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ORIGINAL ARTICLE

Diagnosis Of Porphyria By Measuring Metabolites Of Heme Biosynthesis In Correlation With Clinical Findings

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ABSTRACT

Background: Porphyria is a group of genetic diseases which require specialized laboratory facilities for accurate diagnosis .Facilities for diagnosis of Porphyria have been established in our institution since 1996 with few biochemical investigations in correlation with clinical findings.

Materials And Methods: Quantification of urinary total porphyrins was done by Double beam computerized spectrophotometer. Urine porphobilinogen (PBG) is screened by Hoesch test (1) and confirmed by Watson-Schwartz test (2). Presence of PBG in urine was further confirmed by wavelength scanning by spectrophotometer.

Results And Discussion: Normal value of total porphyrins in urine: <35nmol/µmol of creatinine. In last 12 years, we have diagnosed four cases of Porphyria. The two cases were diagnosed as Acute Intermittent Porphyria (AIP) on the basis of increased levels of urine total porphyrins, increased urine PBG with symptoms of acute neurovisceral attack. In one case, there was increased level of total urine porphyrins up to 91.6 nmol/µmol of creatinine with history of photosensitivity, skin bullae with scars since childhood. The case was diagnosed as Congenital Erythropoetic Porphyria (CEP). In the other one, urinary total porphyrins was increased up to 1695 nmol/µmol of creatinine and urine PBG was absent with signs of cutaneous manifestations due to precipitating factors. This case was diagnosed as Porphyria Cutanea Tarda (PCT).

Conclusion: Any Biochemistry Laboratory having spectrophotometer can diagnose porphyria with clinical correlation.

Keywords: Biochemical investigations, clinical correlation, Porphyria diagnosis.

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Introduction

Porphyria is the group of genetic diseases resulting due to defect in heme biosynthesis. Defect in each enzyme gives rise to different porphyria with variable clinical manifestations.

Pinpoint diagnosis of porphyria requires well equipped laboratory facility but few front line laboratory investigations should be done whenever clinical findings suggest porphyria. [Table/Fig 1]

Aims and Objectives

To diagnose porphyria by measuring metabolites of heme biosynthesis in correlation with clinical features.

Materials and Methods

Simple methods using technique of spectrophotometry have been used in the study for initial investigations of the patients who are clinically suspected for porphyria.

Instruments

1. Spectrophotometer (ELICO, Model No. BL-198)
2. Centrifuge
3.Vortex mixer

(Table/Fig 1) Laboratory and clinical findings that we follow to diagnose porphyria cases (1)

Porphyrino-pathies	Enzyme defect	Precipitating factors	Laboratory Findings			Inheri-tance	Clinical Feature
			blood	Urine	Faeces		
Hypochromic anaemia	ALA dehydratase / PBG Synthase (E.C. 4.2.1.24)	Exposure to harmful chemicals & drug lead poisoning	Zn-proto porphyrins	ALA	absent	AR	Acute abdominal pain,acute motor polyneuropathy.
Acute intermittent porphyria (AIP)	PBG deaminase / Hydroxymethyl-bilane synthase (E.C. 2.5.1.61)	Anytime after puberty	absent	PBG	absent	AD	Severe acute abdominal pain, sympathetic over activity due to axonal degenerations, seizure, anxiety, disorientation, hallucination
Congenital erythropoetic porphyria (CEP)	Uroporphyrinogen-III synthase / Uroporphyrinogen synthase (E.C. 4.2.1.75)	(Symptoms persist throughout life).	Coproporphyrin-I &Uroporphyrin-I	Uroporphyrin-I, Coprophyrin-I increase total porphyrins	Coproporphyrin-I	AR	Erythrodontia (reddish brown discolored teeth), fragile skin, bullae, crust, scars, infection, mutilating deformity, deformities of cornea
Porphyria cutanea tarda (PCT)	Uroporphyrinogen-III decarboxylase (E.C. 4.1.1.37)	Hepatic iron overload, HIV, HCV infections, alcohol intake.	absent	Uroporphyrinogen-III, increased total porphyrins.	Coproporphyrinogen-I, Uroporphyrin-III	15-20% AD	Fragility bullae, scars, infections over sun exposed areas
Hereditary coproporphyria (HCP)	Coproporphyrinogen oxidase (E.C. 1.3.3.3)	Prevalent in females' fasting, OC pills, leuteal phase of menstrual cycle.	absent	PBG, coproporphyrin-III	Coproporphyrin III	AD	Acute neurovisceral attack, cutaneous fragility of sun exposed areas, photosensitivity
Variegate porphyria (VP)	Protoporphyrinogen-IX oxidase / Protoporphyrinogen oxidase (E.C. 1.3.3.4)	Steroid, nutritional alterations.	absent	PBG, ALA, uroporphyrins.	Proto-porphyrin , Copro-porphyrin-III	AD	Acute neurovisceral attack,cutaneous fragility photosensitivity of sun exposed areas, hyperpigmentation of skin.
Erythropoetic porphyria(EP)	Ferrochelatase (E.C. 4.99.1.1)	Absent	Protoporphyrin	Absent	Proto-porphyrin s-IX	AD	Solar urticaria, nail changes, sideroblastic anaemia

