



A CASE REPORT OF FALSE POSITIVE FERRIC CHLORIDE TEST

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Abstract :

Phenylketonuria(PKU) is one among the most well known of the aminoacidurias due to primary or cofactor related defect of the enzyme phenylalanine hydroxylase activity causing accumulation of phenylalanine, phenylketones and phenylamines resulting in mental retardation. Increased level of phenylketones in urine causes permanent green colour by ferric chloride test, a screening test for diagnosis of phenylketonuria. An increased urinary level of metabolites of phenylalanine is one of the criteria for a biochemical diagnosis of classic PKU. Here we report a false positive ferric chloride test in a case leading to diagnosis of phenylketonuria, but negative dinitrophenylhydrazine test and aminoacid profile within normal limits in tandem mass spectrometry.

Keyword : ferric chloride test, false positive phenylketonuria.

A CASE REPORT OF FALSE POSITIVE FERRIC CHLORIDE TEST CASE REPORT:

A 1/365 day old male preterm neonate (35 weeks), delivered after premature rupture of membranes, presented with seizures, rashes all over the body and fever, admitted in NICU. No H/O consanguineous marriage. There is history of previous deceased sibling, details not known.

We received Blood sample for estimation of glucose, calcium, bilirubin and urine sample collected on second day for metabolic screening. Peripheral smear examination and CRP was also done.

RESULTS: Serum glucose-38 mg/dl (50-80 mg/dl) Ionized calcium-1.2mmol/l (1.15-1.33mmol/l) Total serum bilirubin-13.5 mg/dl. (6-12mg/dl for preterm) Direct bilirubin -7.8 mg/dl. (<0.2mg/dl) C reactive protein-6 mg/l. (<5mg/l)

Peripheral blood smear report:

Neutropenia with increase in band forms (immature neutrophils)

Urine metabolic screening:

Done with 3 years old normal child urine as control.

Physical Examination:

Colour - Dark yellowish. Volume - Not known Odour - Aromatic odour. pH -Acidic (by litmus paper) Specific gravity -Not measured (Very small quantity)

Chemical examination:

Ninhydrin test _ Negative (Amino acids) Fer-
ric chloride test: – Positive (Phenyl pyruvic
acid) Dinitrophenylhydrazine test – Negative.
(ketoacids) Silver nitrate test _ Negative
(alkaptonuria) Cetrimide test _ Negative
(mucopolysaccharidosis) Benedict's test –
Negative(reducing sugars)

Cyanide –nitroprusside test -- Negative
(sulphur containing aminoacids) Ehrlich's
test – Negative (porphobilinogen) As urine
was dark yellowish in colour Hay's test and
Fouchet's test were done to rule out jaun-
dice.

Hay's test _ positive. Fouchet's test _ posi-
tive.

With the above findings for further confirma-
tion, Direct blood spot on filter paper taken
on the 2nd day was sent to NeoGen Labs,
Bangalore for tandem mass spectrometry
screening. Aminoacid profile including
phenylketonuria was within normal limits.
Scanned report of newborn screening test by
MS/MS was attached with this scientific pa-
per.

TMS Report Form

Diagnosis:

Neonatal hyperbilirubinemia due to sep-
sis was confirmed.

DISCUSSION:

Phenylalanine is an essential amino-
acid. Dietary intake in excess of ana-
bolic needs is converted to tyrosine by
phenylalanine hydroxylase and further
degraded via a ketogenic pathway(1).A
primary or cofactor tetrahydrobiopterin-
related defect of phenylalanine hydroxy-
lase activity causes accumulation of
phenylalanine, phenylketones and
phenylamines(1).

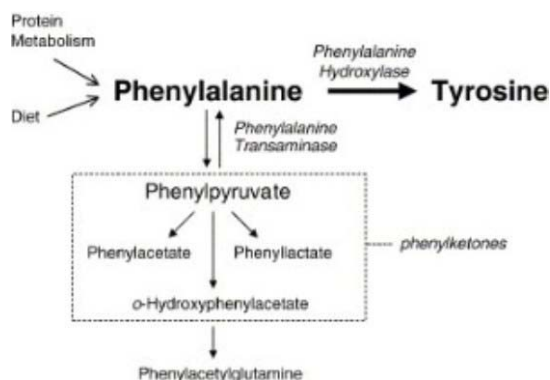
The phenylalanine metabolites have no
role in the pathogenesis of central nerv-
ous system damage in patients with
phenylketonuria.The CNS damage in
patients with PKU is caused by the ele-
vated concentration of phenylalanine in
brain tissue. The high blood levels of
phenylalanine saturate the transport
system across the blood-brain barrier,
causing inhibition of the cerebral uptake
of other large neutral aminoacids such
as tyrosine and tryptophan(2).

