Pterins Workshop

ERDIM Virtual Meeting
October 21-22, 2021

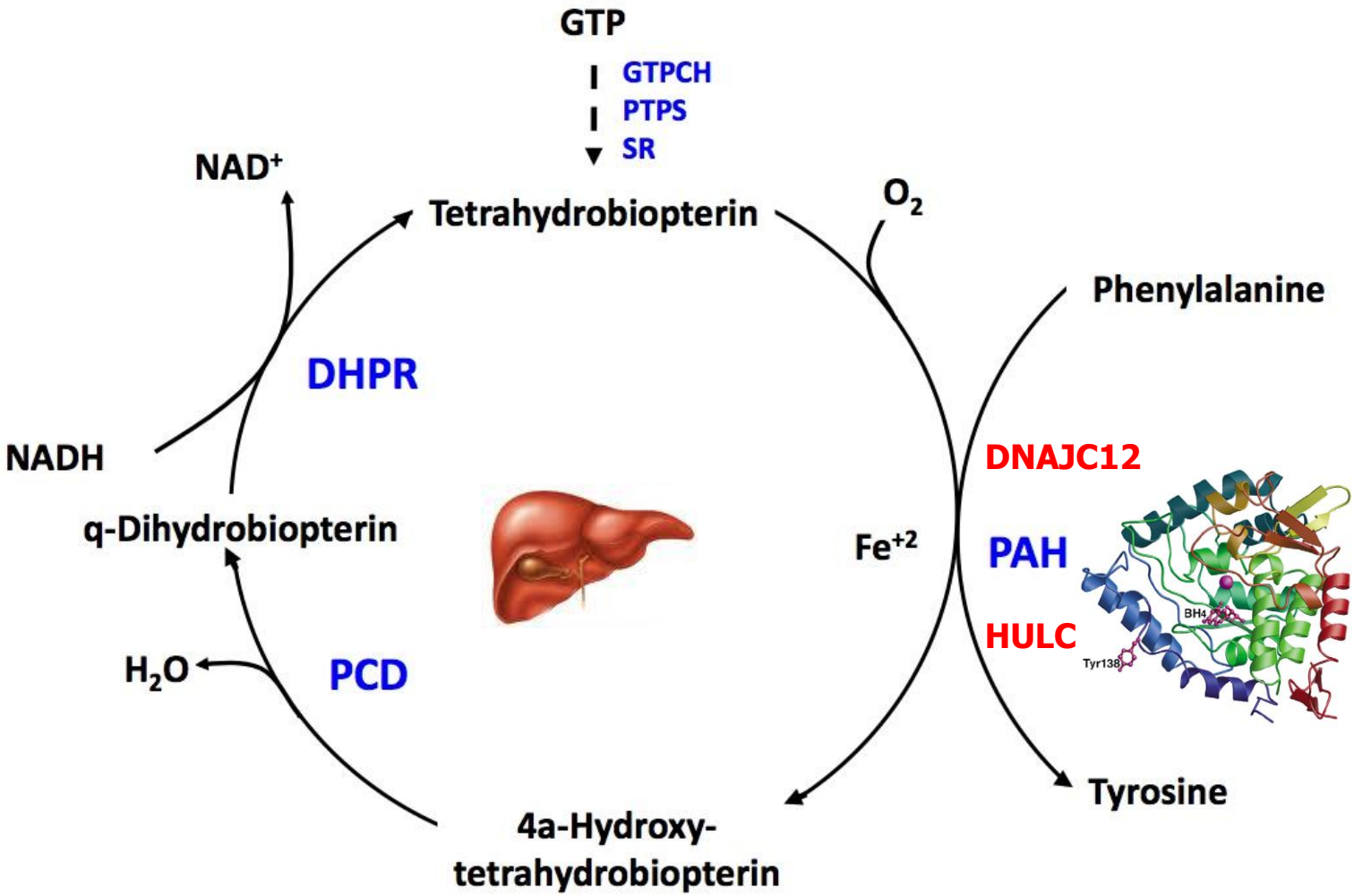
Contents

Tetrahydrobiopterin (BH_4) pathway and disorders
Nenad Blau (Zurich)

Methods and samples
Claudia Carducci (Rome)

Interpretation and ERNDIM PTU scheme
Alessio Cremonesi (Zurich)

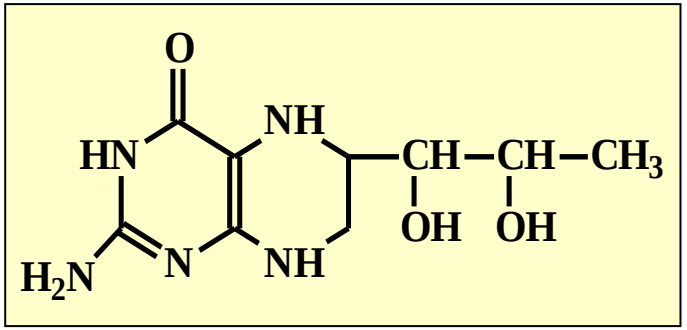
Phenylalanine hydroxylating system



DHPR: Dihydropteridine reductase; GTPCH: GTP cyclohydrolase; PAH: phenylalanine hydroxylase; PCD: Pterin-4-alpha-carbinolamine dehydratase 1; PTPS : 6-pyruvoyl-tetrahydropterin synthase; SR: Sepiapterin reductase.

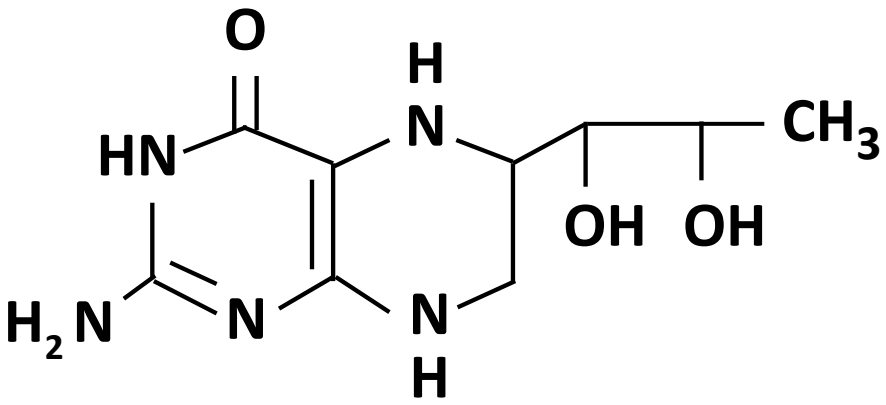
Blau N, et al. (2010) Lancet

Biological Functions of BH_4



- Co-factor / co-substrate for:
  - Phenylalanine hydroxylase **Phe → Tyr**
  - Tyrosine hydroxylase **Tyr → L-Dopa → Dopamine**
  - Tryptophan hydroxylase **Trp → 5-OH-Trp → Serotonin**
  - Nitric oxide synthase (NOS) **Arg → Cit + NO·**

Oxidation forms of BH_4



5,6,7,8-Tetrahydrobiopterin (BH_4)

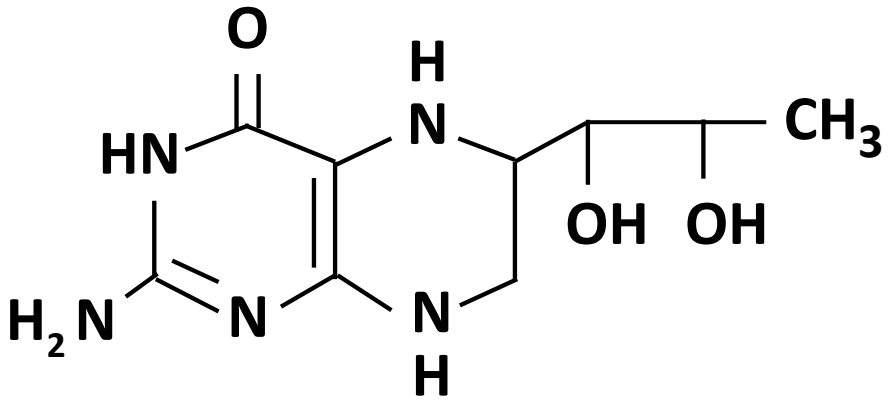
↓ Ox.

q oder7,8-Dihydrobiopterin (BH_2)

↓ Ox.

Biopterin (Bio)

Regeneration of BH4



5,6,7,8-Tetrahydrobiopterin (BH_4)

DHPR — **Ox.** ↓

q or 7,8-Dihydrobiopterin (BH_2)

Ox. ↓

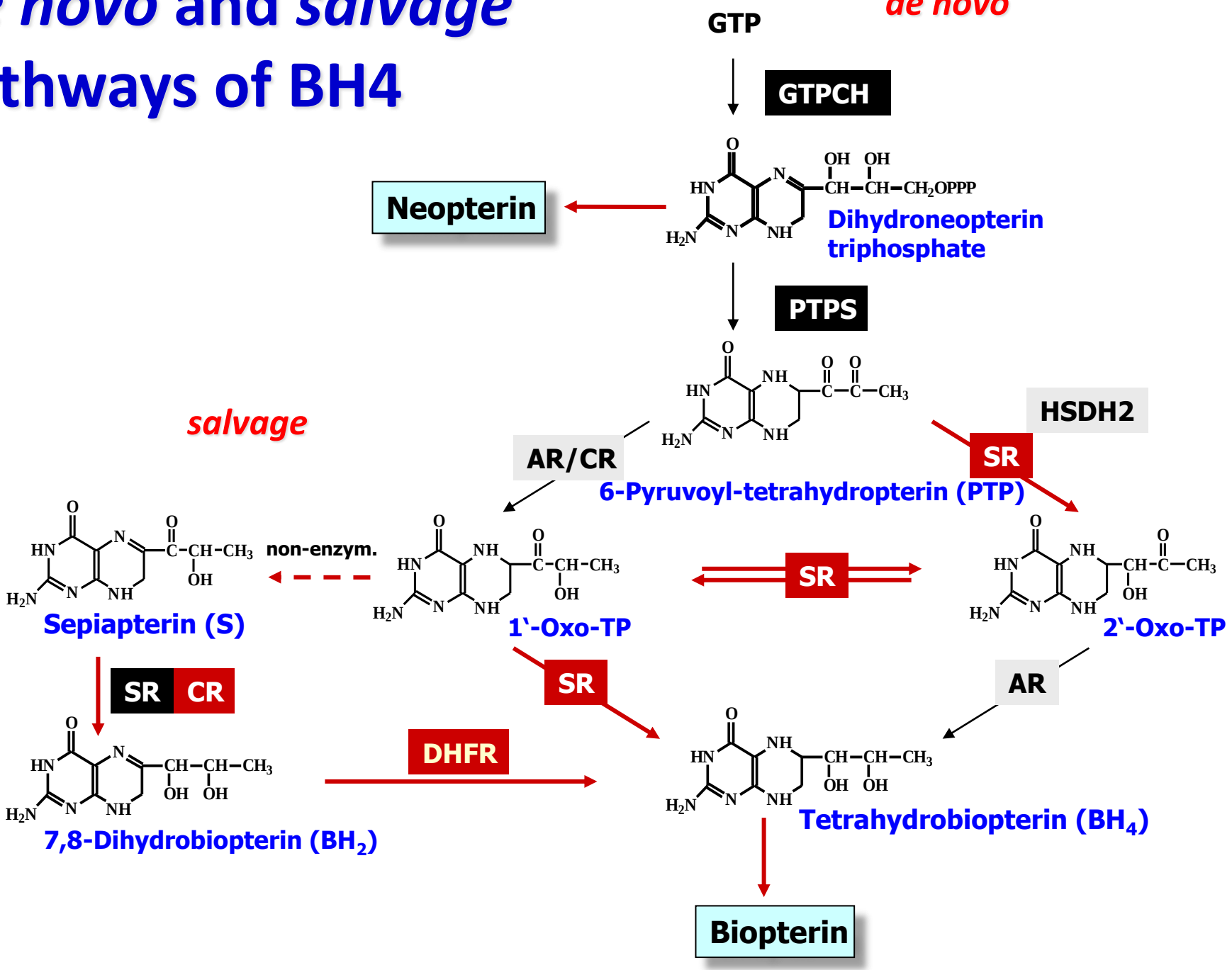
Biopterin (Bio)

