Comparative Study of Two Screening Tests for Urinary Porphyrins in the Diagnosis of Porphyrias

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ABSTRACT

Objective: To evaluate the efficiency of two urinary porphyrins screening tests: routine fluorescent detection and semi quantitative spectrophotometric scanning.

Methods: Minimal-level detection was performed by adding standard coproporphyrin of 0, 25, 50, 100, 250 and 500 μg/L in urine and then screened by the two methods. Urine samples from 39 controls, 7 patients with porphyrias and 20 patients with liver impairment were quantitated for total porphyrins, followed by a comparison of the two qualitative tests.

Results: The fluorescent test detected the minimal porphyrin level at 250 μg/L whereas spectrophotometric scanning could detect a lower level, at 100 μg/L. Total control subjects showed negative results from both tests while all 7 patients with porphyrias and 6 out of 20 cases of liver impairment showed definite positive results.

Conclusion: Urinary screening for porphyrins from both tests revealed the same accuracy from this study. Still, the spectrophotometric method which is simpler, more sensitive and easily interpretable seemed more practical as a screening test in general laboratories.

Keywords: Screening test; Porphyrias; Urinary porphyrins

Siriraj Med J 2005;57:530-535

Porphyrias are a group of inherited or acquired disorders of heme biosynthesis due to A deficiency of the enzymes in the biosynthetic pathway resulting in an excessive accumulation of porphyrins and their precursors.[1,2] Heme, the end product of this pathway, is a tetrapyrrole ring with the chemical name of protoporphyrin IX type III. The heme molecule containing one iron atom resides in the specific pocket in each subunit of hemoglobin.[1,2]

Inherited porphyrias are mainly classified into two groups, hepatic and erythropoietic porphyrias depending on whether the enzyme deficiency occurs in red blood cells or the liver. Hepatic porphyrias are divided into four types: porphyria cutanea tarda (PCT), acute intermittent porphyria (AIP), hereditary coproporphyria (HCP) and variegate porphyria (VP). There are only two types for erythropoietic porphyrias; congenital erythropoietic porphyria (CEP) and erythropoietic protoporphyria (EPP). Each type of porphyrias results in the accumulation of a unique pattern of intermediates due to the deficiency of one of the eight enzymes in the heme biosynthetic pathway, Fig 1.[3,4] In addition, some disturbances in porphyrin metabolism could be found in liver diseases and heavy alcohol drinkers.[5,6] The association between porphyrinuria and liver impairment is well established.[7] The pathogenesis of disturbances as suggested by Doss et al,[8-10] depends on the impaired enzymatic functions in the heme biosynthesis pathway from liver diseases complicated with alcoholic toxicity. The commonly disturbed enzymes are porphyrinogen synthase, uroporphyrinogen decarboxylase, coproporphyrinogen oxidase and ferrochelatase. However, the enzymatic impairment is temporary and clinically asymptomatic for porphyriatic attack. Nowadays the alcohol-liver porphyrinuria syndrome might often be mistaken for porphyrias. It is interesting to compare the levels of increased urinary porphyrins in these patients with cases of congenital porphyrias.

There are two main clinical presentations in a porphyriatic attack: neuropsychiatric symptoms and skin photosensitivity. Increased porphyrin precursors, δ-aminolevulinic acid (ALA) or porphobilinogen (PBG), cause neurovisceral symptoms while increasing porphyrins which are uroporphyrin, coproporphyrin and protoporphyrin, causing skin photosensitivity. The major manifestations of hepatic porphyrias are neurologic, including abdominal pain, neuropathy, and mental disturbances, whereas erythropoietic porphyrias cause a characteristic cutaneous photosensitivity. Cutaneous photosensitivity to sunlight is due to the excitation of accumulated porphyrins in the skin from ultraviolet light which then causes the formation of superoxide radicals. These reactive oxygen species then damage the cell membranes causing the release of destructive enzymes from lysosome. The destruction of cellular components caused by the lysozyme leads to cell damage, skin photosensitivity, scarring and deformation.[4] Moreover, urine from these patients may turn reddish-brown on standing due to the presence of excessive porphyrin intermediates.[11] Since most symptoms found in this rare disease are nonspecific, and appear intermittently, the missed

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TABLE 1. Characteristics of each type of porphyrias