Reagents

1. Ehrlich's reagent (Hoesch test solution): 2 gms of p-dimethylaminobenzaldehyde in 100 ml of 6 M, HCl. Stable for 09 months at room temperature [1].
2. Ehrlich's reagent (Watson-Schwartz test solution): 0.7 gm of p-dimethylaminobenzaldehyde in 150 ml of concentrated HCl, add 100 ml of water and mix [1].
3. Saturated solution of sodium acetate
4. Chloroform
5. Butanol
6. Creatinine estimation kit by Jaffe's kinetic method

Specimen Collection and Stability

All samples must be protected from light; urinary porphyrin concentrations can decrease by up to 50% if kept in the light for 24 hours. Urinary PBG and porphyrins are best analysed in a fresh, random sample (10–20 ml) collected without preservative [Table/Fig 2]. Twenty four hour collections offer little advantage, delay diagnosis, and increase the risk of losses during the collection period. PBG and porphyrins are stable in urine in the dark at 4°C for up to 48 hours and for at least a month at −20°C. Very dilute urine (creatinine < 4 mmol/litre) is unsuitable for analysis [Table/Fig 2].

(Table/Fig 2) Pinkish colored urine suggesting the presence of porphyria

Procedure for Performing the Tests

PBG in urine

Screening test for PBG

Hoesch Test: was used as a screening test for PBG [1]. 2ml of Ehrlich's reagent was placed into a test tube then overlayed with 3 drops of urine. If .PBG was present, an immediate cherry red color developed at the point of mixing . False positive test may be encountered by the presence of indicator dyes derived from foods, soft drinks, medicines etc [3] [Table/Fig 3].

(Table/Fig 3) Hoesch test positive showing cherry red color at the point of mixing

Confirmatory Test for PBG

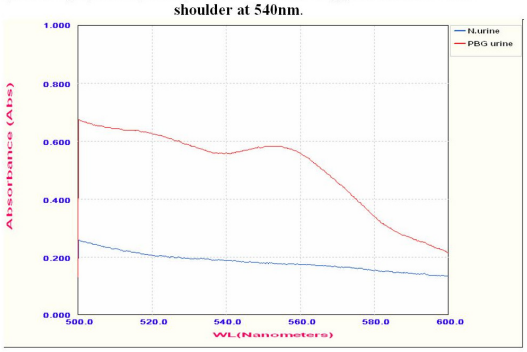
Watson-Schwartz Test was used as a confirmatory test for PBG [1]. This test was performed only in Hoesh test positive samples In 2 ml of urine 2ml of Ehrlich's reagent was added and mixed well, then mixed with 4ml of

saturated sodium acetate solution. The pH of the reagent was set in between to 4-5 with narrow range pH indicator paper. Now, 3.0 ml of chloroform was added and vortexed for 1 min. The tube was kept undisturbed for phases to separate. The upper aqueous layer was examined for magenta color. If magenta color did not develop, then there was no need to proceed further. Lower chloroform layer was removed and 2.0 ml n-butanol was added and vortexed for 01 min. Magenta color was examined in lower aqueous layer which indicated presence of PBG. Butanol extraction procedure was repeated twice. This increased the specificity of PBG detection [Table/Fig 4].