***De novo* Pathway**

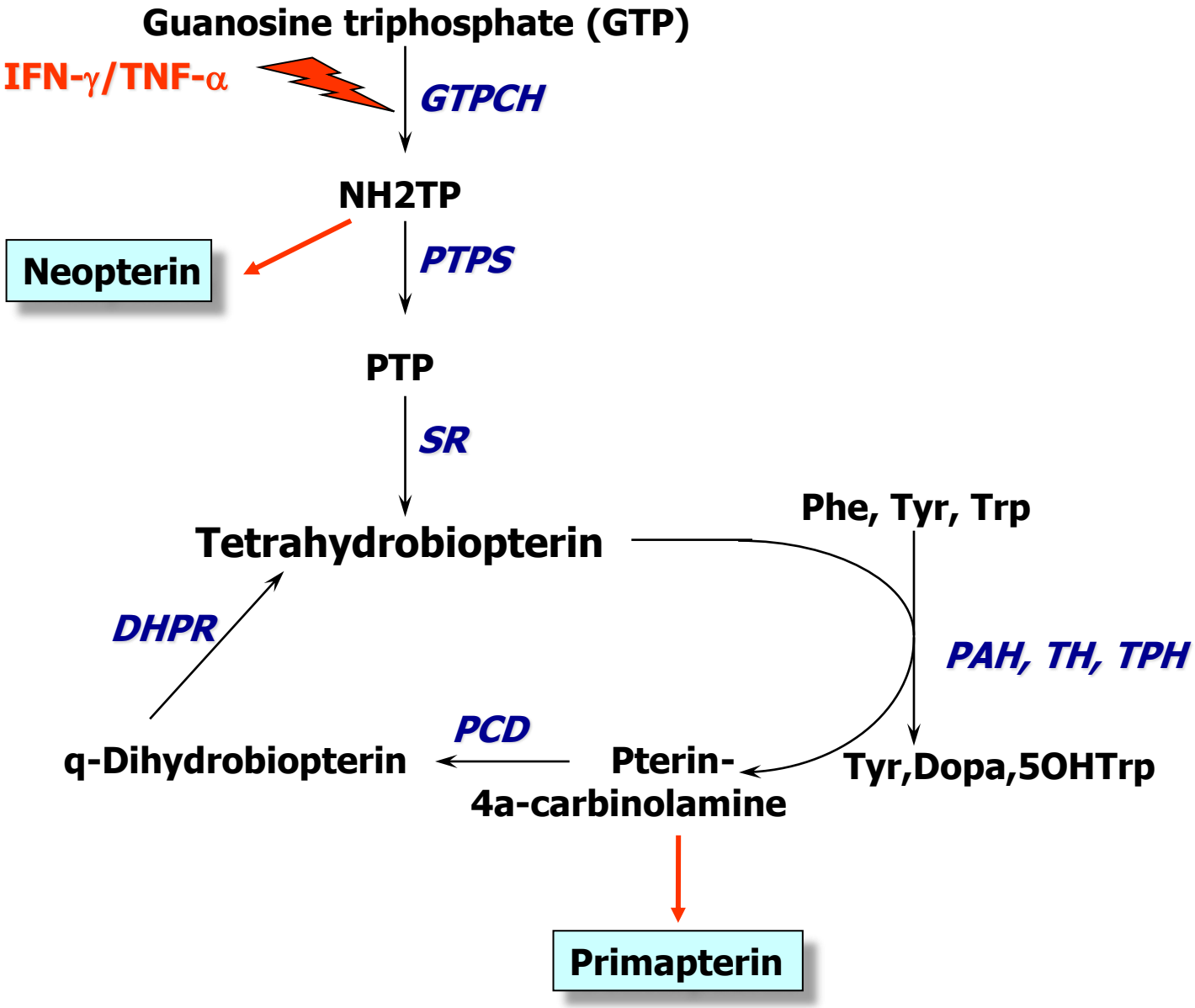
Salvage Pathway — **DHFR**

DHPR = Dihydropteridine reductase
DHFR = Dihydrofolate reductase

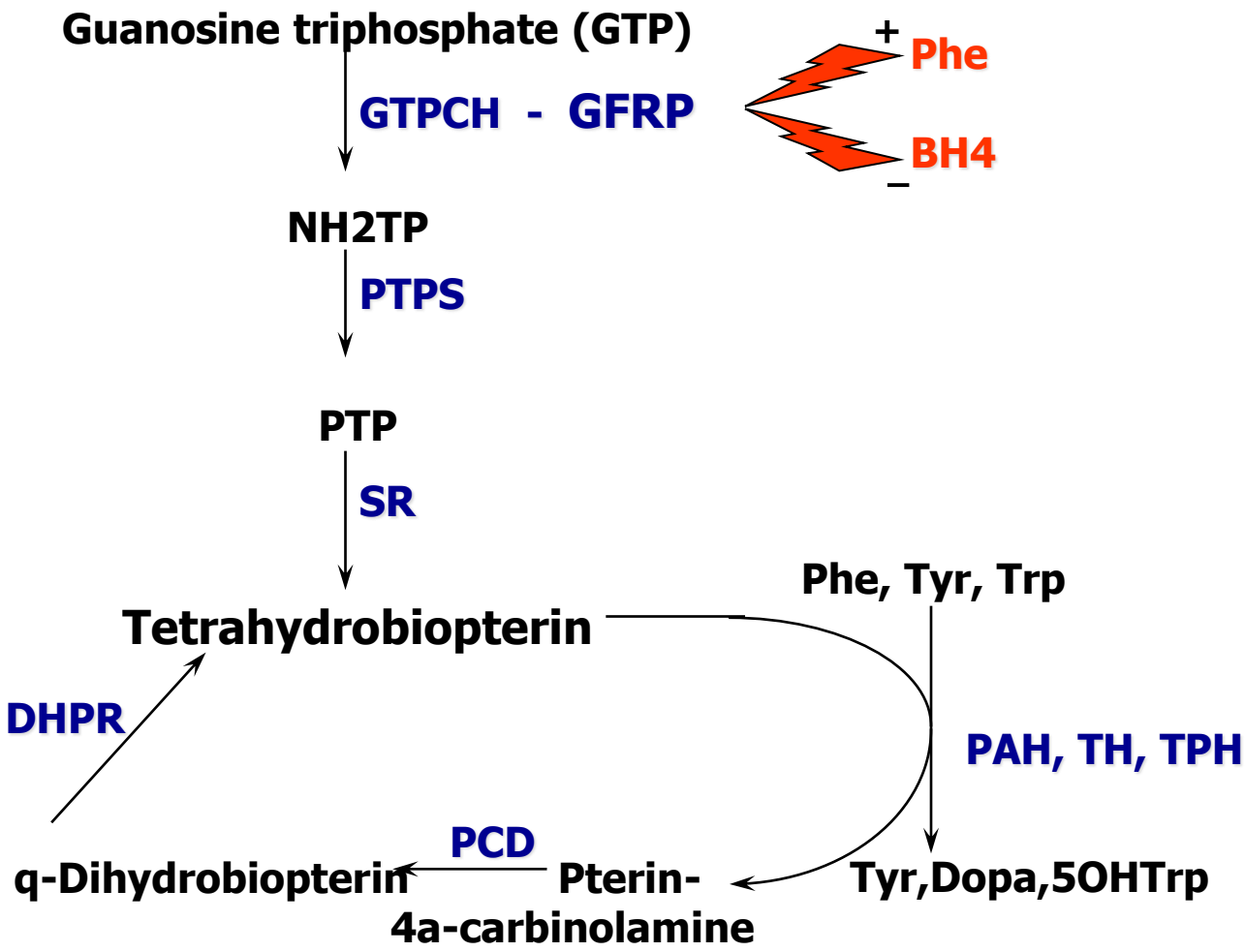
De novo and *salvage* pathways of BH4



Effect of cytokines on BH_4 metabolism



Regulation of BH_4 metabolism by phenylalanine

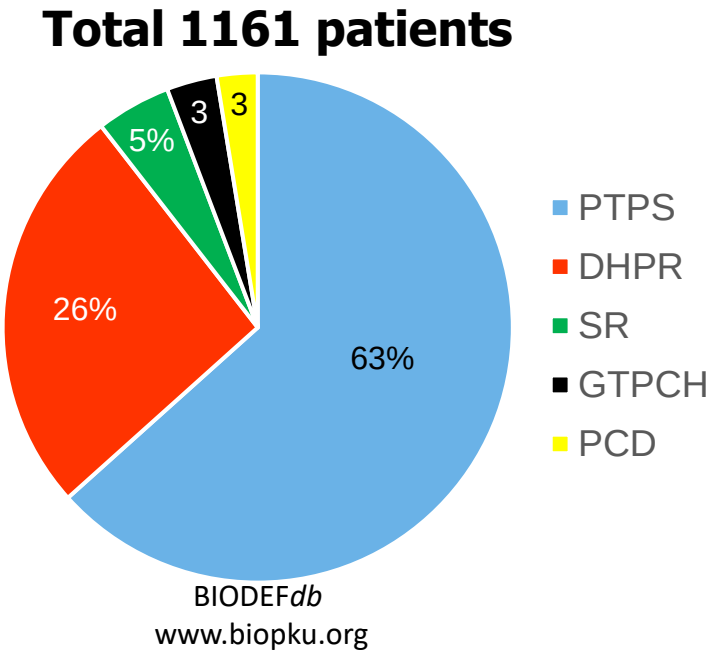


GFRP = GTPCH Feedback Regulatory Protein

Hyperphenylalaninemias (HPA)

	Blood Phe* µmol/L
Phenylalanine hydroxylase (PAH) deficiency	
Mild Hyper-Phe-NT (not requiring treatment)	120-360
Mild Hyper-Phe (gray zone)	360-600
Mild PKU	600-900
Moderate PKU	900-1200
Classical PKU	>1200
Tetrahydrobiopterin (BH_4) deficiencies	
GTP cyclohydrolase (GTPCH) deficiency	55-2900
PTP synthase (PTPS) deficiency	130-2300
Sepiapterin reductase (SR) deficiency (without HPA)	<90
Pterin-carbinolaminedehydratase (PCD) deficiency	180-1900
Dihydropteridine reductase (DHPR) deficiency	130-3200
DNAJC12 deficiency	120-800

BH_4 deficiencies



Take-home messages PTU Workshop

- **Clinical presentation of BH4 deficiencies is more severe than in PKU patients**

- **BH4 deficiency may present with and without hyper-Phe**

- **Early diagnosis of BH4 deficiencies is essential for immediate initiation of appropriate treatment and for beneficial outcome of patients.**

ERNDIM
QUALITY ASSURANCE IN LABORATORY TESTING FOR IEM

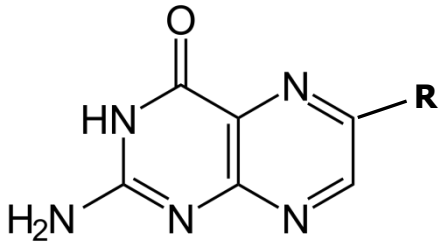
ERNDIMQA-PTU WORKSHOP: 22 OCTOBER 2021

Analytical methods for the determination of pterins in biological fluids

Dr Claudia Carducci, PhD
Sapienza University of Rome

Pterins: chemical structure and properties

Pterins belong to a heterocyclic family formed by a bicyclic pyrimidine–pyrazine (pteridines)

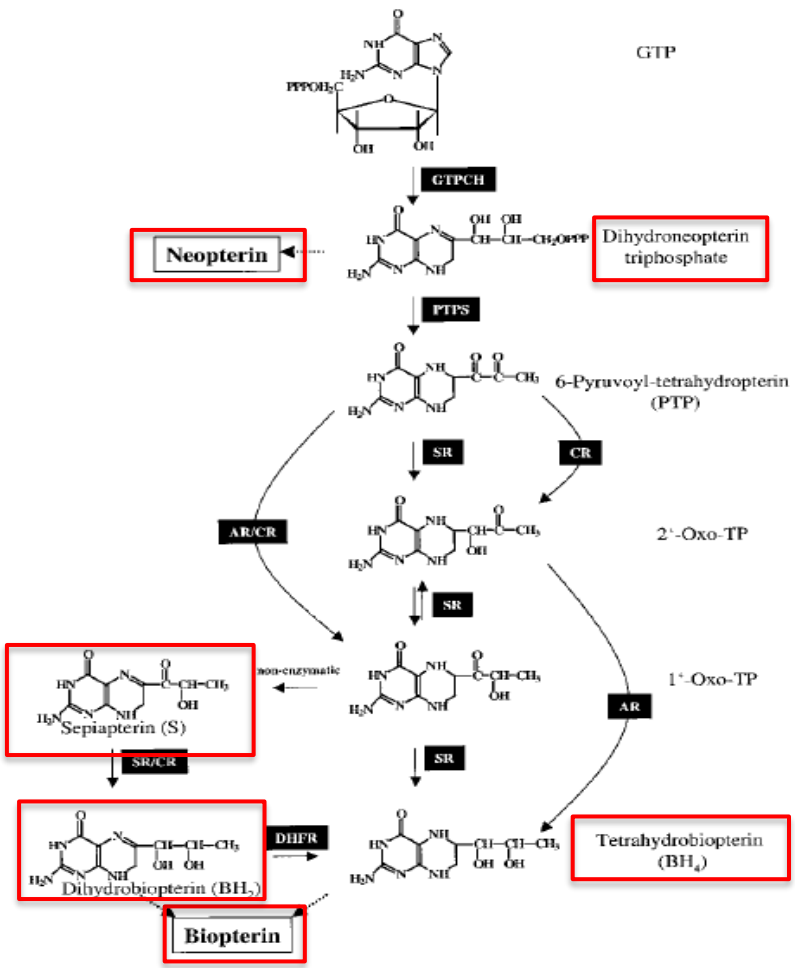


PROPERTIES

1. High polarity
2. Low solubility in water and organic solvents
3. Sensitivity to light
4. Occurrence of forms at diverse oxidation states (tetrahydro, dihydro, and completely oxidized form)
5. Reduced forms are extremely sensitive to oxygen and dissociate rapidly
6. They are present in biological fluids at low concentrations (a few µmol/L in urine; a few nmol/L in serum and cerebrospinal fluid)
7. Oxidized forms are highly fluorescent

Pterins analysed for the diagnosis of BH4 deficiency

Biosynthesis of BH4



Blau N et al Mol Genet Metab 2001;74:172-185

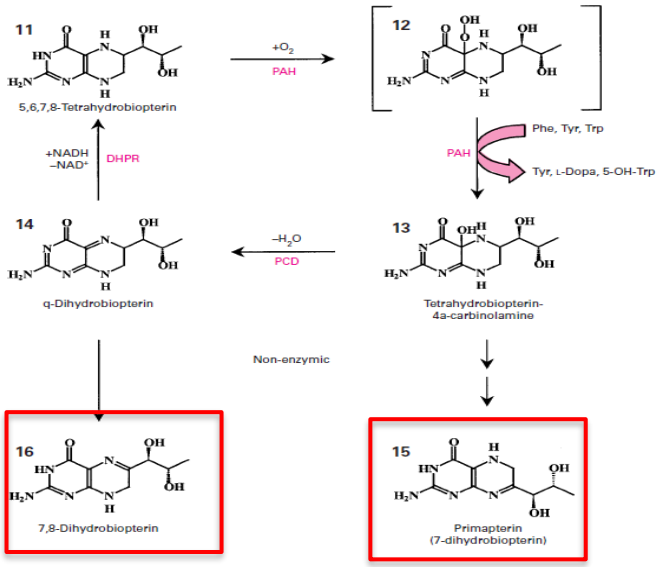
$BH4 + BH2 + B = B_{total}$

$N + NH2 = N_{total}$

Primapterin

Sepiapterin

BH4 regeneration pathway



Thony B et al Biochem J 2000; 347:1-16

Analytical methods for the determination of pterins

Most methods used for pterin analysis involve:

chemical conversion of reduced pterins into the stable, fully oxidized, fluorescent parent compounds

separation by high performance liquid chromatography (HPLC) and fluorescence detection (FD)

Pre-processing of urine samples: oxidation of reduced forms

Acidic oxidation using iodine*

- Fresh random urine (0.5 ml) was adjusted to pH 1.0–1.5 with 6 mol/l HCL.
- Oxidation of reduced pterins was performed by adding 110 µl 0.5%I_2 1%KI in HCl 0.1M
- Samples were incubated for 45 min at room temperature
- Reaction was stopped by the addition of 100 µl of 1% ascorbic acid

**T. Fukushima, J.C. Nixon, Analysis of reduced forms of biopterin in biological tissues and fluids, Anal. Biochem. 102 (1980) 176–188. (*with some modifications)

Oxidation using MnO_2#

- Fresh random urine (1 ml) was adjusted to pH 1.0–1.5 with 6 mol/l HCL.
- Oxidation of reduced pterins was performed by adding 20 mg MnO_2
- Samples were shaken for 5 min at room temperature and centrifuged for 10 min at 4000 rpm

*# A.Niederwieser, et al in H. Wachter., H.-Ch.Curtius and W.Pfleiderer (Editors), Biochemical and Clinical Aspects of Pteridines, Vol1, Walter de Gruyter, Berlin, 1982, pp 81-102 (*with some modifications)

A deproteinization step with ultrafiltration or SPE could be added prior to HPLC injection

MnO_2 vs Iodine oxidation

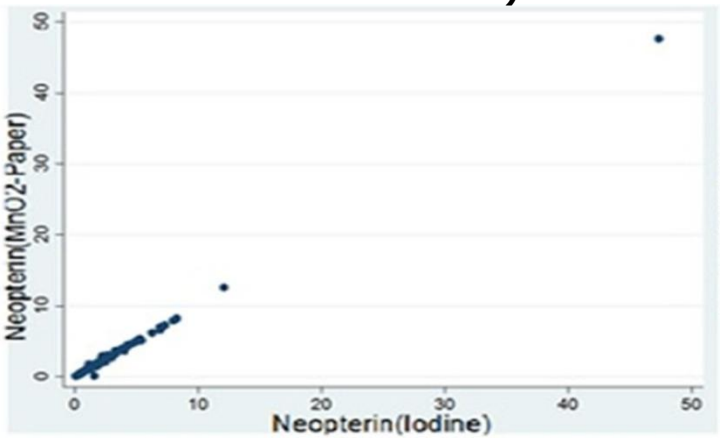
Ind J Clin Biochem (Oct-Dec 2019) 34(4):436–443
https://doi.org/10.1007/s12291-018-0777-3

ORIGINAL RESEARCH ARTICLE

The Comparison of Iodine-Type and MnO_2-Type Oxidation for Measuring the Levels of Urine Neopterin and Biopterin in Patients with Hyperphenylalaninemia: A Descriptive-Analytic Study in Iran

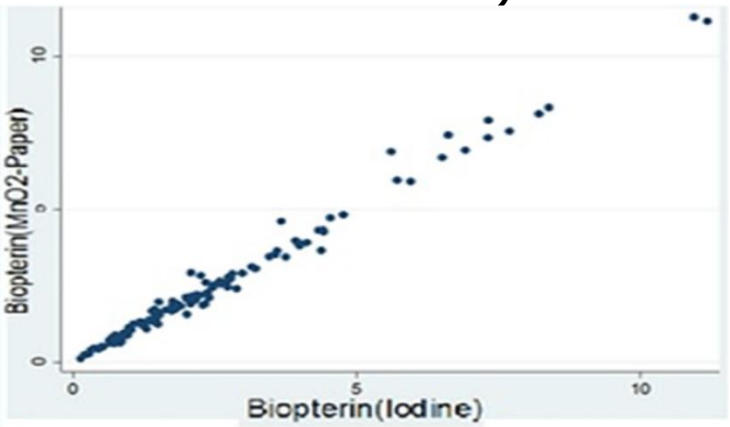
Atena Askarizadeh[1] · Shohreh Khatami[1] · Soghra Rouhi Dehnabeh[1]