Types	Incidence	Enzyme deficiency	Inheritance	Photo sensitivity	Neurovisceral symptoms	Major metabolites		
						Red cell porphyrin	Urine porphyrin	Stool porphyrin
Erythropoietic Porphyrias								
X-linked sideroblastic anemiar (XLSA)	Rare	ALA synthase	XLR	-	-	-	-	-
Congenital erythropoietic porphyria (CEP)	Rare	Uro'gen Synthase	AR	+++	-	Uro'gen I	Uro'gen I	Copro'gen I, Uro'gen I
Erythropoietic protoporphyria (EPP)	Common in Caucasian	Ferrochelatase	AD	+	-	Proto'gen IX	-	Proto'gen IX
Hepatic Porphyrias								
ALA dehydratase porphyria (ADP)	Rare	ALA Dehydratase	AR	-	+	Pro'gen	ALA Copro'gen lll	-
Acute intermittent porphyria (AIP)	Common in Northern European	HMB synthase	AD	-	+	-	ALA, PBG	-
Porphyria cutanea tarda (PCT)	Most common	Uro'gen Decarboxylase	AD	+++	-	-	Uro 1,lll 7-carboxylate porphyrin	Isocopro porphyrin
Hereditary copropophyria (HCP)	Common in Britain, Europe	Copro'gen oxidase	AD	+	+	-	ALA,PBG Copro'gen lll	Copro'gen lll
Variegate porphyria (VP)	Common in South Africa (3/1,00)	Proto'gen oxidase	AD	+	+	-	ALA,PBG Copro'gen lll	Proto'gen lX, 5-carboxylate porphyrin

Uro'gen, Uroporphyrinogen; Copro'gen, Coproporphyrinogen; Proto'gen, Protoporphyrinogen,
AR, Autosomal recessive; AD, Autosomal dominant

diagnosis for porphyrias often occurs. Thus the effective management of this rare disease requires prompt and accurate diagnosis.

Laboratory tests can mostly confirm or exclude the diagnosis of porphyrias. The accumulation of porphyrins or precursors can differentiate each type of porphyrias. Qualitative and quantitative measurement of porphyrins excreted in urine is an essential tool for the investigation and differential diagnosis of each type of porphyrias. Several techniques are available for the analysis of porphyrins in biological samples, such as spectrophotometry,[12] fluorometry,[13-15] thin layer chromatography,[16] ion exchange chromatography,[17] high performance liquid chromatography (HPLC)[18-29] and liquid chromatography-mass spectrometry.[30-32]

The standard analytical methods of porphyrins are expensive, time consuming and are generally performed in centers with specialized laboratories. Therefore, a simple, rapid and inexpensive screening test should be very useful in providing initial evidence to support or exclude the diagnosis of porphyrias in suspected cases. In this study, the effectiveness of two different screening tests for porphyrins was evaluated. One was a conventional screening test, which was qualitative in nature and based on fluorescent detection of porphyrins in urine under a UV lamp. The other more recent one was a semiquantitative test based on spectrophotometric scanning of porphyrins in urine. This technique was developed by Deacon et al in 1988 and seemed simpler and less tedious than the conventional test.[12]

MATERIALS AND METHODS

Sample collection

The urine samples recruited in the study were from 39 normal controls, 7 patients with various types of porphyrias and 20 patients with liver impairment. Random urine from each subject was freshly collected and protected from light before being refrigerated at -20^{0}C. All urine was analyzed within 24 hours for total porphyrins by the two screening tests.

The control group included physically healthy subjects with no history of chronic or severe illness at the time of the study. They were 25 males and 14 females aged from 5 to 24 years.

Patients with porphyrias were clinically proven cases who were admitted in Siriraj Hospital. They were 2 cases of congenital erythropoietic porphyria (CEP) aged 2 and 3 years old, 4 cases of porphyria cutanea tarda (PCT), 10, 45, 54, 65 years old and 1 acute intermittent porphyria (AIP), 36 years old.

Patients with liver impairment, aged from 29 to 77 years old, were those attending or admitted to Siriraj Hospital. They were classified into 2 groups according to the severity of their liver functions as classified by the criteria of Child and Pugh.[34] There were 3 patients with mild impairment, with scores of less than 7, while another 17 cases were in the moderate to severe group with scores higher than 7. The scores were counted from the signs of liver impairment such as ascites, encephalopathy and the liver function test.