(Table/Fig 4) Watson-Schwartz test positive showing lower magenta color

Absorption spectra of PBG: PBG reacts with Ehrlich's aldehyde reagent (of Watson-Schwartz test) in acidic medium to give red condensation product with characteristic absorption spectrum that has peak at 553 nm and preceded by a shoulder at 540 nm. To generate this absorption spectrum of PBG, magenta colored aqueous layer of Watson-Schwartz test was used [3] [Table/Fig 4].

(Table/Fig 5) Absorption spectra of PBG showing peak at 553nm and shoulder at 540nm.



Jaffe's kinetic method for creatinine estimation: Urine dilution: to 9.9ml of analytical grade water 100µL urine was mixed to get 100 times diluted urine. To 500µL of alkaline picrate reagent (250µL of picric acid reagent was added to 250µL of sodium hydroxide solution) 100µL of 100 time diluted urine was mixed. Reading was taken in analyzer for 60 seconds with lag of initial 20 seconds at 492nm.

Total porphyrins in urine:4ml of centrifuged urine was mixed with 1 ml of concentrated HCl. Undissolved materials were removed by centrifugation. 0.5 ml of supernatant was taken in cuvette and wavelength scanning was done in spectrophotometer in between 350 nm to 450 nm against air blank. If peak absorbance was found near to 400 nm (Soret band) then presence of porphyrins was confirmed [3].

$$\text{Total urinary porphyrins} = \frac{A \times 2500}{\mu\text{mol of creatinine/liter of urine}} \quad (3)$$

"A" is the peak absorbance above the base line between two suitable points as shown in [Table/Fig 4].

The factor 2500 was derived from volume of urine, the volume of acid (thus the dilution of the sample is 5 times) and millimolar extinction coefficient i.e. 500 which was approximately that of a 7:1 (mol: mol) mixture of coproporphyrin-III and uroporphyrin-III in 2.3M HCl. The porphyrin composition of this mixture resembled that of normal urine [3].

Results

The normal value of total pophyrins in urine: < 35 nmol/m mol of creatinine [3]. Biochemical investigation results were correlated with clinical findings to diagnose different types of porphyria [1].

In two cases of AIP, patients having history of acute neurovisceral attack, very severe ill defined abdominal pain, pain in extremities, sensory loss with complaints of seizure, anxiety and hallucinations. They had sympathetic over activity characterized by raised blood pressure and sweating. They were asymptomatic till puberty but had sudden onset of symptoms after puberty. There were no cutaneous manifestations. Increased urinary PBG was detected by Watson-Schwartz test and confirmed by wave-length scanning with spectrophotometer. So, the patients were diagnosed as cases of AIP [1].

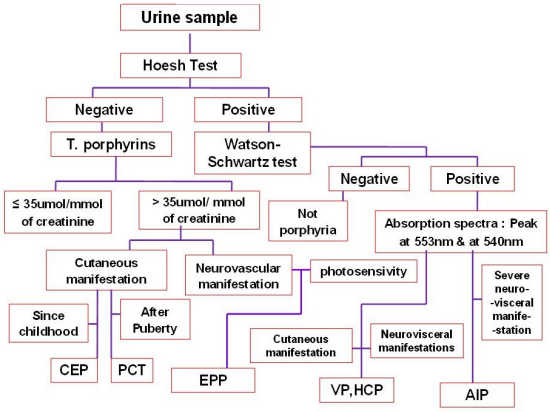
In case of CEP, the child had a history of severe photosensitivity since birth, fragile skin with bullae, infections and scars (Case-2) over skin persisted and having features of mutilation of digits (Case-2 (a)) and facial features like Erythrodontia i.e. red brown colored teeth; there was a history of pink staining diapers during infancy due to porphyrin excretion through sweat and cornea was damaged due to photosensitivity [1].The total urine porphyrin was 91.6 nmol/µmol of creatinine and the urinary PBG was absent. So, the patient was diagnosed as a case of CEP [1].