Several distinct forms of phenylketonu-
ria exist. All are inherited as autosomal
recessive traits with classic phenylke-
tonuria being the most common entity
(1).There is also an unusual form of hy-
perphenylalaninemia / phenylketonuria.
These patients have non-
phenylketonuric PKU with phenylalanine
hydroxylase deficiency. These patients
have hyperphenylalaninemia in the ab-
sence of phenylketones in urine due to
deficiency of phenylalanine transamina-
tion.

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| TEST NAME | TEST RESULT | REFERENCE RANGE |
|---------------------|---------------|----------------------|
| Phenylalanine (Phe) | 0.02 mmol/mol | 0.02 - 0.06 mmol/mol |
| Tyrosine (Tyr) | 0.02 mmol/mol | 0.02 - 0.06 mmol/mol |
| Valine (Val) | 0.02 mmol/mol | 0.02 - 0.06 mmol/mol |
| Leucine (Leu) | 0.02 mmol/mol | 0.02 - 0.06 mmol/mol |
| Isoleucine (Ile) | 0.02 mmol/mol | 0.02 - 0.06 mmol/mol |
| Threonine (Thr) | 0.02 mmol/mol | 0.02 - 0.06 mmol/mol |
| Proline (Pro) | 0.02 mmol/mol | 0.02 - 0.06 mmol/mol |
| Glycine (Gly) | 0.02 mmol/mol | 0.02 - 0.06 mmol/mol |
| Alanine (Ala) | 0.02 mmol/mol | 0.02 - 0.06 mmol/mol |
| Aspartic acid (Asp) | 0.02 mmol/mol | 0.02 - 0.06 mmol/mol |
| Glutamic acid (Glu) | 0.02 mmol/mol | 0.02 - 0.06 mmol/mol |
| Asparagine (Asn) | 0.02 mmol/mol | 0.02 - 0.06 mmol/mol |
| Glutamine (Gln) | 0.02 mmol/mol | 0.02 - 0.06 mmol/mol |
| Protein (Total) | 0.02 mmol/mol | 0.02 - 0.06 mmol/mol |

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Phenylalanine Metabolic Pathway

There is normal postnatal attenuation of transaminase activity in neonates particularly preterm infants and can be a cause of missed cases if detection of phenylpyruvic acid in urine (by the ferric chloride test) is the basis of the screening test (3). Phenylketonuria is estimated to occur 1 in 10,000 to 20,000 births, if undetected results in severe mental retardation. Phenylketonuria was first identified in Norway by Ivan Folling in 1934. When a mother with mentally retarded child reported a peculiar mousy odour to her child's urine. Analysis of the urine showed increased amounts of phenylpyruvate (4). Classical Phenylketonuria is caused by failure to inherit the gene to produce the enzyme phenylalanine hydroxylase. The gene is inherited as an autosomal recessive trait with no noticeable characteristics or defects exhibited by heterozygous carriers. Patients with classic PKU are clinically silent at birth and neurological manifestations typically do not become evident until a few months of age (1).

Screening tests are available for early detection of the abnormality. Once diagnosed dietary treatment that eliminate phenylalanine must occur before the child is 3 weeks of age to prevent the excessive build up of serum phenylalanine and can thereby avoid damage to the child's

mental capabilities (6). Phenylketonuric infants may lose about 50 points in their adult IQ if left untreated until the end of the first year of life. Those who are diagnosed later require institutional care because of severe mental retardation and hyperactivity (1).

- fair skin and blue eyes,
- microcephaly,
- prominent maxilla with widely spaced teeth,
- enamel hypoplasia and growth retardation.
- Some have seborrheic or eczematous rash.
- Most infants are hypertonic, about one fourth have seizures and more than 50% have abnormal EEG findings (1).

The criteria for a biochemical diagnosis of untreated classic Phenylketonuria are

- (1) a plasma phenylalanine level above 20mg/dl.
- (2) a phenylalanine/tyrosine ratio >3.
- (3) increased urinary levels of phenylalanine metabolites (i.e., phenylpyruvic acids and 2 hydroxyphenylacetic acids)
- (4) a normal concentration of tetrahydrobiopterin (BH4) (1).

Identification and measurement of phenylketones in the urine by ferric chloride may offer a simple test for diagnosis of infants with developmental and neurological abnormalities (2). Infants with a positive screening test need additional metabolic testing to confirm or exclude the diagnosis (6).

FERRIC CHLORIDE TEST:

To 1 ml of fresh urine in a tube slowly add 5 drops of 10% ferric chloride. Observe the colour (11). Permanent green colour – phenyl pyruvic acid. (4) (5).