P<0.001 and R = 0.99)



(a)

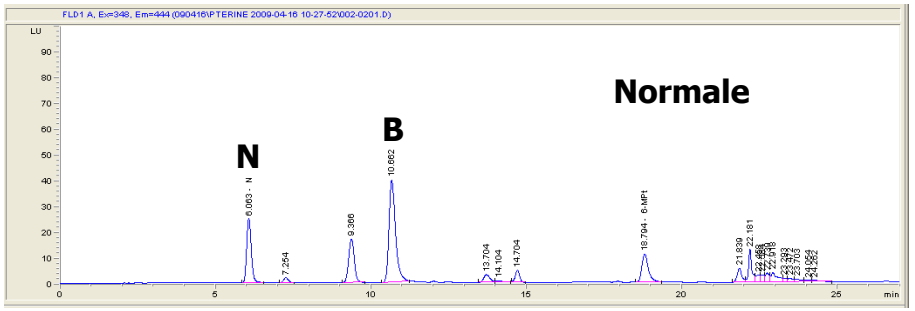
P<0.001 and R = 0.99)

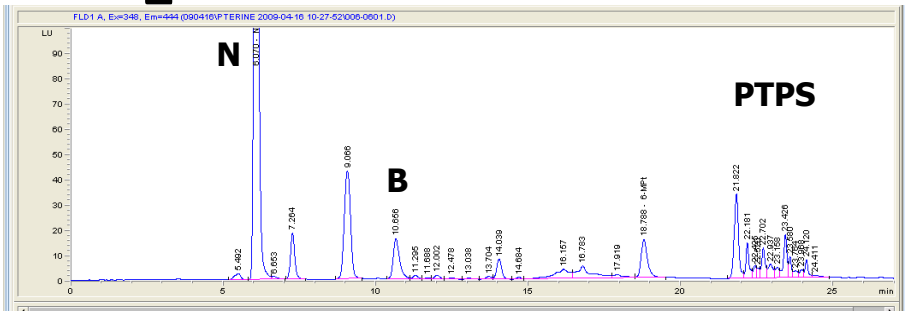


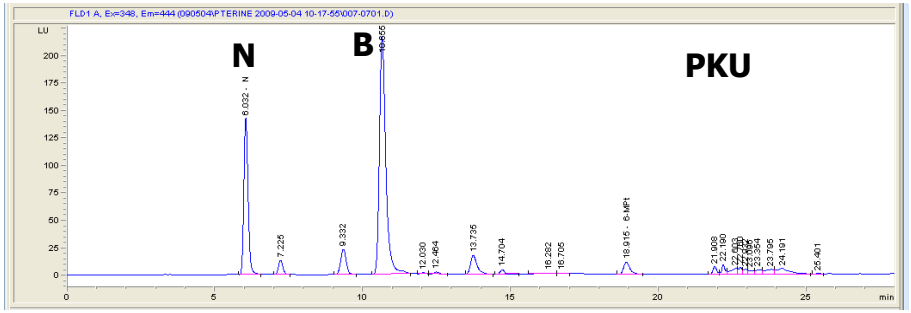
(b)

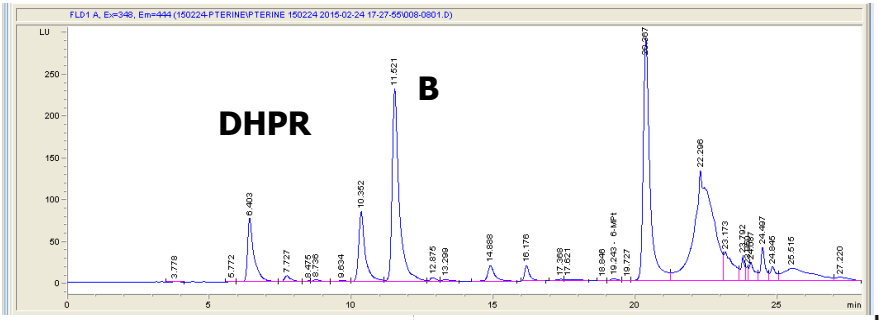
Fig. 1 Correlation between ($neopterin_{MnO2}$ and $biopterin_{MnO2}$) oxidation and ($neopterin_{iodine}$ and $biopterin_{iodine}$) oxidation tests

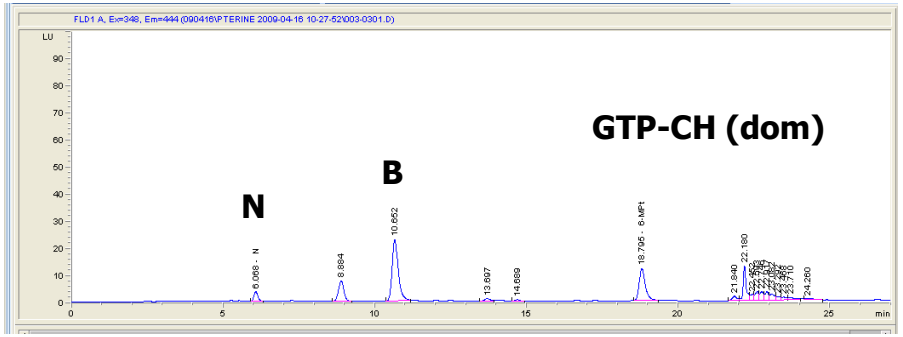
HPLC ANALYSIS AFTER I_2 OXIDATION











Mobile phase A : KH_2PO_4 24mM pH 5.0
Mobile phase B : CH_3OH:H_2O (70/30, v/v)
Gradient elution
Injection volume 20 µl

Flow rate 1.1 ml/min
Ex 348 nm Em 444 nm
Column Spherisorb S5 ODS1 5µm, 4.6x250mm
Column temperature: 38° C

Table 1 Genotype and biochemical phenotype in CGH1 mu

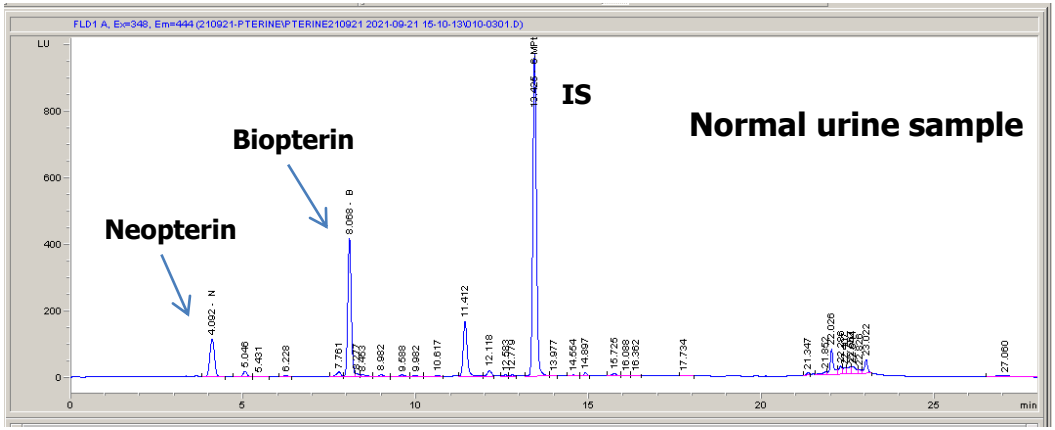
ID	Genotype	Sex	Age (years)	Urine NEO[a] (r.v.)[c]	Urine BIO[a] (r.v.)[c]	Ref.
1a	c.631-632 del AT [p.M211fs]	F	11	**0.27** (0.30–1.84)	1.21 (0.53–2.05)	Pedigree 1: proband
1b	c.631-632 del AT [p.M211fs]	M	36	0.23 (0.19–0.91)	0.69 (0.28–0.86)	Pedigree 1: father of 1a
1c	c.671A>G [p.K224R]	M	8	**0.22** (0.30–1.84)	0.65 (0.53–2.05)	Pedigree 1: brother of 1a
1d	c.671A>G [p.K224R]	F	34	0.41 (0.19–0.91)	0.36 (0.28–0.86)	Pedigree 1: mother of 1a
2a	Exon 1 deletion	F	7	**0.23** (0.30–1.84)	**0.42** (0.53–2.05)	Pedigree 2: proband
2b	Idem	F	36	**0.13** (0.19–0.91)	**0.16** (0.28–0.86)	Pedigree 2: mother of 2a
2c	Idem	M	5	**0.25** (0.30–1.84)	0.60 (0.53–2.05)	Pedigree 2: brother of 2a
2d	Idem	F	58	**0.13** (0.19–0.91)	**0.16** (0.28–0.86)	Pedigree 2: maternal grandmother 2a
3	c.68 C>T [p.P23L]	M	25	**0.11** (0.19–0.91)	**0.19** (0.28–0.86)	Leuzzi, unpublished case
4	c.671A>G [p.K224R]	M	17	**0.16** (0.30–1.84)	**0.47** (0.53–2.05)	Leuzzi et al. 2002
5	c.262 C>T [p.R88W]	F	45	**0.14** (0.19–0.91)	0.4 (0.28–0.86)	Leuzzi, unpublished case

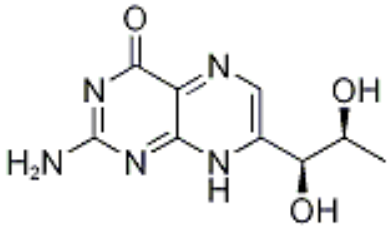
Leuzzi V, Carducci C, Chiarotti F, D'Agnano D, Giannini MT, Antonozzi I, Carducci C. Urinary neopterin and phenylalanine loading test as tools for the biochemical diagnosis of segawa disease. JIMD Rep. 2013;7:67-75.

PRIMAPTERIN

Marker of pterin-4a-carbinolamine dehydratase (PCD) deficiency

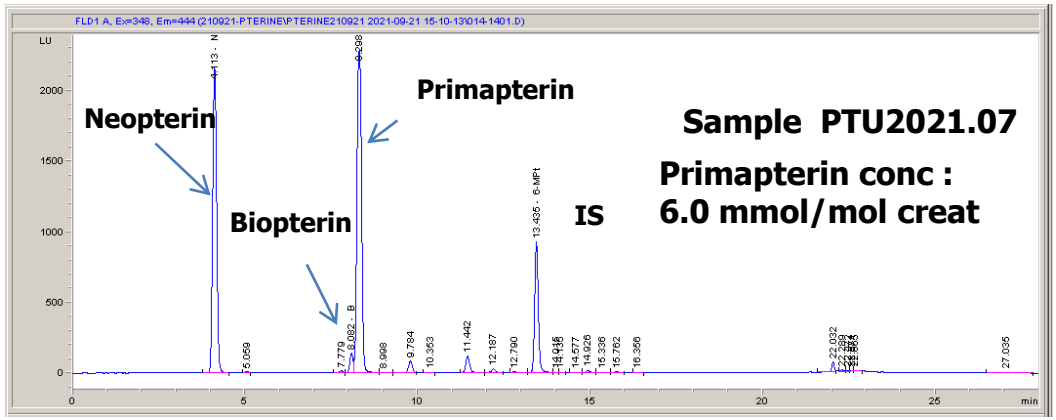
Pathological range: 0.1–1.9 (N=5)*





L-Erythro-7-isobiopterin

ACIDIC OXIDATION USING I_2/KI



HPLC ANALYSIS

Mobile phase A : KH_2PO_4 24mM pH 5.0
Mobile phase B : CH_3OH:H_2O (70/30, v/v)
Gradient elution
Injection volume 20 µl
Flow rate 1.1 ml/min
Ex 348 nm Em 444 nm
Column Pororshell HPH-C18, 4µm, 4.6x250mm
Column temperature: 38° C

**Opladen T, Hoffmann GF, Blau N. An international survey of patients with tetrahydrobiopterin deficiencies presenting with hyperphenylalaninaemia. J Inherit Metab Dis. 2012 Nov;35(6):963-73*

METHOD FOR THE DETERMINATION OF SEPIAPTERIN IN URINE

Molecular Genetics and Metabolism 115 (2015) 157–160

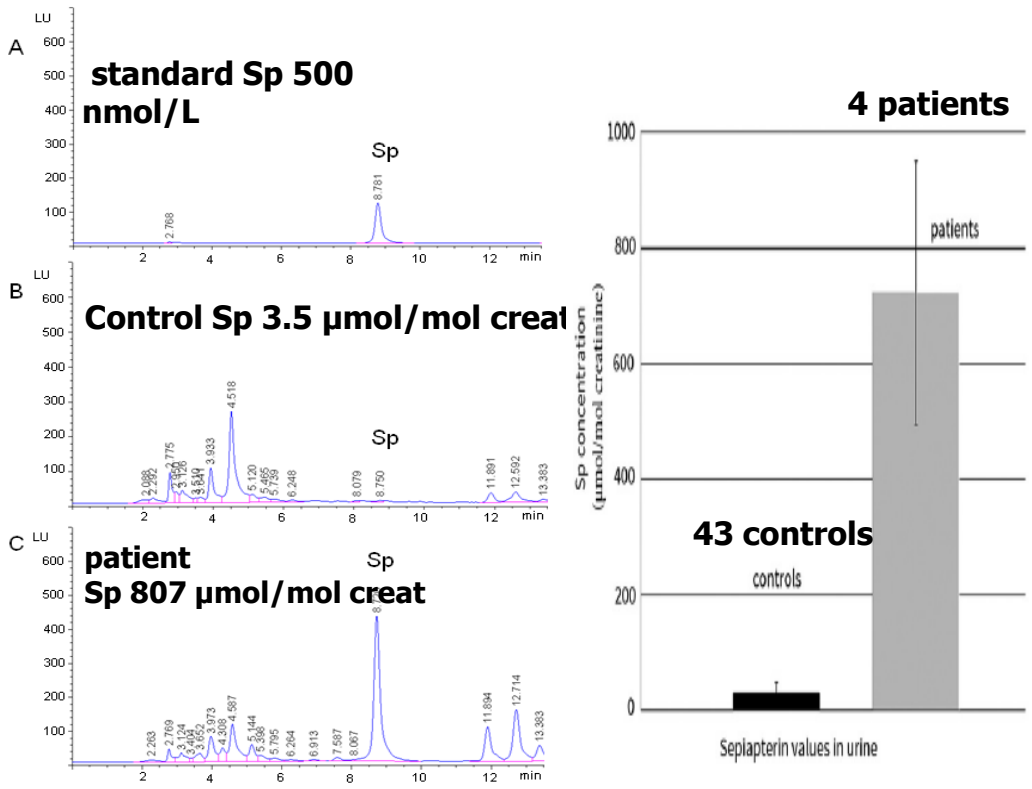
Contents lists available at ScienceDirect

Molecular Genetics and Metabolism

journal homepage: www.elsevier.com/locate/ymgme

Urine sepiapterin excretion as a new diagnostic marker for sepiapterin reductase deficiency

Claudia Carducci [a], Silvia Santagata [a], Jennifer Friedman [b,c], Elisabetta Pasquini [d], Carla Carducci [a], Manuela Tolve [a], Antonio Angeloni [e], Vincenzo Leuzzi [f,*]