Test for the minimal detection limit of the two screening tests

Various concentrations of standard coproporphyrin were added into normal urine to get the serial concentrations from 0, 25, 50, 100, 250 and 500 μg/L. Then each sample was tested in order to check for the minimal detection limit of the 2 screening methods. The serial concentrations of standard coproporphyrin were prepared twice in a new set of reagents to check for the accuracy and the

TABLE 2. Results of total urinary porphyrins in 39 normal controls, 7 patients with porphyrias and 20 patients with liver impairment according to the 2 screening tests, fluorometric and spectrophotometric scanning.

Subjects (number)	Total porphyrins(μg/L) Mean ± SD (range)	Qualitative fluorescent screening method	Qualitative spectrophotometric scanning method
Normal controls (39)	44 ± SD (8-129)	All negative	All negative
Patients with porphyrias (7)	17,180 (24-111,578)	All positive	All positive
Patients with liver impairment (20)	204 (30-1,267)	6 positive	6 positive

reproducibility of the results.

Quantitative analyses of coproporphyrin and uroporphyrin, the main substances of total porphyrins, were performed by the method of Schwartz and Talman.[35,36]

Coproporphyrin assay

Five milliliters of urine were placed in a separating funnel. Five milliliters of acetate buffer (pH 4.8), 10 ml of distilled water and 50 ml of ethyl acetate were then added to the urine. After the funnel was shaken for two minutes, the mixture was allowed to separate. The lower urine phase containing uroporphyrin was collected for the assay which is described later. The upper ethyl acetate phase containing coproporphyrin was mixed with 10 ml of 1% sodium acetate by vigorous shaking for two minutes. The lower phase was pooled together with the previous extract of uroporphyrin. Then 10 ml of 0.005% iodine was added to the upper phase, followed by vigorous shaking for 1 minute. The lower phase was discarded. The urine coproporphyrin was extracted from the upper phase with 5 ml of 1.5 M HCl 4 times. All acid extracts were pooled and adjusted to a total volume of 25 ml with 1.5 M HCl.

Coproporphyrin was analyzed by spectrofluorometry at the excitation and emission wavelengths of 405 and 595 nm, respectively, using 1.5 M HCl as a blank. To generate a standard curve, the standard coproporphyrin I solution (5 μg/ml) was diluted with 1.5 M HCl to obtain the serial concentrations of 0.01, 0.02, 0.03, 0.04, and 0.05 μg/l. The fluorescence intensity was plotted against the coproporphyrin I concentration (μg/l) and urine coproporphyrin concentration was calculated as follows;

Urine coproporphyrin (μg/L)

$$= \frac{\text{Concentration} \times V \times 1000 \times 0.925}{D}$$

Concentration (μg/l) of urine sample was derived from the calibration curve

V was the volume of acid extraction (i.e. 25 ml)

D was the volume of the urine specimen (i.e. 5 ml)

0.925 was the correction factor of coproporphyrin I as its fluorescence in 1.5 M HCl and ethyl acetate is approximately 8% higher than in 1.5 M HCl alone.

Uroporphyrin assay[35]

The lower aqueous phase of uroporphyrin, collected during the assay for coproporphyrin, was then placed in a 50 ml centrifuge tube containing 0.5 g of aluminum oxide to adsorb uroporphyrin. The aluminium oxide was thoroughly stirred and then centrifuged at 1,500 rpm for 5 min. The supernatant was discarded and the aluminum oxide pellet with adsorbed uroporphyrin was washed twice with 20 ml of half-saturated sodium acetate followed by 30 ml of distilled water. The uroporphyrin was then eluted from aluminum oxide by resuspending it in each 5 ml of 1.5 M HCl four times. The acid extracts were pooled and adjusted to 25 ml with 1.5 M HCl.

The uroporphyrin was also analyzed by spectrofluorometry at the same excitation and emission wavelengths of 405 and 595 nm, respectively. The results were read from the same standard curve of the coproporphyrin assay. The concentration of urine uroporphyrin was calculated as follows;

Urine uroporphyrin (μg/l)

$$= \frac{\text{Concentration} \times V \times 1000 \times 0.925 \times 0.75}{D}$$

Concentration (μg/l) was derived from the calibration curve

V was the volume of acid extraction (i.e. 25 ml)

D was the volume of the urine specimen (i.e. 5 ml)

0.925 was the correction factor of coproporphyrin I in 1.5 M HCl alone

0.75 was the correction factor of uroporphyrin as its fluorescence was higher than the standard coproporphyrin I.