In case of PCT, 24 year male patients with severe cutaneous manifestations over exposed areas on the skin were precipitated by first-time alcohol intake (case-1) without any neurovisceral symptoms. Total urinary porphyrins raised upto 1695 nmol/µmol of creatinine and the urinary PBG was absent. So, the patient was diagnosed as a case of PCT [1].

Discussion

Urine sample

We have formulated this flowchart by which we can simplify the diagnosis of porphyria with the tests described above correlating with the relevant clinical findings. This flow chart will be helpful to all those who are willing to do the diagnosis of porphyria [Table/Fig 6].



(Table/Fig 6)

In this we have incorporated Hoesch test as a screening test for the urinary porphobilinogen (PBG).

Out of four cases, two cases were AIP and one case was PCT and other one was CEP.

In two cases, patients having very severe ill defined abdominal pain, pain in extremities, sensory loss with complaints of seizure, anxiety and hallucinations. They had sympathetic over activity characterized by raised blood pressure and sweating. They were asymptomatic till puberty but had sudden onset of symptoms after puberty. There were no cutaneous manifestations. Thus their clinical findings were suggestive of Acute Intermittent Porphyria. So, they were first screened with Hoesch test urinary PBG. When found positive, were further detected by Watson-Schwartz test for increased PBG and confirmed by wave-length scanning with spectrophotometer. Thus the patients were diagnosed as cases of AIP.

In another case, a 24 year male patient with severe cutaneous manifestations over exposed areas were precipitated by alcohol intake (case-

1) without any neurovisceral symptoms; so, they were first screened with Hoesch test urinary PBG. When found negative, total urinary porphyrins were estimated, which was raised upto 1695 nmol/µmol of creatinine. So, the patient was diagnosed as a case of Porphyria Cutanea Tarda.

In a case , the child had a history of severe photosensitivity since birth, fragile skin with bullae, scars and infections (Case-2) over skin persisted and having features of mutilation of digits (Case-2 (a) and facial features; there was a history of pink staining diapers during infancy due to porphyrin excretion through sweat and cornea was damaged due to photosensitivity.

So, he was first screened with Hoesch test urinary PBG. When found negative, the total urine porphyrins were estimated which were found to be 91.6 nmol/µmol of creatinine. So, the patient was diagnosed as a case of Congenital Erythropoetic Porphyria.

In the similar way all other types of porphyria can be diagnosed by following the same protocol.

These 04 cases were diagnosed in our laboratory with simple test and by double beam spectrophotometer. Individual porphyrins estimation requires further solvent fractionation of 24 hour urine followed by identification and quantitification by spectrophotometer. As we have limited facility, we diagnosed the cases correlating our laboratory finding with clinical features.

If magenta color develops in lower layer that indicates presence of urobilinogen Secondary coprporphyrinuria is seen in liver dysfunction alcoholism and lead poisoning, but without typical clinical features of porphyria. Fluorescence spectroscopy is required for differentiation between porphyria and psudoporphyria caused by non steroidal anti inflammatory drugs and some other drugs in which the porphyrin metabolism is normal. Differentiation of VP and HCP is difficult with our laboratory but this can be achieved by plasma fluorescent emission scan by spectrofluorophotometer to pinpoint diagnosis.

Conclusion

With limited laboratory facility we could diagnose three different varieties of porphyria. Our message is any laboratory having spectrophotometer with wavelength scanning mode can diagnose porphyria cases with clinical correlation as we did.

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