Other false positives are Green colour – bilirubin. (5)(7). Transient green colour – Tyrosyluria. Transient blue colour – Alkaptonuria. Grey-black colour – Melanuria. Green-grey – Maple syrup urine disease. Violet blue – Indicanuria.

Drugs such as clioquinol (a halogenated quinolone- topical antiinfective) salicylates, para-aminosalicylic acid and phenothiazine also give false positive result .(5).

DINITROPHENYLHYDRAZINE TEST:

To 1 ml of urine add 0.5ml of DNPH reagent (0.1% of dinitrophenylhydrazine in 2 N hydrochloric acid). A yellow precipitate after 10 minutes indicates a positive result.

HAY'S TEST:

Take 2 small test tubes
To one small test tube 3/4th full of distilled water sprinkle fine powder of sulphur.
To other small test tube 3/4th full of urine sprinkle fine powder of sulphur.
If bile salts are present sulphur powder will sink.

FOUCHET'S TEST:

To 10 ml of urine in a test tube add 5 ml of 10% barium chloride solution. mix well. Filter the precipitate through a whatman.no.42 filter paper. Dry the filter paper. To this add 0.5 ml of Fouchet's reagent.(10%ferric chloride in trichloroacetic acid) Blue or green colour indicates presence of bile pigments.

TANDEM MASS SPECTROMETRY:

Mass spectrometry (MS) is a powerful qualitative and quantitative analytical

technique that is used to measure a wide range of clinically relevant analytes(1).

In a tandem mass spectrometer, two or more mass analyzers are connected in tandem. In the first the targeted compounds is selectively ionized and its characteristic ions are separated from others in the mixture. The selected primary ions then collide with molecules of a neutral gas to produce fragments that are separated and identified in the second spectrometer. Using two mass spectrometers, separated by a zone in which the collision-induced fragmentation takes place permits the selective and specific analysis for many compounds of various structural classes. It is applicable to the rapid assay of specific analytes in complex biological fluids. The need for a chromatographic step is eliminated because separation and analysis takes place simultaneously within the tandem mass spectrometer(7). Clinically MS/MS has been used to screen neonates for amino acid metabolic disorders as phenylketonuria, maple syrup urine disease, tyrosinemia, hypermethioninemias and homocystinuria, acylcarnitine profile and biochemical profile including G6PD deficiency, cystic fibrosis, congenital hypothyroidism, congenital adrenal hyperplasia, galactosemia and biotinidase deficiency. Compared with older methods MS/MS offers greater analytical sensitivity, accuracy and precision and has higher clinical specificity producing fewer false negative and false positive results(7).

Effective and relatively inexpensive methods for mass screening of newborn infants have been developed and used in USA and several other countries. The method of choice is tandem mass spectrometry, a few drops of blood which are placed on a filter paper and

mailed to a central laboratory, are used for assay. Diagnosis must be confirmed by measurement of plasma phenylalanine levels. Blood phenylalanine in affected infants with PKU may rise to diagnostic levels (>20 mg/dl) as early as 4 hours after birth even in the absence of protein feeding. It is recommended that the blood for screening be obtained in the 1st 24-48 hours of life after feeding protein to reduce the possibility of false-negative results, especially in the milder forms of the condition(2). In the developing countries where economic constraints limit the diagnosis of inborn errors of metabolism, it is recommended to provide these simple urine screening tests (Ninhydrin test, ferric chloride test, dinitrophenylhydrazine test, silver nitrate test, cetrimide test, cyanide-nitroprusside test, Ehrlich's test,..) to broadly classify and identify inborn errors of metabolism(9).

CONCLUSION:

In this case scenario, ferric chloride test being positive, misguided the diagnosis of PKU, but turned to be false positive after the results from tandem mass spectrometry revealed normal amino acid profile. Here bilirubin interference produced a false positive ferric chloride test which was then confirmed by tandem mass spectrometry. Hence we wish to alert clinical biochemists about these interferences (tyrosine, keto acids, histidine, bilirubin, lactate, melanin, xanthurenic acid, acetoacetic acid, drugs like para-aminosalicylic acid, phenothiazines and salicylates) in ferric chloride test and to stress the necessity of confirming the validity of all screening tests for PKU by using tandem mass spectrometry.

All neonates with abrupt deterioration of clinical conditions should be screened for metabolic disorders by simple urine tests which are rapid, easy and inexpensive for early diagnosis and early

treatment of these disorders with better outcome(9). About one percent of all babies tested prove to be false positive. So specific confirmatory tests of those with positive results must be performed (10). To summarize the benefits of early diagnosis and treatment in ameliorating the clinical impact of phenylketonuria is well established as classic example of euphenic therapy (Lederberg's term) where a normal phenotype is restored without modification of the mutant genotype (3).

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