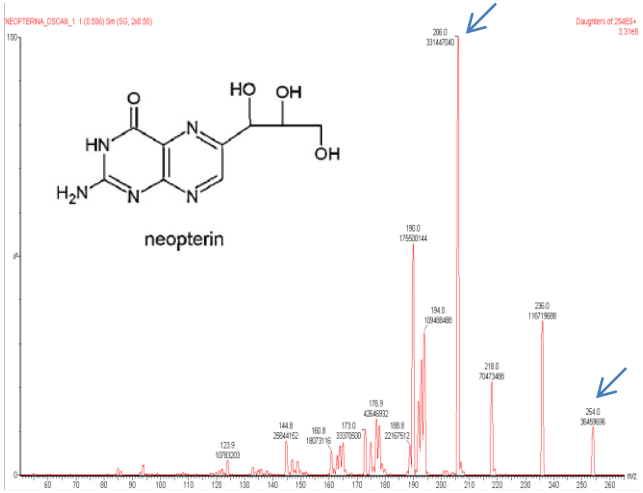
- First morning urine samples were collected in darkened containers then put into melting ice and stored at −80° C until analysis.
- 360 µl urine + 40 µl of ascorbic acid 1%

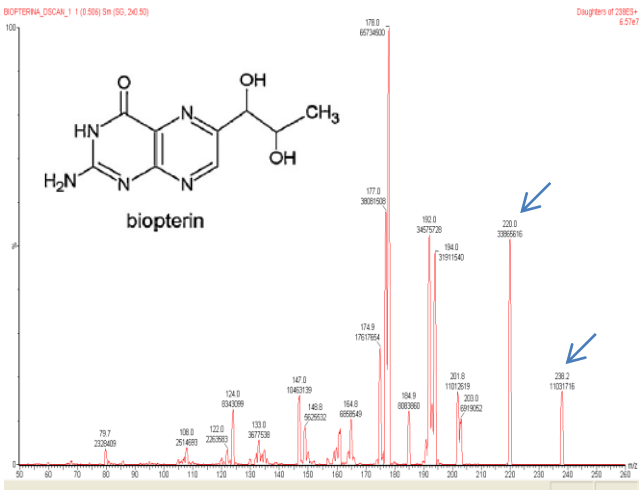
- *Injection volume:* 20 µl
- *Column:* Spherisorb S5 ODS1 250 × 4.6 mm I.D., 5 µm
- *Mobile phase A*: 24mM KH_2PO_4 pH 5.0
- *Mobile phase B* : CH_3OH/H_2O(70:30)
- *Flow-rate:* 1.1 ml/min
- Gradient elution
- *Temperature of column*: 38 ° C
- *FD*: Ex 425 nm Em 530 nm

	HVA	5HIAA	BH2	B	N	Sp	Phe
CSF	↓	↓	↑	↑	n	↑	n
urine				n	n	↑	n

NEOPTERIN AND BIOPTERIN ANALYSIS BY UPLC-ESI-MS/MS

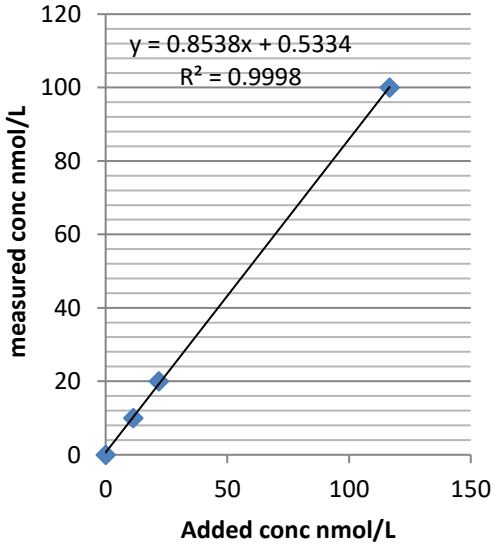
Daughter ion scan



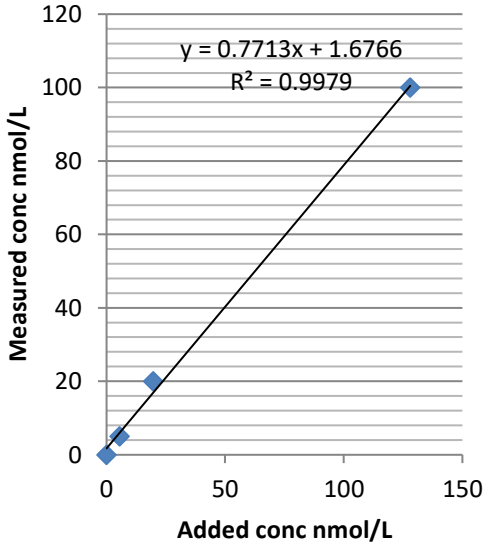


	MRM Transition (m/z)
N	254.02→206.01
B	238.08→220.08
$^{13}C_5$-N	259.02→210.01
D_3-B	241.08→223.08

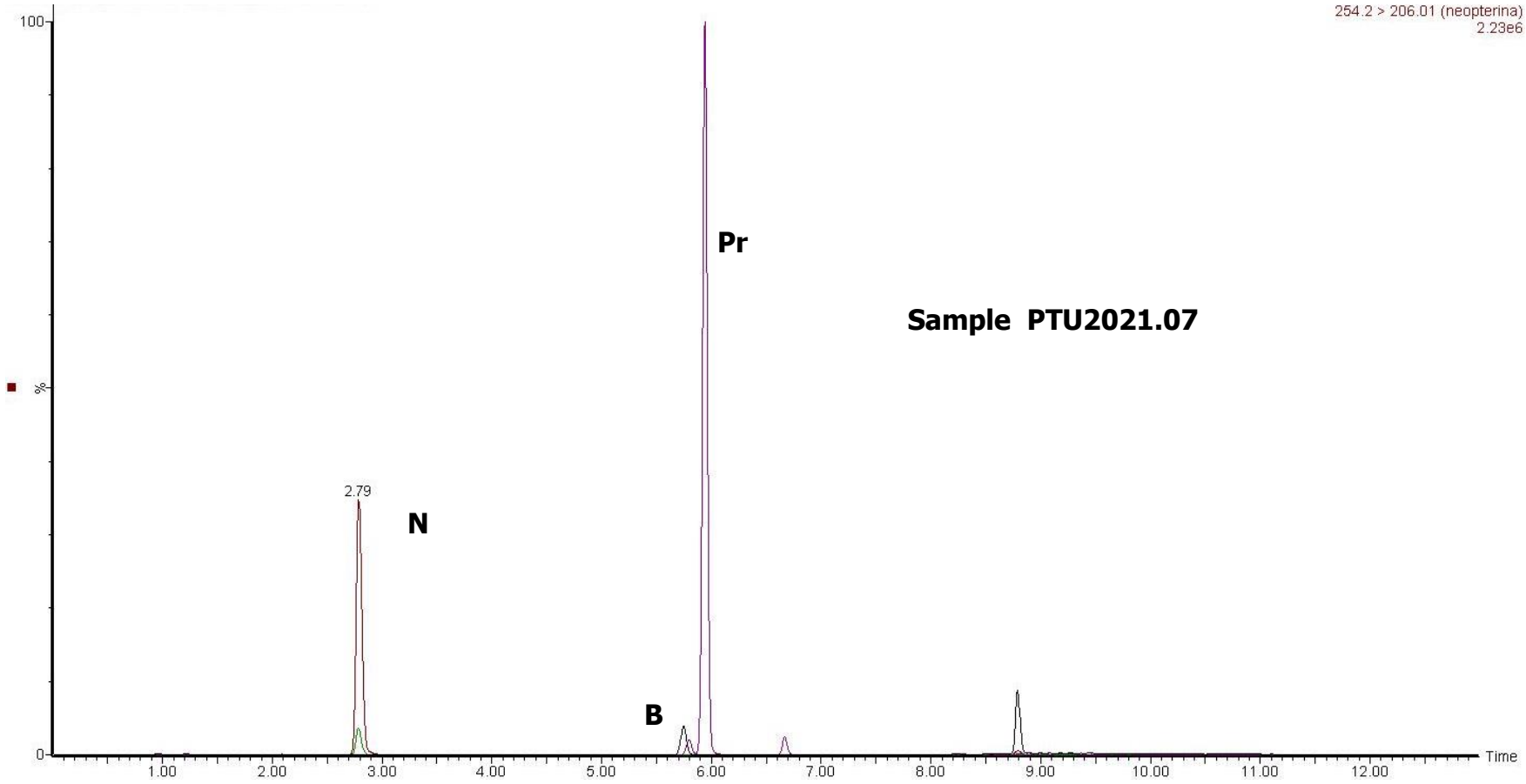
Biopterin



Neopterin



OXIDIZED PTERINS ANALYSED BY UPLC-ESI-MS/MS



DETERMINATION OF PTERINS IN DBS

Available online at www.sciencedirect.com
SCIENCE DIRECT
Molecular Genetics and Metabolism 86 (2005) S96–S103
Molecular Genetics and Metabolism
www.elsevier.com/locate/ymgme

Screening for tetrahydrobiopterin deficiencies using dried blood spots on filter paper

Marcel R. Zurflüh [a], Marcello Giovannini [b], Laura Fiori [b], Betina Fiege [b], Yasemin Gokdemir [c], Tolunay Baykal [c], Lucja Kierat [a], Konrad H. Gärtner [a], Beat Thöny [a], Nenad Blau [a,*]

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[c] Istanbul Faculty of Medicine, Children's Hospital, Department of Nutrition and Metabolism, Istanbul, Turkey

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Available online 7 November 2005

J Inherit Metab Dis (2011) 34:819–826
DOI 10.1007/s10545-011-9300-1

ORIGINAL ARTICLE

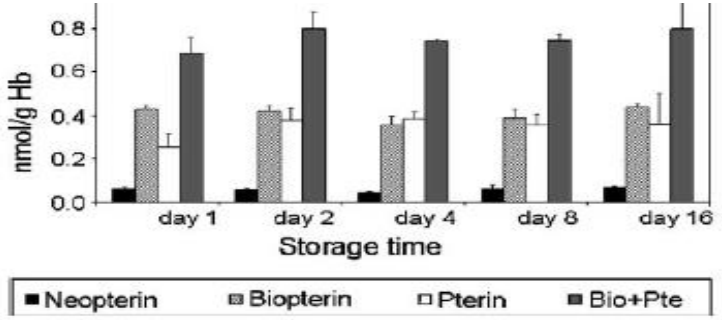
Diagnosis of tetrahydrobiopterin deficiency using filter paper blood spots: further development of the method and 5 years experience

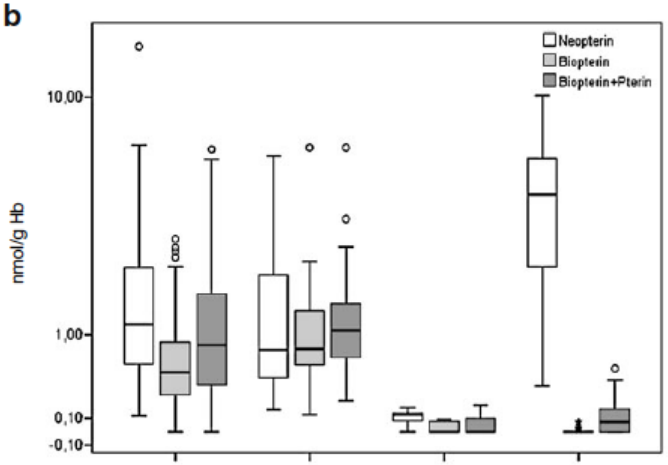
Thomas Opladen · Bettina Abu Seda · Anahita Rassi · Beat Thöny · Georg F. Hoffmann · Nenad Blau

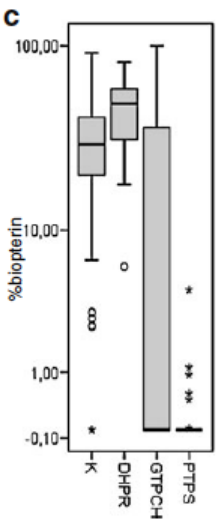
- DBS samples collection, handling and conservation procedures are by far more convenient if compared to urine: samples are stable at room temperature up to 16 days
- The method is based on the determination of fully oxidised N and B (and Pt) (originally present in the sample and produced by natural oxidation)
- Sensitivity:

  GTP-CH N 100% (urine 100%) - B 71% and 100% expressed as nmol/g Hb (urine 100%)

  PTPS N 44% (urine 95%) – B 85.5 and B 96% expressed as nmol/g Hb (urine 100%)







DETERMINATION OF B AND N IN DBS BY UPLC-ESI-MS/MS

Clinica Chimica Acta 466 (2017) 145–151

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Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Development of a new UPLC-ESI-MS/MS method for the determination of biopterin and neopterin in dried blood spot

Silvia Santagata [a], Emanuele Di Carlo [c], Carla Carducci [a], Vincenzo Leuzzi [b], Antonio Angeloni [c], Claudia Carducci [a,*]

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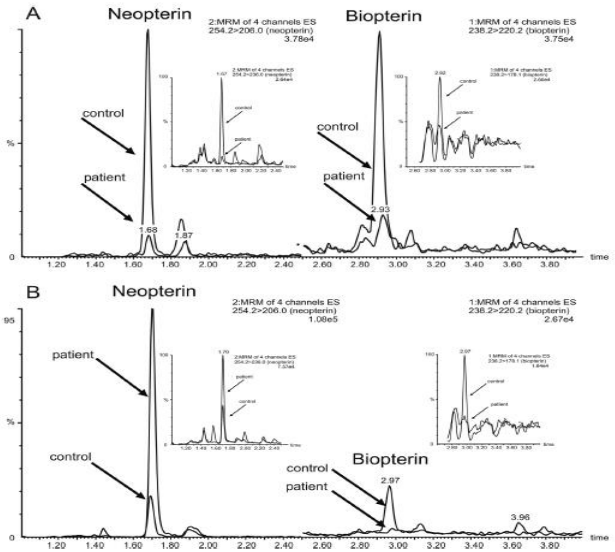
[b] Department of Pediatrics, Child Neurology and Psychiatry, Sapienza University of Rome, Via dei Sabelli 108, 00185 Rome, Italy
[c] Department of Molecular Medicine, Sapienza University of Rome, Viale del Policlinico 155, 00161 Rome, Italy



A -GTPCHd deficiency (Neo 3.8 nmol/l, Bio 5.2 nmol/l)
B -PTPS deficiency (Neo 128.1 nmol/l, Bio 0.8 nmol/l)

1. **Extraction in 150 µl of 13C5-Neopterin, D3-Biopterin (50 nmol/l) in HCl 20 mmol/l by sonication for 10 min**
2. **ultra-filtration using Nanosep filter at 15000 g for 15 min**
3. **10 µl were injected**

2 spots , 6 mm diameter

Column: ACQUITY UPLC HSS T3 1.8 µm 2.1 × 50 mm (40° C)
Mobile phase A: water 0.2% formic acid
Mobile phase B: methanol 0.2% formic acid
Flow rate: 0.3 ml/min

Table 2
Neo and Bio concentration in DBS of patients affected by PTPS, GTP-CH (dominant form), DHPR, HPA and normal controls.

	PTPS[a]	PTPS[b]	DHPR[c]	GTP-CH[d]	HPA[e]	Controls		
N of subjects	6 (23 samples)	1	2	3	15	98	42	30
Median age	11 y	7 m	4 y	10–18 y	14 y	3 d	29 d	12 y
(min-max)	(7 m-41 y)		13 y		(3–52 y)	(2–4 d)	(16–55 d)	(2–39 y)
Bio nmol/l	40.9 ± 31.3	<LoQ	**57.5**	19.0	29.0 ± 17.0 (6.7–64.4)*	17.5 ± 3.9	16.2 ± 4.1	15.2 ± 5.7 (8.8–26.9)
(2.5–97.5%ile)	(12.7–123.3)		15.9	**6.7**		(6.8–24.2)	(8.0–24.4)	
				5.2				
				5.9				
Neo nmol/l	43.7 ± 30.3	**128.1**	22.1	**0.6**	32.1 ± 9.5 (17.2–57.2)*	64.6 ± 20.0 (28.7–105.7)	31.8 ± 11.8 (15.8–62.6)	22.5 ± 10.0 (8.1–39.5)
(2.5–97.5%ile)	(9.2–147.7)		10.5	**6.6**				
				3.8				
				6.5				

*Min-max; single values out of control ranges are in bold; [a]supplemented with BH4; [b] before therapy; [c]under therapy; [d]autosomal dominant form; [e]Phe 115-1587 µmol/l of blood.