Qualitative fluorescent screening for porphyrin[36,37]

The increased porphyrins after extraction from acidified urine, when exposed to UV light, showed a pink to coral red fluorescence. The assay was performed as follows. Five ml of urine was placed in a separating funnel with a stopper glass. Three drops of glacial acetic acid and 5 ml of ether were then added to the urine. After

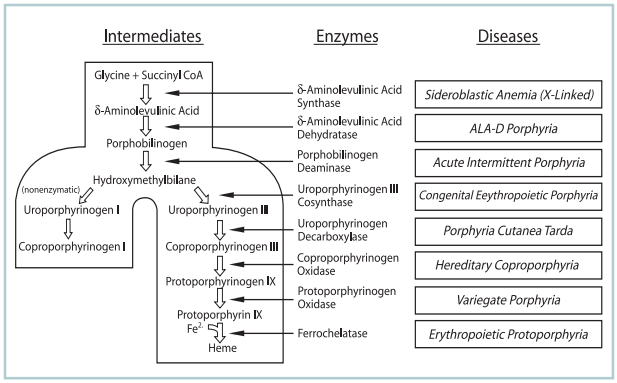

Fig 1. The heme biosynthetic pathway showing types of porphyrias due to specific enzyme deficiency 4.

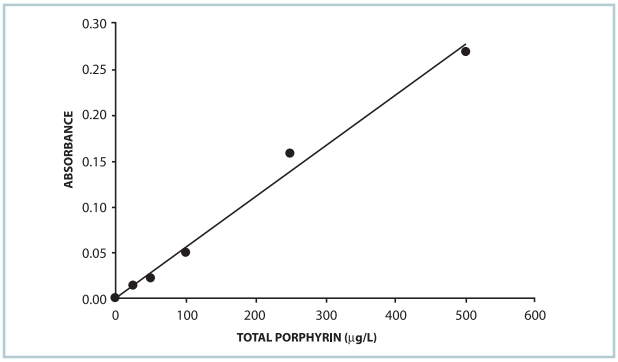

Fig 2. Serial concentrations of standard coproporphyrin from 0-500 μg/ L detected by the semiquantitative spectrophotometric scanning method.

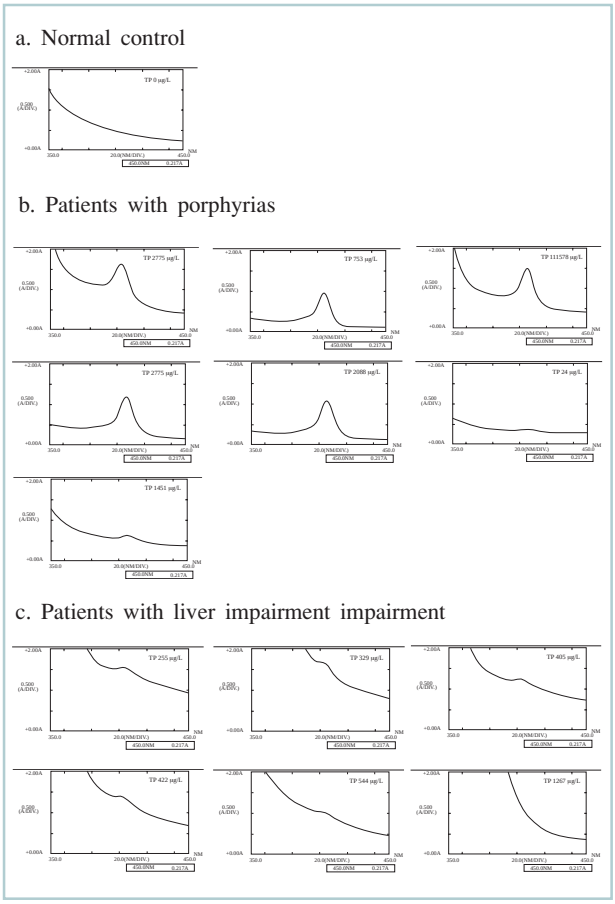

Fig 3. Spectrophotometric scanning for total porphyrins (TP) in urine showing negative results in all normal controls (a), definitely positive in all 7 patients with porphyrias (b), and slightly positive in 6 out of 20 patients with liver impairments (c).

shaking vigorously for 1 minute and allowing for separation, the lower part was discarded. The upper part containing extracted porphyrin in ether was added with 5 ml of 1.5 M HCl and was then shaken again. If there was an increased porphyrin in the urine, the lower layer of acid extract would appear pink to red fluorescence under Wood's lamp. Some normal urine may show blue or lavender to violet fluoresces but not a pink- red color.

