient with BH_4 -responsive PAH genotype (A403V/S41IX) on two separate loading tests. During the first test (slow response), T_4 blood spot BH_4 levels were 9.7 nmol/g Hb while during the second test (good response) BH_4 levels were ~ 3 times higher (33.2 nmol/g Hb).

for measurement of DHPR activity in patients with HPA [6]. These are routine tests for the screening of disorders in BH_4 metabolism and diagnosis is completed with the analysis of BH_4 metabolites in urine. Dried blood spots on Guthrie cards were also used for simultaneous measurement of total biopterin and DHPR activity. It used the *Crithidia fasciculata* bioassay, which may not detect biopterin levels in patients with partial or peripheral defects and was never tested for other BH_4 disorders [7].

Depending on the profile of neopterin and biopterin in urine, enzyme defects in BH_4 metabolism can be localized. However, urine samples need to be oxidized or sent frozen on dry ice. We developed a method that allows measuring amino acids, DHPR activity, and pterins from a single Guthrie card (eight 6 mm diameter spots).

One of the main problems with the analysis of pterins in body fluids is their sensitivity to oxygen and light. Particularly, BH_4 is readily oxidized and degraded to biopterin and pterin, and depending where the degradation takes place, pterin is further oxidized to isoxanthopterin and xanthopterin by the xanthine dehydrogenase [8]. BH_4 is present in blood, CSF, and urine mainly in tetrahydro form [9] and in the circulating blood it is probably bound to albumin. In solutions, BH_4 is stable at acidic pH while at basic pH the side chain of BH_4 is split off, producing a pterin ring [9]. We have previously shown that addition of ascorbic acid and DTE to plasma samples prevents BH_4 from both oxidation and degradation to pterin [10]. Using differential oxidation of plasma samples with iodine at

Table 3
Reference values for neopterin and biopterin + pterin in blood spots (nmol/g Hb) of patients with HPA, corrected for the reported recovery (65%)

| n = 70 | Neo | Bio + Pte | %Bio + Pte |
|-----------------|-----------|-----------|------------|
| Median | 1.08 | 0.77 | 31.3 |
| 5–95 percentile | 0.31–4.45 | 0.15–2.91 | 13.9–78.3 |