ANALYSIS OF REDUCED FORMS

- Most of pterins is present as reduced forms (about 70% of total N in CSF were present as NH2; about 80% of the biopterins in plasma is present as BH4)
- They are important for the study of BH4 homeostasis (increase in the BH2/BH4 ratio leads to NOS dysfunction of cardio-vascular system)
- Important for pharmacokinetic / pharmacodynamic studies (HPA treatment with sepiapterin* and sapropterin[§])
- Reduced forms could be more sensitive for the diagnosis of BH4 deficiencies (could BH2 be a good marker for DHPR deficiency?)

*N. Smith, N. Longo, K. Levert, K. Hyland, N. Blau. Phase I clinical evaluation of CNSA-001 (sepiapterin), a novel pharmacological treatment for phenylketonuria and tetrahydrobiopterin deficiencies, in healthy volunteers. Mol Genet Metab 126 (2019) 406-412

§ Fiege B, Ballhausen D, Kierat L, Leimbacher W, Goriounov D, Schircks B, Thöny B, Blau N. Plasma tetrahydrobiopterin and its pharmacokinetic following oral administration. Mol Genet Metab. 2004 Jan;81(1):45-51

DETERMINATION OF REDUCED FORMS: INDIRECT METHODS

Acidic and alkaline oxidation with I_2/KI:

- Acid iodine oxidation BH4 +BH2 $\rightarrow B_a$ (HPLC-FD analysis)
- Alkaline iodine oxidation BH2$\rightarrow B_b$ (HPLC-FD analysis)
- B_a-B_b= BH4

T. Fukushima, J.C. Nixon, Analysis of reduced forms of biopterin in biological tissues and fluids, Anal. Biochem. 102 (1980) 176–188.

DETERMINATION OF REDUCED FORMS: ELECTROCHEMICAL OXIDATION

- Reduced forms are electroactive compounds and could be detected by ECD
- To determine oxidated and reduced forms in one run, ECD could be coupled with FD
- BH4 is detected electrochemically, whereas BH2 and dihydroneopterin are measured via fluorescence after post-column coulometric oxidation into biopterin and neopterin.
- Electrochemical detection is difficult to perform in complex biological mixtures, and therefore its application focused mainly on cerebrospinal fluid, a relatively pure matrix.

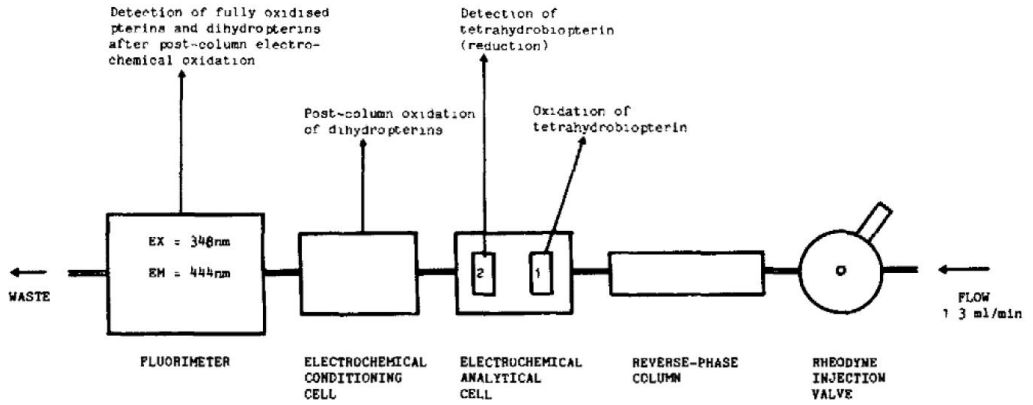


Fig. 1. Equipment configuration.

Howells DW, Smith I, Hyland K 1986 Estimation of tetrahydrobiopterin and other pterins in cerebrospinal fluid using reversed phase HPLC with electrochemical and fluorescence detection. J Chromatogr 381:285-29

DETERMINATION OF OXIDIZED AND REDUCED FORMS IN URINE BY UPLC-ESI-MS/MS

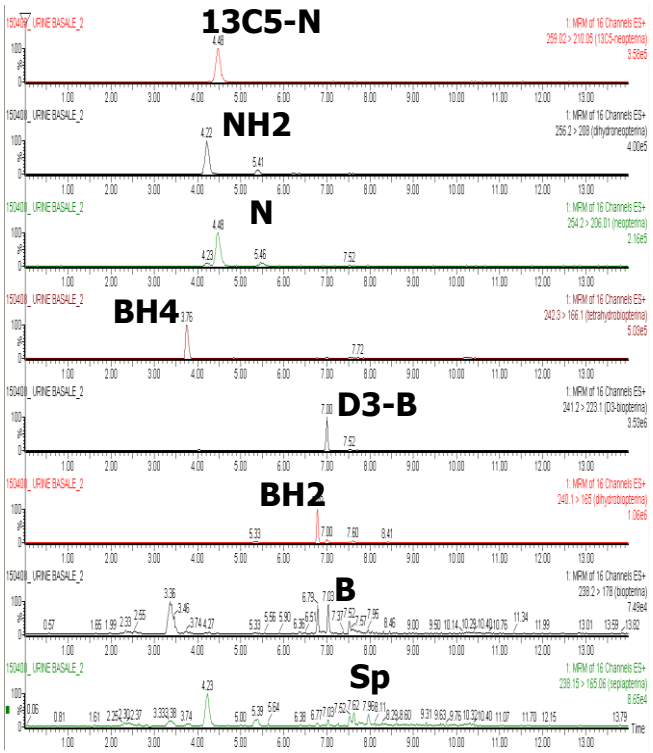
PROS

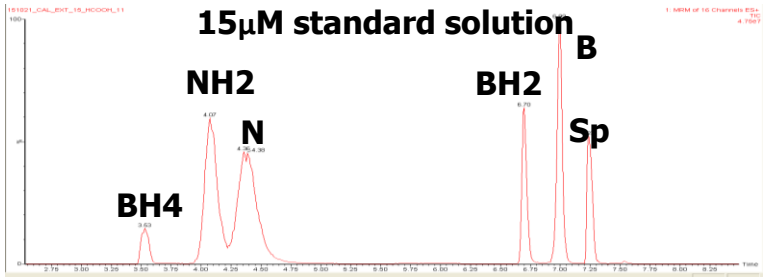
- Contemporary analysis of reduced and oxidized forms, Sp and Pr
- High sensitivity

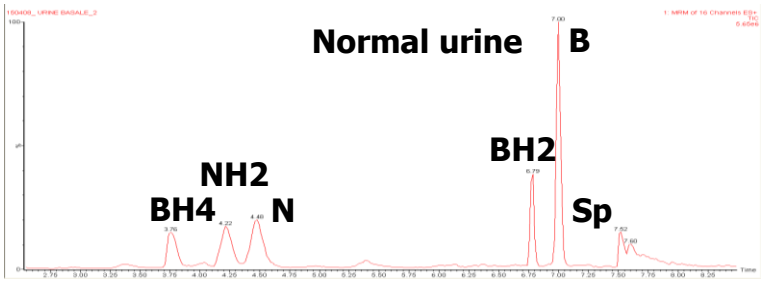
CONS

- Complexity and cost of the instrument
- Low availability of homologous standards labeled with stable isotopes
- Matrix effect (ion suppression)

Normal urine





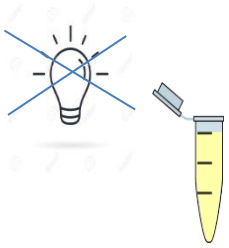


OPTIMIZATION OF THE SAMPLE PREPARATION PROCEDURE

Stability of BH_4

- 88% of a 32.13 µmol/L BH4 solution oxidized to BH2 and B within 20h at 4° C but no degradation was found if 5.7mmol/l ascorbic acid was added and gassing with argon; in this conditions 41.5 nmol/l BH4 solution was protected for 4h at 4° C*
- In biological samples oxidation of BH4 produces biopterin, BH2 and other pterins

Dithiothreitol (DTT) and ascorbic acid are added to prevent BH4 oxidation and impeding side-chain cleavage of reduced pterins.



100 µL urine

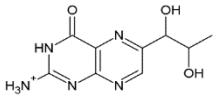
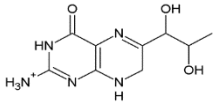
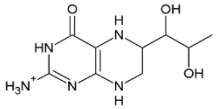
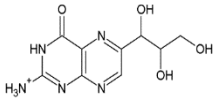
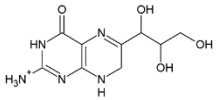
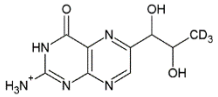
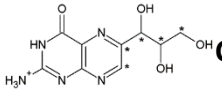
Dilution 1:10 con
H_2O (degassed)
18 mM DTT
0.4% (w / v) ascorbic acid
0.32% formic acid
0.5 µM internal standard
($^{13}C_5$-N, D_3-B,^{15}N-BH4)

Shake for 5 minuts

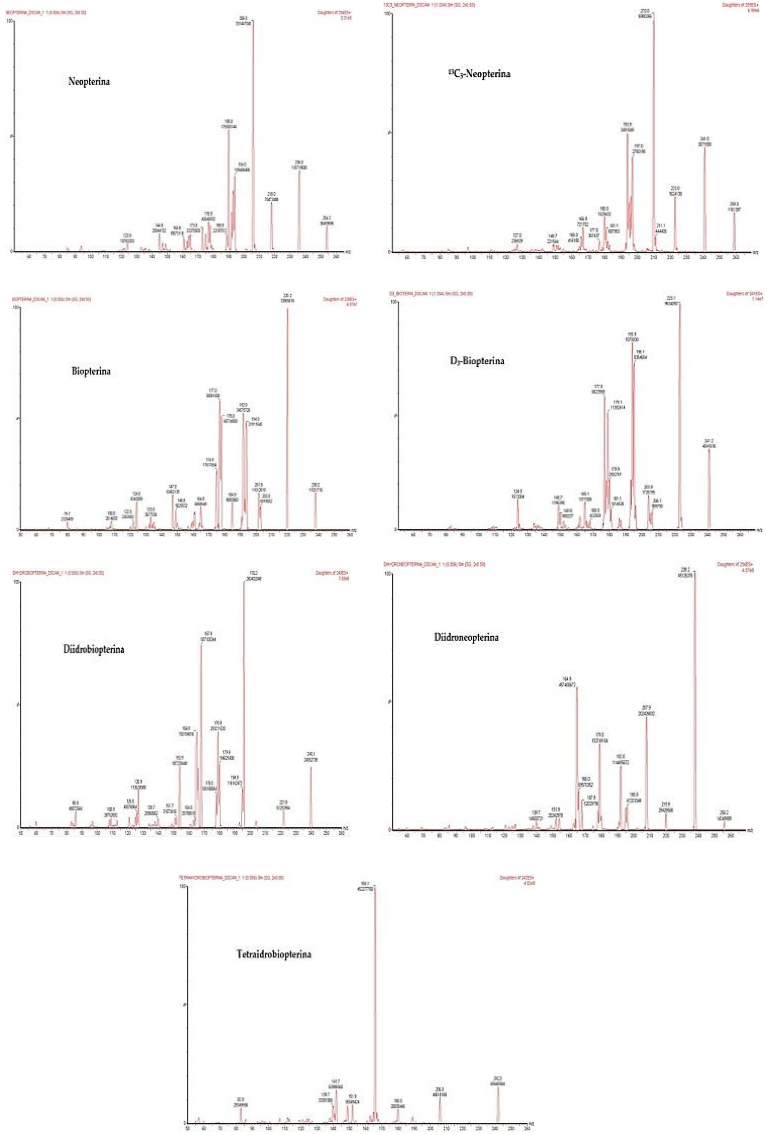
Inject 10 µL in HSS T3 C18 column

*Howells DW, Hyland K. Direct analysis of tetrahydrobiopterin in cerebrospinal fluid by high-performance liquid chromatography with redox electrochemistry: prevention of autoxidation during storage and analysis. Clin Chim Acta. 1987 Jul 30;167(1):23-30.

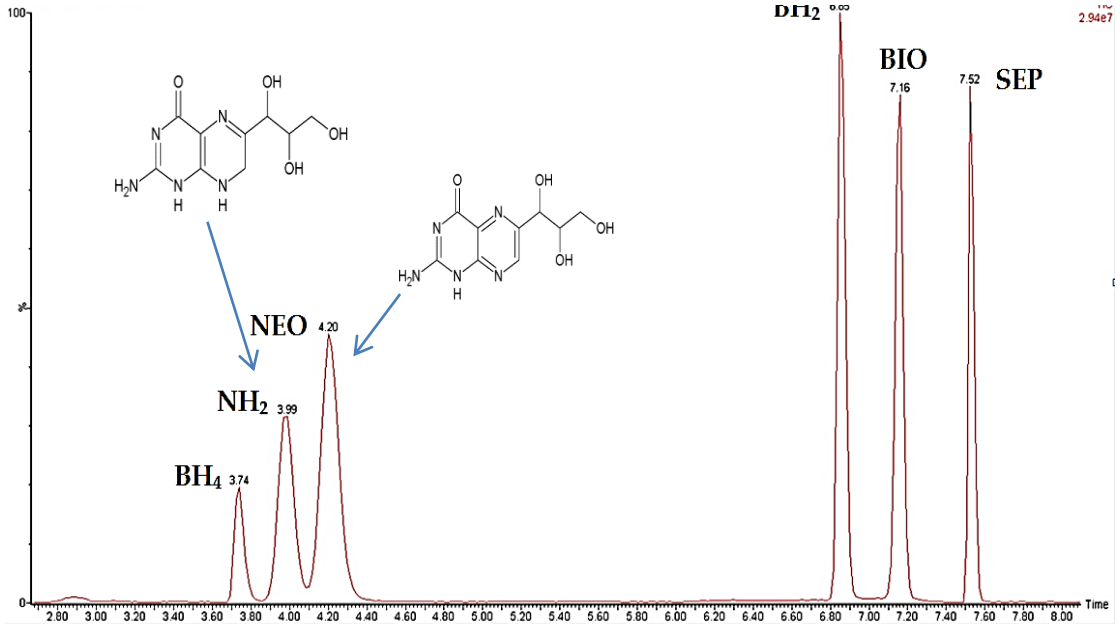
OPTIMIZATION OF MS/MS INSTRUMENTAL PARAMETERS

Compound	Parent ion	Chemical Formula	Molar mass	m/z Transitions
Biopterin (BIO)		$C_9H_{11}N_5O_3$	237.22	238.20 → 220.20
dihydrobiopterina (BH_2)		$C_9H_{13}N_5O_3$	239.23	240.10 → 196.20
tetra-hydrobiopterina (BH_4)		$C_9H_{15}N_5O_3$	241.25	242.30 → 166.10
Neopterin (NEO)		$C_9H_{11}N_5O_4$	253.22	254.20 → 206.01
dihydroneopterina (NH_2)		$C_9H_{13}N_5O_4$	255.23	256.20 → 238.20
D_3-Biopterin (D_3-BIO)		$C_9H_8D_3N_5O_3$	240.23	241.20 → 223.10
$^{13}C_5$-Neopterin ($^{13}C_5$-NEO)		$C_4(^{13}C)_5H_{11}N_5O_4$	258.18	259.02 → 210.05

Setting of MRM transitions: daughter scan



OPTIMIZATION OF CHROMATOGRAPHIC SEPARATION AND METHOD VALIDATION



Validation tests:

