



Review Article

Detection of Alkaptonuria by simple, effective and precise chemical methods: A technical review

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ARTICLE INFO

Article history:

Received 21-08-2020

Accepted 27-08-2020

Available online 28-09-2020

Keywords:

Alkaptonuria

Homogentisic acid

Ochronosis

Rare disease

Homogentisate dioxygenase

Chemical reactions

ABSTRACT

Alkaptonuria is a rare error in tyrosine metabolism. Due to genetic variants severity of disease vary. Lack of local expertise and difficulty in detection of AKU has been expressed in literature. Onset of clinical symptoms and ochronosis starts before adult age but timely detection at paediatric age is useful in prevention of ochronotic arthropathy.

Objective of this technical review is to provide simple, quick and precise chemical methods with reagent and technical details so as to detect HGA in urine at every primary health care setups to pick up AKU at early age.

Conclusion: Present technical review explains eight precise, time tested, simple and effective chemical methods. They are technically less demanding and can be used at primary centre to even at bed side clinics. Review may increase screening of the disease by medical practitioners and may encourage budding researchers to contribute in creating data base for prevalence of AKU in India.

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1. Introduction

Alkaptonuria (AKU) is a rare inborn error of Tyrosine (amino acid) metabolism. It is due to congenital deficiency of Homogentisate 1-2 dioxygenase (HGD) [EC.1.13.11.5].¹ or loss of gene function as a result of missense mutation.² Enzyme HGD is normally present in Liver, Kidney, Intestine and prostate. In alkaptonurics homogentisic acid (HGA), an intermediate in tyrosine metabolism, is not further converted to maleyl acetoacetic acid and its level rises in blood. Slow accumulation of HGA polymer (Benzoquinone or Benzoquinone acetate)³ in connective and cartilaginous tissue causes arthritis, also imparts bluish black discolouration of cheek skin, nose, sclera and ear called Ochronosis.⁴ A detectable quantity of HGA gets excreted in urine. Dark black stain in diapers can be an early indicator of disorder in infancy but if missed out, it remains asymptomatic, untold until about fourth decade of life. Due to available genetic variants different concentrations of

HGA can be found in serum and urine of affected person expressing different level of severity of disease.⁵

AKU is so rare that it is unknown to the general public and overlooked by many clinicians. A prevalence of 1 in 250,000 to 1 in 500,000 live births has been reported from UK and US population. However, higher incidences of 19 to 25 in 500,000 live births are detected in Slovakia, Dominican Republic and Middle East countries.⁶ Though there is a lack of such systemic survey, few scattered cases have been reported from various parts of India.⁷⁻¹¹ Localised higher incidence of AKU among Romani community at Vellore, Tamil Nadu¹² and Dharwad district of Karnataka^{13,14} are reported from South India.

In a developing country, due to lack of health education and unawareness of neonatal screening program, Inborn error of metabolisms including alkaptonuria are missed-out unnoticed. Till recent past, diagnosis of metabolic disorders was made by positive findings with chemical tests. Due to lack of expertise erroneous results were reported. Due to rare disorder, precise diagnosis was restricted at specialised centers only.¹⁵ Today owing to availability of more easy

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automated methods these chemical tests have lost their value and remained in the books. It is important to bring them back in practise at every level with proper understanding so that disease can be detected at early age.

Biochemical tests performed towards diagnosis of AKU disorder are hard core chemical tests based on reducing nature of HGA or oxidative polymerization of HGA. Though sophisticated advanced methods like HPLC,^{16,17} MRI, X-ray and genetic study are available for diagnosis at higher centers having sharp edge but, due to lack of any definite treatment they remain time consuming and uneconomical for a patient. Though the test have importance at research labs, a routine clinical laboratory ignore them due to lack of expertise. A clinician's interest towards diagnosis of this condition is to protect patient from arthropathy and Ochronosis. Hence it is important for clinical laboratory to provide confirm results at minimum cost and short possible time to support clinician in the palliative treatment when needed.