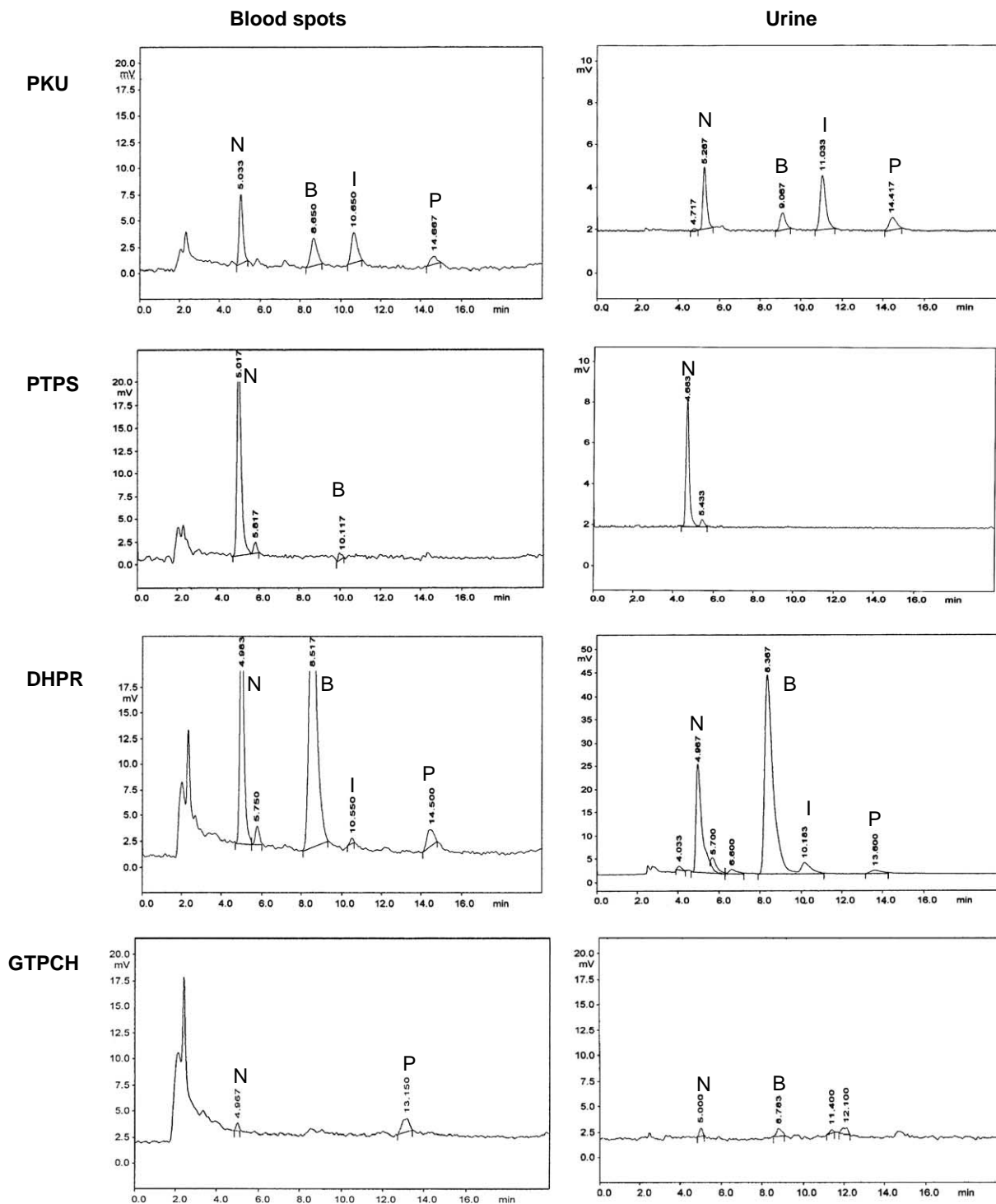


Fig. 8. HPLC profiles of pterins in blood spots and urine from patients with PKU and PTPS, DHPR, and GTPCH deficiencies. N, neopterin; B, biopterin; I, isoxanthopterin; P, pterin.

acidic and basic pH, we were able to show that about 80% of total biopterin is present as BH_4 . In dried blood spots on filter paper, BH_4 is already fully oxidized to biopterin and partially degraded to pterin. While the percentage of pterin is rather low in patients who are not on BH_4 , it can be up to 50% after administration of BH_4 . Once oxidized, pterins are stable in blood spots for up to 16 days at room temperature, which is the time estimated to be sufficient to

send samples to the laboratory. Over longer period of time (up to 18 months), neopterin (102%) and biopterin (105%) are more stable than pterin (67%). There was a positive correlation between plasma and blood spots for biopterin + pterin ($r=0.883$) and neopterin ($r=0.842$) and similar correlation was found between urine and blood spots ($r=0.852$ for biopterin + pterin and $r=0.874$ for neopterin).

Elution and extraction of neopterin, biopterin, and pterin was optimized with respect to the eluent and time. Pterins are generally well soluble in hydrochloric acid and at the acidic pH and when stored in the dark, they are stable over a longer period of time. Addition of organic solvent, as used for the extraction of amino acids or acylcarnitines, did not improve extraction of pterins from dried blood spots. Recovery was calculated to be 63–69%. Concentrations of pterins in blood spots were expressed per hemoglobin content because of a relative high amount of neopterin and biopterin in red blood cells. We have previously shown that concentrations of biopterin are about two times higher in red blood cells than in plasma and this ratio is even higher for neopterin (eight times higher in red blood cells) [11].

Reproducibility is similar to those found for plasma pterins with a relative high run-to-run imprecision (24.0–52.9%) for the low-value sample, while the high-value run-to-run imprecision (14.3–17.0%) and both low (5.5–12.2%) and high-value (3.34–13.1%) within-run imprecision fulfill all diagnostic criteria.

Blood spots biopterin + pterin were used to monitor BH₄ pharmacokinetics in healthy controls and patients with HPA. Preliminary pharmacokinetic of BH₄ was already done in healthy volunteers by monitoring biopterin concentrations in plasma following oral administration of BH₄ [10]. As already mentioned, about 80% of total biopterin was found as BH₄ when analyzed immediately in antioxidant pre-treated plasma and without antioxidants no BH₄ was detected. Maximal BH₄ concentrations were found 1–4 h after BH₄ administration and the elimination half-life time was estimated to be 3.3–5.1 h [10]. We investigated blood spot pterins during the course of loading test with BH₄ in 53 patients with HPA (to be published elsewhere). Two loading tests are presented in this paper and blood spot biopterin + pterin were compared with plasma biopterin (Fig. 6). In the first loading test, two dosages of BH₄ (2 × 20 mg/kg) were administered and biopterin + pterin was monitored up to 48 h after administration. As expected, maximal levels were found at T₄ and T₃₂ hours and blood spots and plasma levels were comparable. Similarly, in the second loading test (combined 100 mg/kg phenylalanine and 20 mg/kg BH₄ challenge) biopterin + pterin slightly increased in blood spots and plasma 3 h after phenylalanine administration and peaked 4 h after BH₄ administration. Thus, measurement of pterins in blood spots seems to be useful for monitoring BH₄ pharmacokinetics. This was also demonstrated in a patient who was based on the genotype (A403V/S411X) predicted to be a BH₄-responder. As shown in Fig. 7, there was only slow decrease in blood phenylalanine concentration after administration of BH₄ (20 mg/kg). At that time, blood spot biopterin + pterin levels were 9.7 nmol/g Hb. The test was repeated several weeks later and the patient was found to be a good BH₄-responder with blood spot biopterin + pterin levels of 33.2. Obviously, intra-individual variation in BH₄ absorption may contribute to BH₄ blood levels as documented by monitoring blood spot biopterin + pterin.

In a pilot study of more than 70 patients with HPA, we measured blood spot pterins before the BH₄ loading test and compared results with the standard screening in urine. Four patients were detected to be BH₄-deficient; one patient with the GTPCH deficiency, two with PTPS deficiency, and one with DHPR deficiency. The profile of pterins in blood spots was identical with that found in urine collected at the same time (Fig. 8). Although, the number of patients tested is rather small and no patients with PCD deficiency were detected so far, preliminary results suggest that blood spots on filter paper may be a practical alternative option for the differential diagnosis of common forms of BH₄ deficiency. Eight 6 mm spots are sufficient for the analysis of pterins (four spots), DHPR activity (two spots), and amino acids (two spots).

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