- **Linearity**
- **Recovery**
- **LoD**
- **LoQ**
- **Precision**
- **Matricx effect**
- **Carry-over**

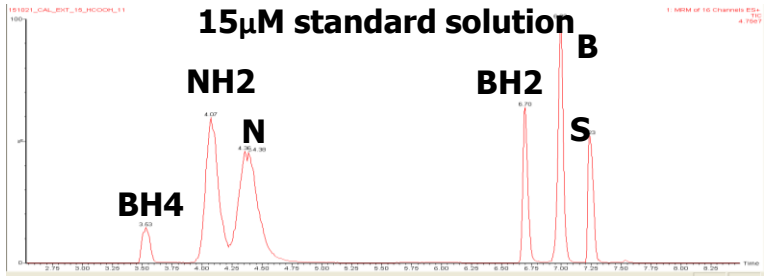
Mobil phase A
(99.8% H_2O, 0.2% HCOOH)

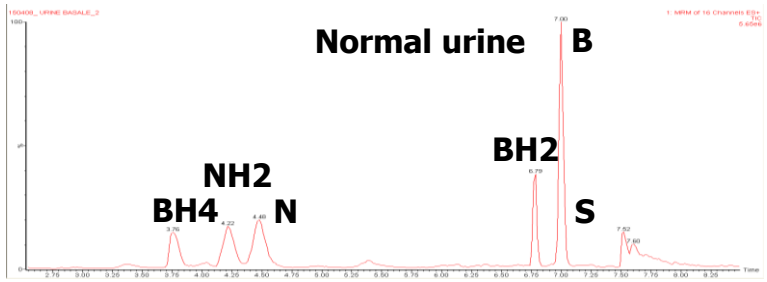
Mobil phase B
(99.8% CH_3OH, 0.2% HCOOH)

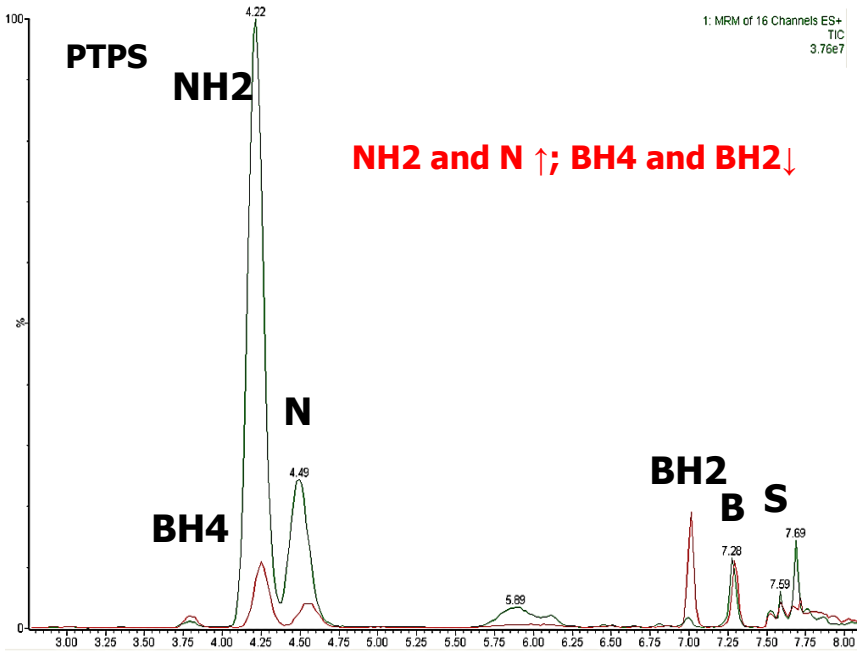
Column HSS T3 C18 (1,8 µm, 2,1x150 mm)

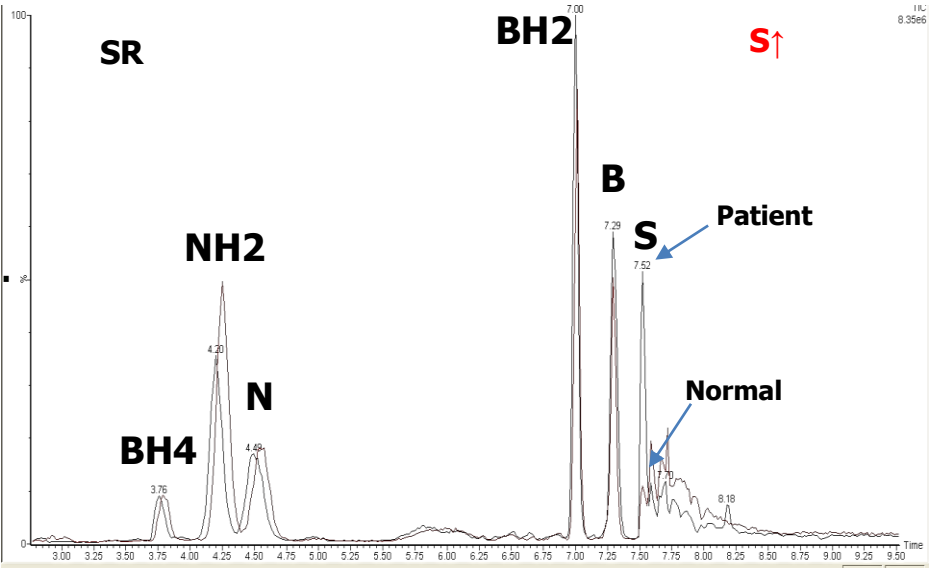
Flow rate 0.3 mL/min

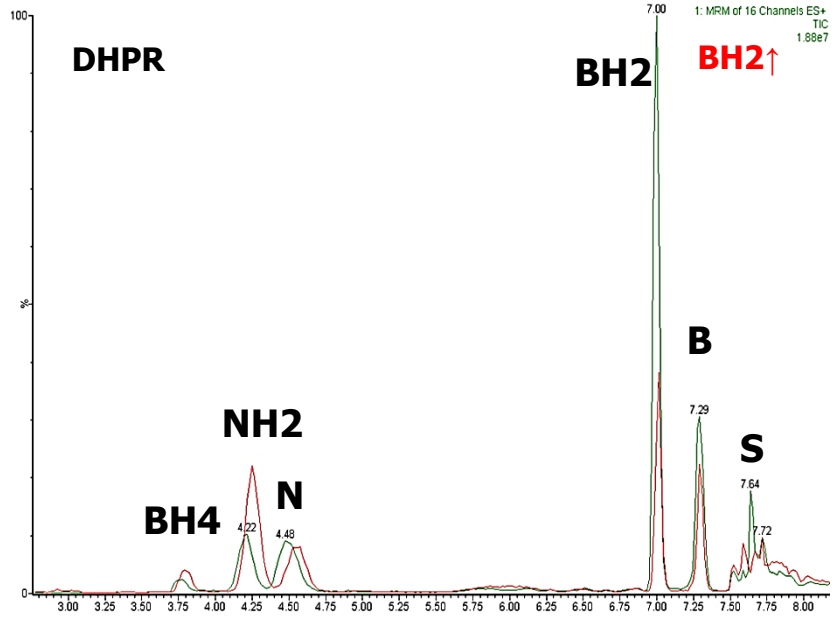
Gradient elution


15μM standard solution
NH2
BH2
B
N
S
BH4


Normal urine
B
BH2
NH2
BH4
N
S


PTPS
NH2
NH2 and N ↑; BH4 and BH2↓
N
BH2
BH4
B
S


SR
BH2
S↑
B
Patient
S
NH2
N
Normal
BH4


DHPR
BH2
BH2↑
B
NH2
N
S
BH4

Take-home messages PTU Workshop

- The determination of total **B, N** and **P** in **urine** by HPLC with fluorescence detection is reliable and robust, and it is the primary tool for the diagnosis of BH4 deficiencies.

- The implementation of the method for the analysis of **Sepiapterin** in urine is feasible.

- Although the analysis of **B** and **N** in **DBS** has demonstrated a lower sensitivity compared to urine, it could be a useful tool to decrease the age of diagnosis of patients affected by BH4 deficiencies

- The analysis of pterins in **CSF** is essential for the confirmation of BH4 deficiencies and for identification of different forms (severe v. mild).

- Particular attention should be paid in the pre-analytical steps: collection, conservation, deproteinization and adding of anti-oxidant agent.

UNIVERSITÄTS-
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ERNDIM
QUALITY ASSURANCE IN LABORATORY TESTING FOR IEM

Pterin Workshop

ERNDIM Virtual Meeting
October 21-22, 2021

Alessio Cremonesi, PhD

Aim of the PTU scheme

Aim:

- **To evaluate the ability of the participating laboratories to quantitatively analyse the concentrations of the 3 (+ creatinine) analytes* included in the scheme**
- **To educate and assess the ability of laboratories to diagnose inborn errors of BH4 metabolism**

Status:

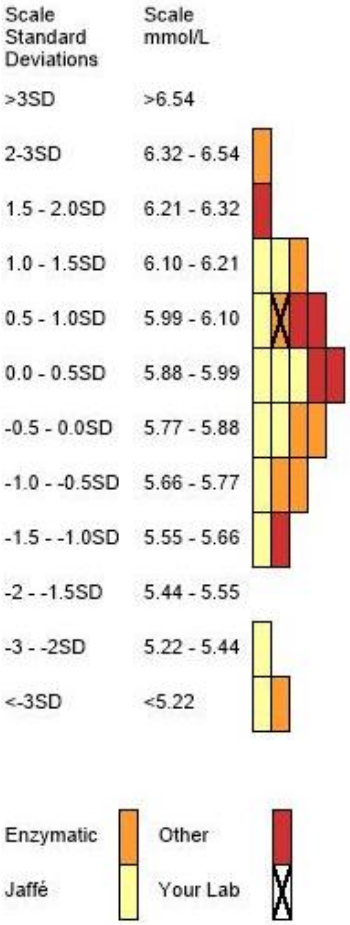
- **Pilot scheme: 2014 – 2016**
- **Full quantitative scheme: since 2017**

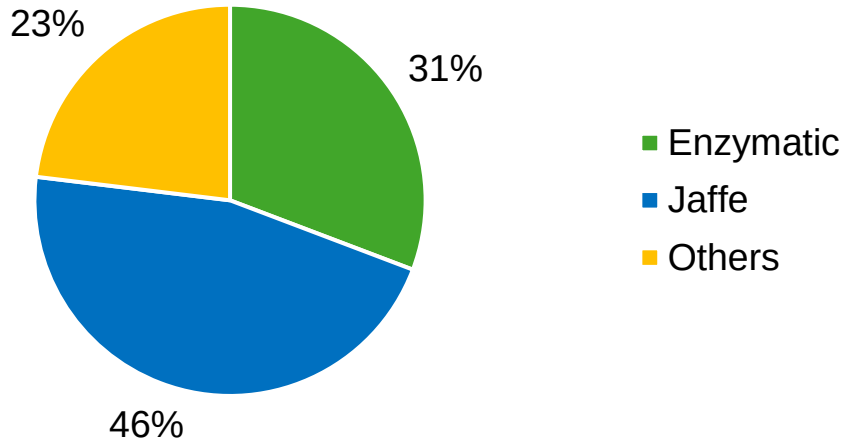
*** creatinine, biopterin, neopterin and primapterin**

Overview of the PTU scheme - 2021

Scheme type	Quantitative
Analytes	Biopterin (µmol/L & mmol/mol creat.) Neopterin (µmol/L & mmol/mol creat.) Primapterin (µmol/L & mmol/mol creat.) Creatinine (mmol/L)
Number of Specimens/Year	8
Volume/Specimen	1 mL/vial
Specimen type	Spiked lyophilised human urine
Geographic Area	Worldwide

Methodology – Creatinine





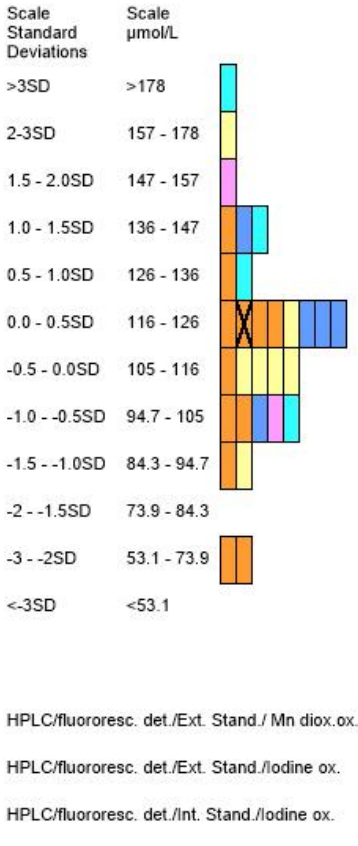
Brief Communication

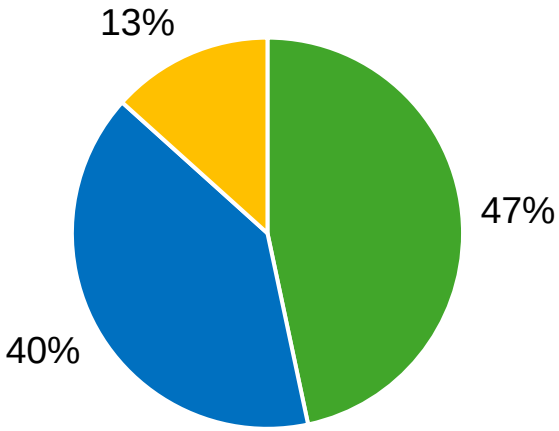
Homogentisic acid interference in routine urine creatinine determination

Perry R. Loken [a], Mark J. Magera [a,*], Wendy Introne [b], Silvia Tortorelli [a], Dimitar Gavrilov [a], Devin Oglesbee [a], Piero Rinaldo [a], Dietrich Matern [a], Kimiyo Raymond [a]

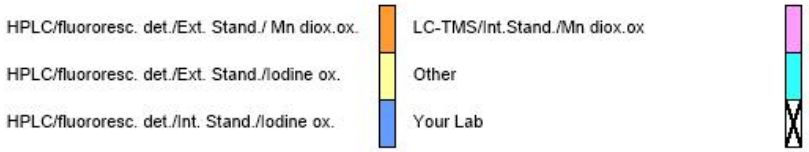
[a] Biochemical Genetics Laboratory, Mayo Clinic College of Medicine, Rochester, MN, United States
[b] National Human Genome Research Institute, National Institutes of Health, Bethesda, MD, United States

Methodology – Pterins





- HPLC-Fluorescence (MnO2)
- HPLC-Fluorescence (I2/KI)
- Others



Cycle review – The Z-Score

Analyte	Your Lab	Med All Labs	n	ZScore	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%
Biopterin (µmol/L)	7.51	7.69	25	0.1		7	1 6		2 3	4 5				
Biopterin (mmol/mol Creat)	1.30	1.40	26	0.4		7	6	1	2 3 5	4				
Creatinine	5.80	5.46	26	0.9						1 2	3 5	4 7	6	
Neopterin (µmol/L)	94.7	87.6	25	0.7					1 6	3 7		2 4 5		
Neopterin (mmol/mol Creat)	16.3	16.0	26	0.2				6	1 7	3	4 5	2		
Primapterin (µmol/L)	0	0	16						1 2 3 4 5 6 7					
Primapterin (mmol/mol Creat)	0	0	16					7	2 3 4 5 6	1				

- **It tells how many standard deviations a control result is from the mean value expected for that sample.**
- **It is calculated by taking the difference between the control result and the expected mean, then dividing by the standard deviation observed for that control material:**

$$Z = \frac{X - \mu}{\delta}$$

X = partecipant's reported result
µ = assigned value (mean of the group)
Δ = SD of the proficiency test

Z = 0	Perfect
-2.0 ≤ Z ≤ 2.0	Acceptable
-3.0 < Z < -2.0 2.0 < Z < 3.0	Questionable
Z ≤ -3.0 Z ≥ 3.0	Unsatisfactory