Aim of this paper is to provide comprehensive technical details of methods with reagent preparations so that any primary health centres or small labs can be able to detect positive sample with confidence and contribute to the diagnosis of rare disease. Also, these detailed tests may encourage technical persons to contribute in making prevalence data-base for AKU in Indian subjects which is unavailable at present. Also simple quick and precise chemical methods described for detection of HGA in urine will remove phobia and increased awareness among people for detection of the disease so that any complications of ochronosis or arthropathy can be avoided in latter age.

2. Materials and Methods

2.1. Sample collection

Presented Biochemical tests can be performed on fresh urine samples.¹ Sample collection is important in order of get proper test results. Random urine sample may be used for screening, but first morning urine sample is concentrated and ideal for confirmation. All chemicals required are available with chemical suppliers.

Following tests are performed on first morning / random fresh urine sample to get correct and reliable results.

2.2. Chemical method for detection of Homogentisic acid in urine/ body fluids

2.2.1. Observation for development of Black / Brown colouration on Freshly voided urine sample

2.2.1.1. Principle. Homogentisic acid in alkaline medium undergoes oxidative polymerization to quinone derivatives which is black to brown in colour.

2.2.1.2. Reagent not required.

2.2.1.3. Test protocol. Take 5 to 10ml freshly voided urine sample [preferably first morning sample] in clean test tube and keep aside for 10-12 hours. Fresh urine sample will be usually pale yellow in appearance, but top layer gradually turns to brown black on keeping aside for 12hrs.

Generally urine is acidic. Acidic urine takes long time to produce colour change (sometimes due to highly acidic urine, one may not get the result). When urine is kept for 10-12hrs at room temperature, urine gradually turns alkaline due to degradation and bacterial action. Atmospheric oxygen and alkaline pH favours urine to produce colouration.

(Tip for General Practitioner: Take 5ml fresh urine sample and add washing soap solution 2-5 drops if lab facility is not available, washing soap solution being alkaline it takes less time to produce colour)

2.2.1.4. Note. Misinterpretations of test result are because of less time given for reaction to produce colour on freshly voided or random urine sample. When urine contains high concentration of Ascorbic acid, oxidation is prevented. For confirmation use first morning urine sample.

2.2.2. Ferric chloride test

2.2.2.1. Principle. Phenols of HGA forms a violet complex with Fe(III), which is intensely coloured. The urine containing HGA yields a transient blue colour after adding few drops of Ferric Chloride solution. Test is useful for diagnosis and monitoring treatment of AKU.

2.2.2.2. Reagent required. 10% Ferric chloride solution: 2gm Ferric chloride salt is dissolved in 100ml distilled water. To this add 1ml 2N HCl.¹⁸

2.2.2.3. Test protocol. To 1ml urine sample add 2-4 drops of 10% Ferric chloride solution. Mix gently and observe the colour change.

A transient blue colour which rapidly fads within 1 min indicates positive test. Any other colour should be ignored.

2.2.2.4. Note. Avoided performing Ferric chloride test on icteric urine from jaundiced patient.

2.2.3. Test with Sodium Hydroxide solution

2.2.3.1. Principle. Homogentisic acid in alkaline medium undergoes oxidative polymerization to quinone derivatives which is black to brown in colour.

2.2.3.2. Reagent required. 1M/L Sodium Hydroxide solution: Dissolve 4gm NaOH in 100ml distilled water. Reagent is stable can be stored at RT. [Caution: Keep the flask in cold water bath as dissolution of NaOH in water is exothermic it produces heat].

2.2.3.3. Test protocol. Take 5ml of patients' urine sample and make distinctly alkaline by adding 1-2ml of 1M/L

NaOH and observe. [one can use KOH or K_2CO_3].

Urine will turn black within 20 to 60 seconds due to presence of HGA. Similar procedure when performed on normal urine as control will not produce any colour.

2.2.3.4. Note. It is important to note that the test is pH dependant i.e when the urine is made distinctly alkaline by adding NaOH, black Alkapton pigment are formed from HGA. Results are instant within 60seconds.

Advantage: Quick test and even dilute urine can give colouration.

Caution: Higher concentration of Uric acid may give gray colour.