Semiquantitative spectrophotometric scanning method[12,38]

Total porphyrins in urine can be detected by spectrophotometry when scanning the absorption spectrum between 350 and 450 nm. The approximate vertical peak of absorption for increased porphyrin is seen at 400-405 nm.

Four ml of urine was acidified with 1 ml of concentrated HCl and then screened for the absorption spectrum between 350 and 450 nm. If the peak was seen in the 400 nm region, the increased porphyrins could be calculated as follows:[38]

A x 2500 nmol/L

A was the peak absorbance above a base line drawn between the two suitable points 2,500 was a factor derived from the data of Rimington when using the volume of urine (4 ml), volume of acid (1 ml) and a millimolar extinction coefficient of 500 for total porphyrin. This coefficient was derived from the approximation of a 7/1 (mol/mol) mixture of coproporphyrin to uroporphyrin in 2.3 M HCl that resembled the ratio in normal urine.

RESULTS

Test for the minimal detection limit of the two screening tests

Both tests were performed in the serial concentrations of standard coproporphyrin from 0 - 500 μg/L added in the normal urine sample. The qualitative fluorescent screening showed positive results of pink fluorescence at a minimal level of 250 μg/L whereas the spectrophotometric scanning could detect more sensitivity at a lower level of 100 μg/L.

Urinary porphyrin levels from normal control subjects and patients were quantitated and compared with the 2 screening methods. Table 2 shows all the analyzed data and Fig 3 shows the results from the spectrophometric scanning.

Normal control

Thirty-nine urine samples from the control subjects were quantitated for coproporphyrin, uroporphyrin and total porphyrins. The mean values and ranges in bracelets were 38 (5-115), 6 (0-21) and 44 (8-129) μg/L, respectively. Both qualitative and scanning methods gave negative screening results in all normal control subjects.

Patients with porphyrias

Urine from 7 patients with porphyrias were quantitated to get the markedly increased values of means (ranges) of coproporphyrin, uroporphyrin and total porphyrins of 7,899 (8-50,875), 9,281 (16-60,703) and 17, 180 (24-111, 578) μg/L, respectively. All these urine samples showed strongly positive tests from both screening methods as shown in Fig 3.

Patients with liver impairment

Urine from twenty patients with liver cirrhosis were quantitated for coproporphyrin, uroporphyrin and total porphyrins to get the values of means (ranges) of 176 (13-1,144), 28 (5-124), and 204 (30-1,267) μg/L, respectively. Both screening tests showed the same positive results in 6 out of 20 patients. These 6 patients were all in the moderate to severe group of liver impairment. Their slightly increased amounts of urinary total porphyrins were 255, 329, 405, 422, 544 and 1,267 μg/L.

DISCUSSION

Total quantitated urinary porphyrin levels in 39 normal controls ranged from 8-129 μg/L with the mean and standard deviation of 44.3 μg/L and 33.6 μg/L, respectively. The values were not significantly different among male and female subjects at various ages, 5 - 24 years. The results were similar to other reports.[5,11,13,19,20,37] From our data we presumed the total urinary porphyrins excretion in normal Thais was not greater than 145 μg/L, the calculated value from the mean + 3 SD. However, it was well accepted that the upper reference limit of urinary porphyrins was 250 μg/L or 320 nmol/L if any urine containing more than this level was counted as porphyrinurias.[3]

Six out of 7 patients with porphyrias were in acute attack while only one patient was in remission. The total urinary porphyrin levels from acute porphyrias ranged from 995 to 111, 578 μg/L while one latent case was 24 μg/L. The data verified that the highly increased porphyrin excretion occurred only during acute attack but turned to a normal range in the remission phase. However, the normal quantitated value in this latent case might be a false negative since the results from both screening tests showed a similar mild positive. The

conventional spectrofluorometric results in this study were sometimes probably inaccurate in cases in which there were some interfering substances contained in the urine. High levels of urobilinogen and some drugs possibly resulted in false increased or decreased levels of the detected porphyrins.[35]

The urinary porphyrins levels in patients with liver impairment were about 5 times higher than that in normal subjects, ranging from 30 - 1,267 μg/L with the mean level of 204.2 μg/L and standard deviation of 294. Only 6 out of 20 patients, or 30% of all cases, showed increased urinary porphyrins levels of more than 250 μg/L. These 6 patients were classified as having moderate to severe liver impairment. Even though porphyrinuria was found in their urine, there were no apparent clinical signs or symptoms of acute porphyrias detected. The data suggested that porphyrinuria secondary to liver impairment was not as severe as found in inherited porphyrias. The more the liver impairment, the more the disturbances of the enzymatic function in the heme biosynthesis were found, affecting the increased porphyrin excretion in urine, especially coproporphyrinuria. The other causes of secondary porhyrinuria were alcoholism, some drug therapy and lead poisoning. In lead poisoning, the toxicity could suppress at many steps of the heme biosynthetic pathway, causing the increase of urinary porphyrins, ALA, PBG and erythrocyte protoporphyrin.[6-11]