Cycle review – The % Classes

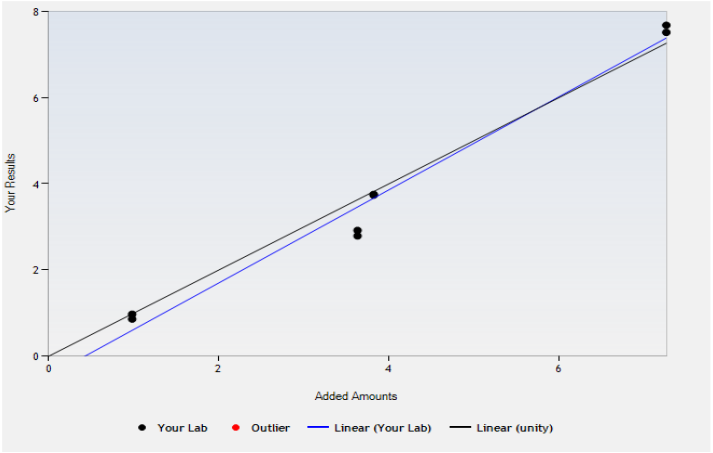
Analyte	Your Lab	Med All Labs	n	ZScore	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%
Biopterin (µmol/L)	7.51	7.69	25	0.1		7	1 6		2 3	4 5				
Biopterin (mmol/mol Creat)	1.30	1.40	26	0.4		7	6	1	2 3 5	4				
Creatinine	5.80	5.46	26	0.9						1 2	3 5	4 7	6	
Neopterin (µmol/L)	94.7	87.6	25	0.7					1 6	3 7		2 4 5		
Neopterin (mmol/mol Creat)	16.3	16.0	26	0.2				6	1 7	3	4 5	2		
Primapterin (µmol/L)	0	0	16						1 2 3 4 5 6 7					
Primapterin (mmol/mol Creat)	0	0	16					7	2 3 4 5 6	1				

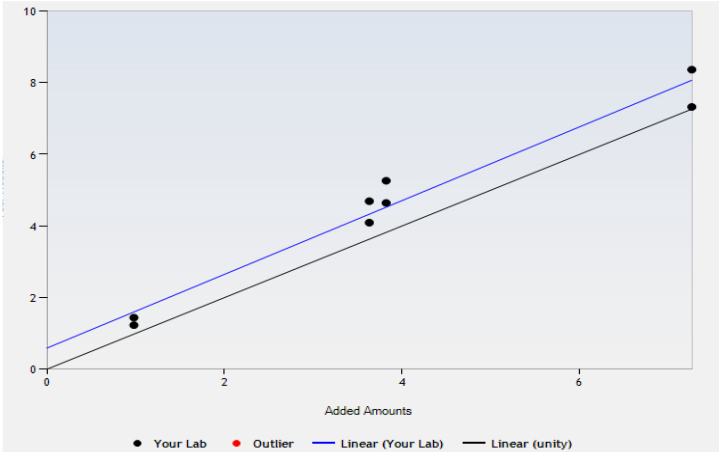
- **Grey box in class 10% class: result belongs to the 10% labs with the lowest outcome**
- **Grey box in the 100% class: result belongs to the 10% labs with the highest outcome**

Rule of thumb
If you have a grey box in the lowest (highest) class and many samples are also in that class you know that outcome of your lab is always too low (high) in comparison to the other labs → structural problem

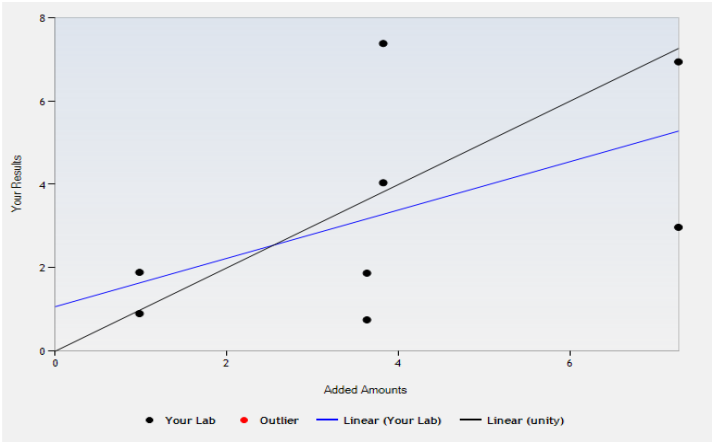
Some examples

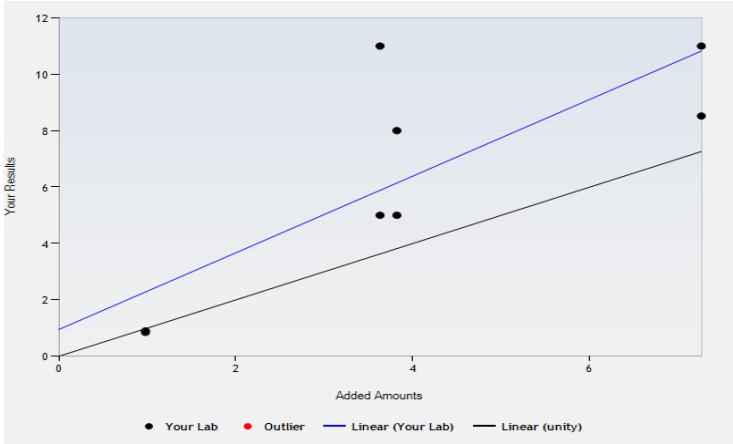
Good precision, correlation and recovery (slope)





Unsatisfactory precision, correlation and recovery (slope)





A short disgression on the scheme precision

2020	Biopterin			Neopterin		
	Mean [µM]	SD [µM]	CV [%]	Mean [µM]	SD [µM]	CV [%]
PTU 1	3.68	1.54	41.8	21.6	6.58	30.5
PTU 2	4.03	1.35	33.5	2.01	0.534	26.6
PTU 3	1.06	0.474	44.7	186	54	29.0
PTU 4	7.4	1.65	22.3	88.4	14.5	16.4
PTU 5	3.69	0.704	19.1	1.99	0.445	22.4
PTU 6	0.959	0.151	15.7	181	19.1	10.6
PTU 7	3.64	0.704	19.3	21.1	3.38	16.0
PTU 8	7.6	0.741	9.8	88.6	9.13	10.3

Observation: constant improvement in the precision during the course of the year!

→ Samples must be measured independently and the obtained concentrations from each pair must not be slightly adapted

Annual Report 2020 - Overview

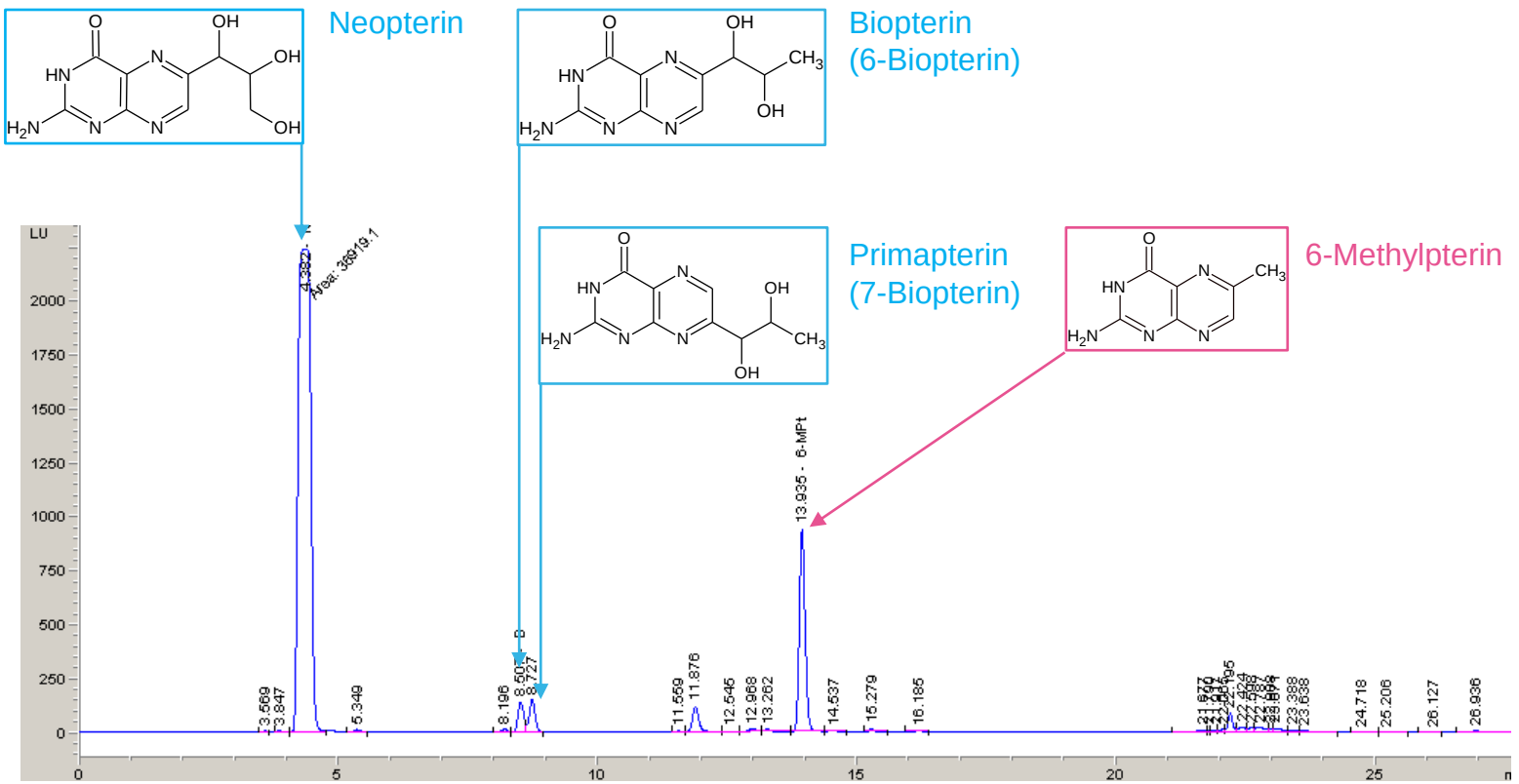
Partecipants: 32 laboratories (28 submissions)

Sample	Abnormalities	Diagnosis
1 and 7	↑ Primapterin	pterin-4a-carbinolamine dehydratase (PCD) deficiency
2 and 5	-	Healthy person Non-BH4 deficiency
3 and 6	↑ Neopterin ↓ Biopterin	6-pyruvoyl-tetrahydropterin synthase (PTPS) deficiency
4 and 8	↑ Neopterin	a non-BH4 deficiency

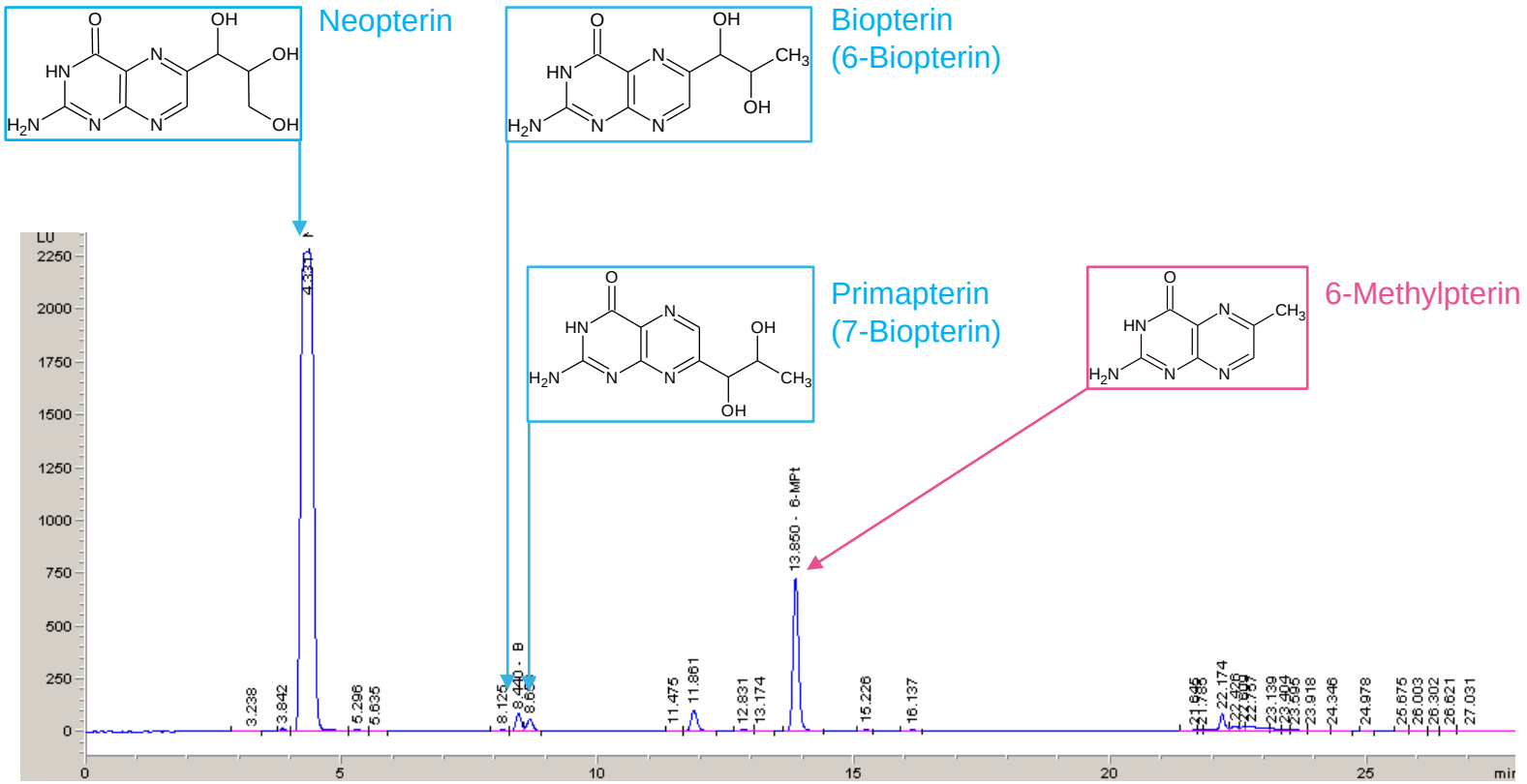
Annual Report 2021 - Overview

Sample	Abnormalities	Diagnosis
1	↑ Primapterin n↓ Neopterin n↓ Biopterin	PCD deficiency GTPCH deficiency
2	↑ Primapterin ↑ Neopterin ↓ Biopterin	PCD deficiency PTPS deficiency
3	↑ Primapterin n↑ Neopterin n↑ Biopterin	PCD deficiency PTPS deficiency on treatment with BH4
4	↑ Primapterin ↑ Neopterin ↓ Biopterin	PCD deficiency PTPS deficiency
5	↑ Primapterin ↑ Neopterin ↓ Biopterin	PCD deficiency PTPS deficiency
6	↑ Primapterin n↓ Neopterin n↓ Biopterin	PCD deficiency GTPCH deficiency
7	↑ Primapterin n↑ Neopterin n↑ Biopterin	PCD deficiency PTPS deficiency on treatment with BH4