Both screening tests, qualitative fluorescent and spectrophotometric scanning, showed negative results in all urine samples of normal controls in contrast to positive tests in all 7 porphyria patients and 6 patients with secondary porphyrinuria due to liver impairment. For the minimal detection limit of the 2 screening tests, the spectrophotometric scanning could detect at the low level of 100 μg/L of porphyrin which was more sensitive than 250 μg/L from the routine qualitative test. However, the increased porphyrin in the urine of patients with porphyrias was remarkably high and was significantly positive from both screening tests. Even in porphyrinuria, secondary to impaired liver, the urinary porphyrin was also higher than 250 μg/L; the positive results from both tests were similarly obtained. Very often in less experienced laboratories the results from borderline porphyrin levels detected by the routine qualitative method may be missed. Also some contamination in the urine causing a dark color or some drugs and chemicals could give false negative or positive results by the routine test. The spectrophotometric scanning that was not influenced by those factors would probably be more accurate than the fluorescent detection. Also the test itself was more simpler; fewer steps and the results were reliable when recorded in the peak area of the positive test. In addition, the positive peak of porphyrins shown by this method could be calculated for the approximate amount of porphyrins that was close to the value from the standard quantitative analysis.[38] These factors support a more practical screening test by the spectrophotometric scanning method, especially in any routine laboratories.

However, the two screening methods could not differentiate types of porphyrias since they measured only the total amount of porphyrins that included mainly coproporphyrin and uroporphyrin. The positive results from each test were significantly reliable for the laboratory diagnosis of porphyrinuria. Thereafter further analysis for porphyrin isomers and precursors by other special techniques is needed in order to identify definite types of porphyrias.

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บทคัดย่อ

การศึกษาเปรียบเทียบวิธีการตรวจกรองหาสารพอร์ฟัยรินในปัสสาวะเพื่อช่วยในการวินิจฉัยภาวะพอร์ฟัยเรีย

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วัตถุประสงค์: เพื่อประเมินประสิทธิภาพของการตรวจกรองสารพอร์ฟัยรินในปัสสาวะ 2 วิธี คือ วิธีประจำ ที่วัดการเรืองแสงของสารพอร์ฟัยริน เปรียบเทียบกับวิธีใหม่ ที่วัดการดูดกลืนแสง

วิธีการ: เปรียบเทียบค่าพอร์ฟัยรินต่ำสุดที่ตรวจพบได้จากทั้ง 2 วิธี โดยเติมสารนี้ในปัสสาวะจำนวน 0 25 50 100 250 และ 500 µg/L ตามลำดับ จากนั้นนำวิธีทั้ง 2 มาตรวจกรองปัสสาวะของคนปกติ 39 ราย ผู้ป่วยพอร์ฟัยเรีย 7 ราย และผู้ป่วยโรคตับเสื่อม 20 ราย

ผลการศึกษา: วิธีแรก ตรวจพบสารพอร์ฟัยรินได้ที่ระดับต่ำสุด 250 µg/L ในขณะที่วิธีหลังได้ค่าต่ำกว่าที่ 100 µg/L ผลการตรวจกรองในปัสสาวะจากทั้ง 2 วิธีได้เหมือนกัน คือในคนปกติทุกรายได้ผลลบ แต่ในผู้ป่วยพอร์ฟัยเรียทั้งหมด 7 ราย และผู้ป่วยโรคตับเสื่อม 6 ใน 20 คน ให้ผลบวก

สรุป: การตรวจกรองสารพอร์ฟัยรินจากทั้ง 2 วิธี ให้ผลแม่นยำเท่ากัน แต่วิธีใหม่ มีความไวและตรวจได้ง่ายกว่า การแปลผลทำได้รวดเร็ว และไม่จำเป็นต้องใช้ผู้เชี่ยวชาญพิเศษในการวิเคราะห์หรือแปลผล จึงเหมาะสมกว่าที่จะใช้ในห้องปฏิบัติการทั่วไป