Samples 2 : PCD- or PTPS deficiency

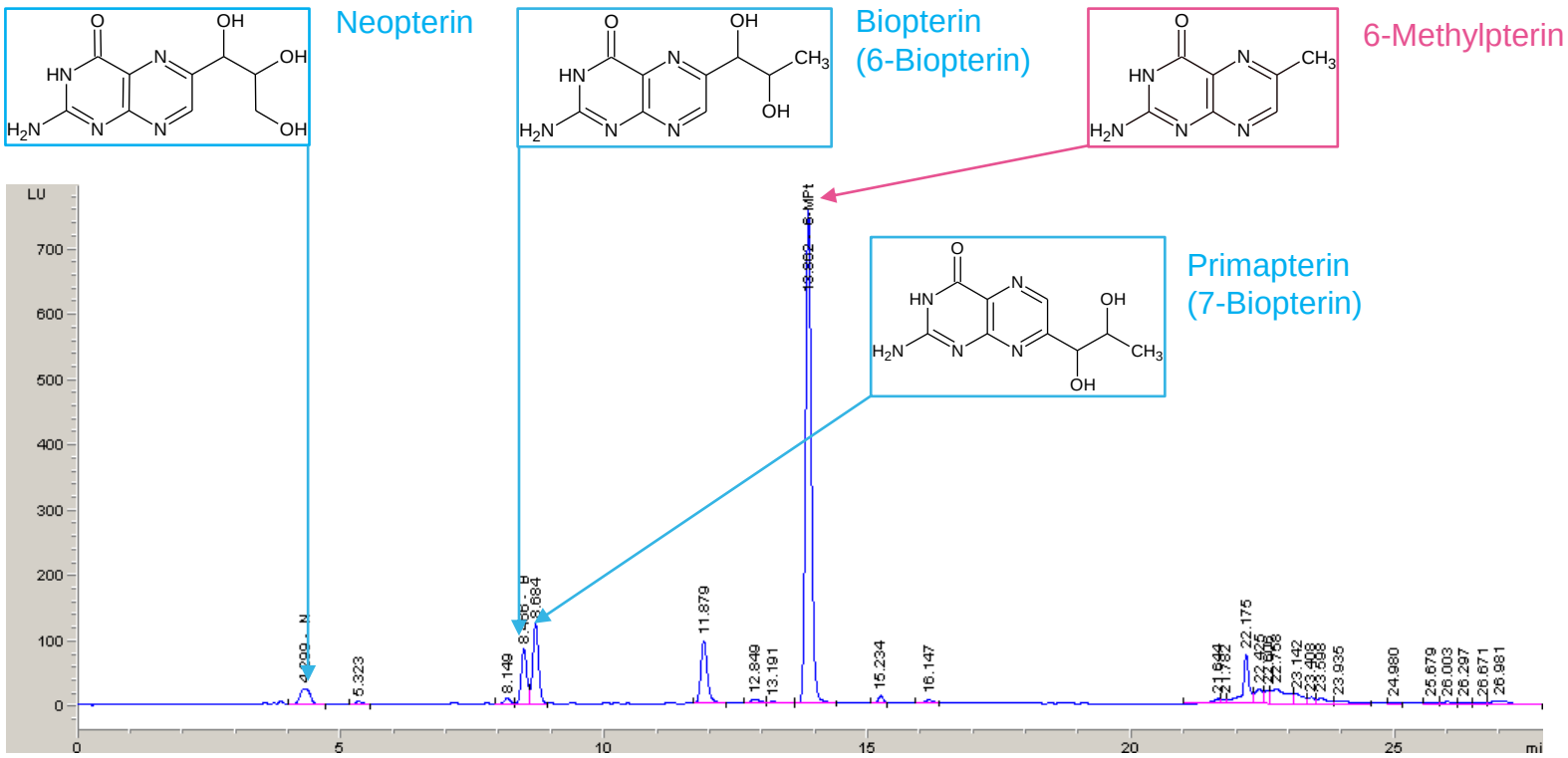


Samples 4 and 5: PCD- or PTPS deficiency



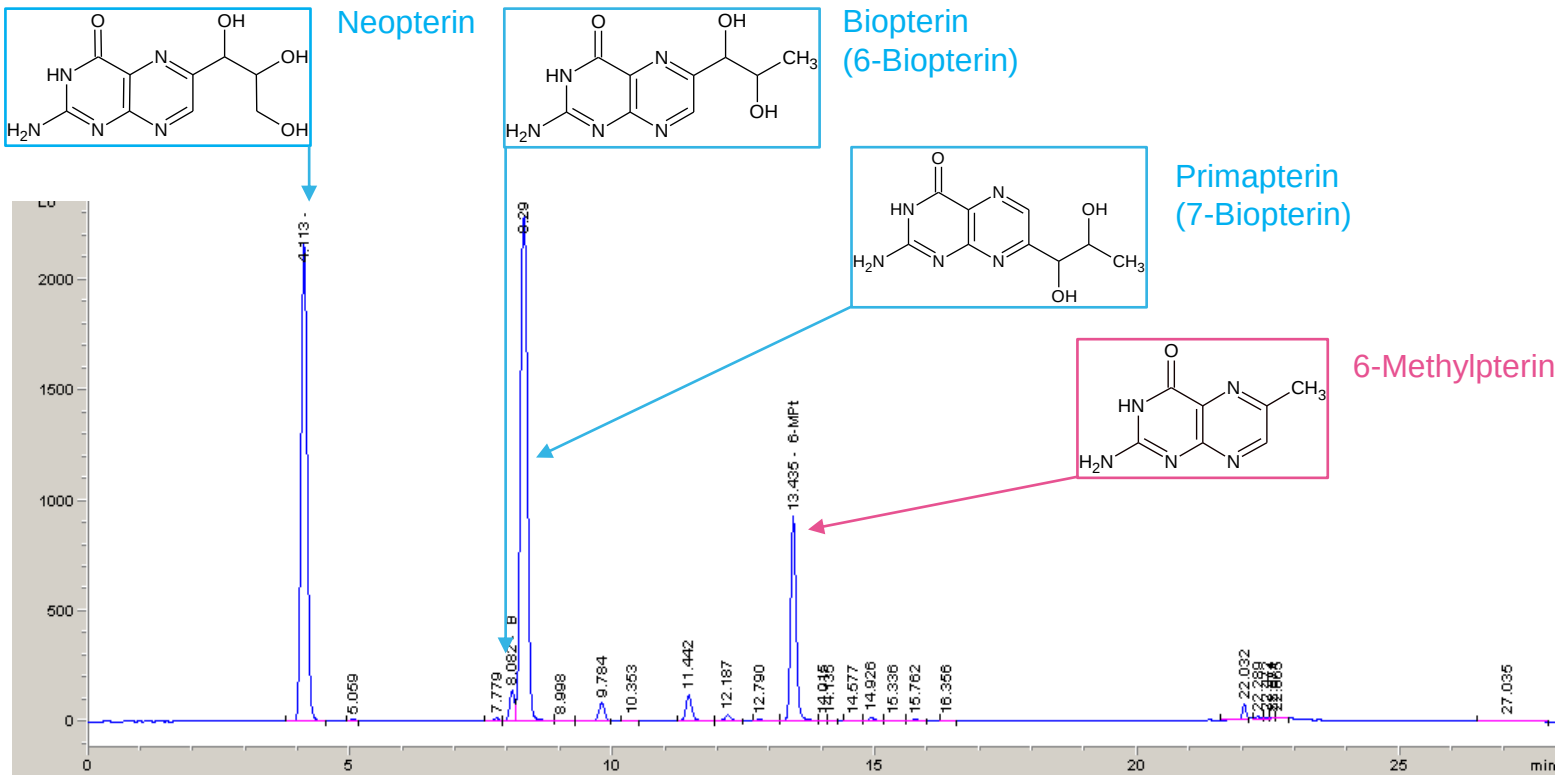
↑ **Primapterin**
↑ **Neopterin**
↓ **Biopterin**

Samples 1 and 6: PCD- or GTPCH-deficiency



↑ Primapterin
n↓ Neopterin
n↓ Biopterin

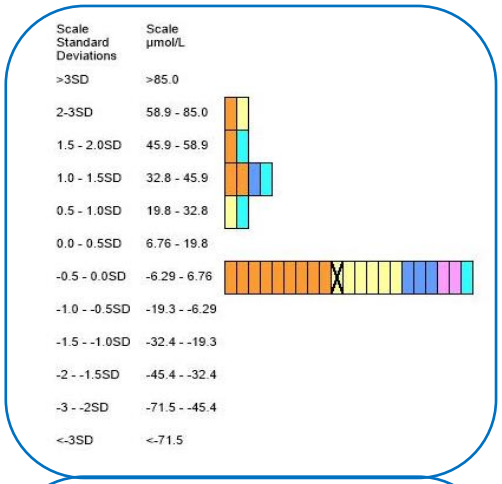
Samples 3 and 7: PCD deficiency or PTPS deficiency on treatment with BH4

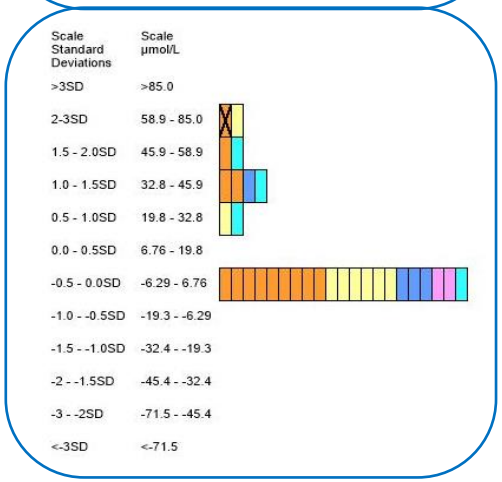


↑ Primapterin
n↑ Neopterin
n↑ Biopterin

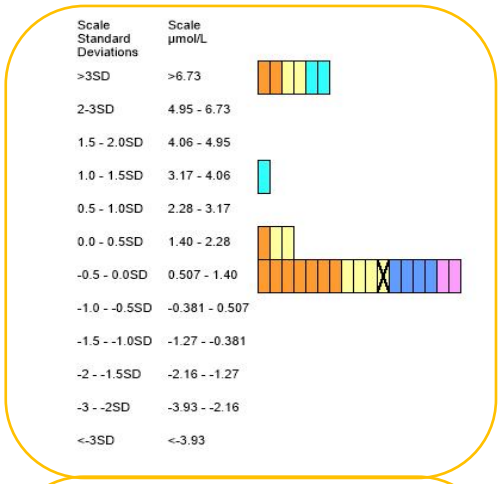
Primapterin measurement can be challenging

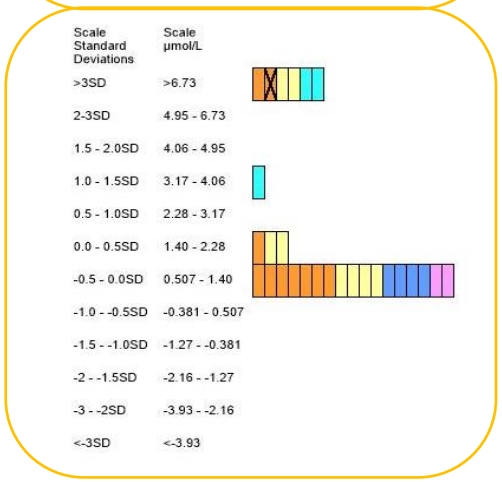
Bio in PTU 3



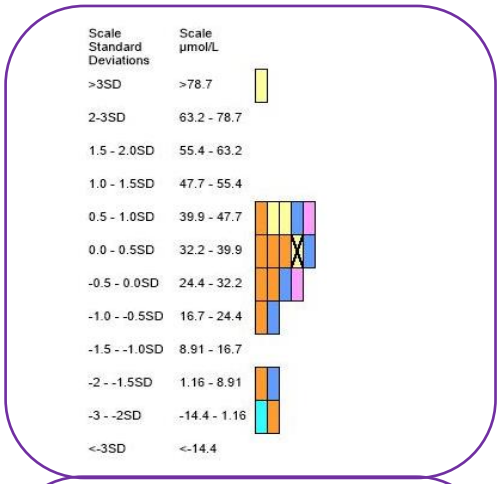


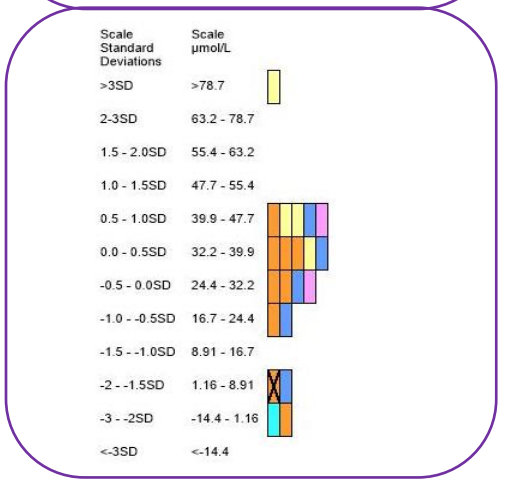
Bio in PTU 7





Prima in PTU 7





The chromatographic separation of biopterin and primapterin is challanging!

Summary PTU 2020 & 2021

- Since 2017 full quantitative scheme involving ~30 laboratories worldwide
- Methodology:
  - HPLC-Fluor (MnO_2) ≈ HPLC-Fluor (I_2/KI) >> LC-MS/MS
- Only 19/27 laboratories reported a value for Primapterin
- Accuracy:
  - Recovery = 97% (Prima) – 104% (Bio)
- Precision:
  - CV = 9.3% (Neo) – 19.2% (Prima)
  - CV = 2.1% (Crea)
- Linearity:
  - r = 0.941 (Bio) – 0.998 (Prima)
- Interlab precision:
  - CV = 24.3% (Neo) – 70.1% (Prima)
  - CV = 6.0% (Crea)

- Due to manufacturing problems, the analyte constellations of the 2021 scheme were sometimes misleading
  - The scoring system was adapted to not penalize the laboratories

Take-home messages PTU Workshop

- **Sample pairs should be measured and quantified as separate and independent samples**

- **All laboratories should strive to detect and quantify primapterin in urine (strong association between PCD deficiency and early onset diabetes!)**

- **Good chromatographic resolution is necessary to resolve biopterin and primapterin from each others**

Case presentation

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Biochemical Genetics

Laboratory
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Case #1 - 1 month 25 days old

Tests conducted	Date sample taken	Results
Newborn Screening (NBS)	08/17/21, 2nd DOL	NORMAL. But because patient is on NPO, repeat sample was done.
2nd Newborn Screening	08/28/21, 13th DOL	on TPN, OGT feeding with breastmilk; Elevated Phe: 309.6 (<250); Phe/Tyr: 7.0 (<4.80)
Plasma amino acid	09/5/21, 21st DOL	NORMAL Phe: 43 (38-137); Not consistent with PKU, hyperphenylalaninemia, PAH deficiency, biopterin cofactor biosynthesis or regeneration defect.
Urine pterin (tests done in a laboratory in Taiwan)	09/4/21, 20th DOL	Normal Neopterin: 5.86 (NV < 7days: 1.2-2.93 | NV 2m-6m: 0.9-7.49); Low Biopterin: 0.47 (NV < 7days: 0.43-1.92 | NV 2m-6m: 1.73-3.68); Low B%: 7.5 (NV < 7 days: 19.8-50.3 | NV 2m-6m: 26.2-68.4); (B+I)% - 11.6 (NV < 7days: >10 | NV 2m-6m: >15)
DHPR (tests done in a laboratory in Taiwan)	09/4/21, 20th DOL	= 5.1; Normal control – 6.0; Deficient control – <0.1; (reference for neonates: 5.2-11.5); The patient may be a PTPS deficiency.

Case #1

- Baby is term 39 weeks, BW: 4900g, LGA and mother had difficulty during delivery. Baby was admitted managed as Meconium aspiration pneumonia, Perinatal asphyxia, LGA, Sepsis, Brachial nerve palsy, R. He was discharged improved on Sept 9, 2021. On recall, baby is asymptomatic except for the brachial nerve palsy and still cannot move her right arm.

- For this case, there should have been a repeat Phe level after 48hours off TPN but due to the result of the urine pterins, plan to do mutational analysis. A repeat DBS was collected last Oct 8, still awaiting results if elevated Phe or normal.