2.2.4. Filter paper spot test

2.2.4.1. Principle. Homogentisic acid in alkaline medium undergoes oxidative polymerization to quinone derivatives which is black to brown in colour. Filter paper impregnated with alkali help polymerisation of HGA to black colour pigment.

2.2.4.2. Reagent required. A] 10% Sodium Hydroxide solution: Dissolve 10gm NaOH in 100ml distilled water to get 10% NaOH solution. Reagent is stable can be stored at RT. [Caution: Keep the flask in cold water bath as dissolution of NaOH in water is exothermic, produce heat]. B] Preparation of Alkali impregnated Filter paper: Take 3X3 square inch cut piece of Whatman filter paper, soak it in 10% NaOH solution and dry it by either hair dryer or keeping it in an incubator at 50 -70°C. Once the paper is totally dry it is ready for the Filter paper spot test.

2.2.4.3. Test procedure. Mark two circles on each 3X3 square inch strip of Whatman filter paper with lead pencil and label as patient urine and normal control urine. Add about 50microlit [2drops] of fresh urine sample into the respectively marked circle, observe for any colour change.

Urine containing HGA will react with alkali to produces black Alkapton pigment and the spot appear black. Whereas normal urine fails to give black spot.

2.2.4.4. Advantage. Quick and Simple test, can be used for population screening.

Caution: High level of uric acid may give a false positive test. Presence of Ascorbic acid will interfere in colour production.

2.2.5. Benedict's test for non-sugar reducing substances:¹⁹

2.2.5.1. Principle. Reducing sugar / non sugar reducing substance in alkaline medium reduces copper sulphate to cuprous oxide. Colour depends on quantity of reducing substance.

2.2.5.2. Reagent required . Benedict's qualitative reagent:

2.2.5.3. Test protocol. Take 5ml of Benedict's qualitative reagent, add eight drops of patient's urine and boil for 2min.

Red brown precipitate at the bottom of test tube suggest strong positive result. But instead of blue supernatant as usually obtained for reducing sugar, AKU patient's urine gives black coloured supernatant due to oxidation of HGA to quinone derivative.

2.2.5.4. Caution. Reducing sugar, Ascorbic acid, High level of uric acid or glucuronide may give Benedict's test positive. Result should be counter checked with Uristrip / Dipstrip for Glucose. Alkaptonuria gives Uristrip test negative.

2.2.6. Ammoniacal silver reduction spot test:[Tollen's reagent test]

2.2.6.1. Principle. HGA reduces alkaline ammoniacal silver nitrate solution to black coloured reduced metallic silver.

2.2.6.2. Reagent required. 5% Ammoniacal Silver Nitrate solution:²⁰ Ammoniacal Silver Nitrate solution is commercially NOT available due to its short shelf life. It should be prepared fresh in the laboratory at the time of test.

A] 4% NaOH solution: Dissolve 4gm NaOH in 100ml distilled water. Reagent is stable can be stored at RT. [Caution: Keep the flask in cold water bath as dissolution of NaOH in water is exothermic, produce heat].

B] 10% Ammonium Hydroxide solution: Dilute 10ml Ammonium Hydroxide solution (Liquor Ammonia) to 100ml with distilled water. [Caution: Strong ammonia solution may cause irritation of eye and nose].

C] 1gm% aqueous Silver nitrate solution: 1gm Silver nitrate salt is dissolved in 100ml distilled water.

2.2.6.3. 5% Ammoniacal Silver Nitrate solution. Preparation involves two steps. Step 1] Few drops of 4% sodium hydroxide solution are added to 5ml of 1% aqueous silver nitrate solution. The OH^- ions from sodium hydroxide react with the Ag^+ ions to give insoluble brown solid precipitates of silver oxide. In step 2] Add drop by drop 10% Ammonium hydroxide (aqueous ammonia solution) until all of the brown silver oxide precipitate is just dissolved. At this point the mixture will be clear. Now dilute the content to 50ml with distilled water. Filtering the reagent before use helps to prevent false-positive results. You can use same reagent for developing paper or TLC chromatogram spot of HGA or quinone.

2.2.6.4. Test protocol. Take 3X3 square inch cut piece of Whatman filter paper, Mark two circles with lead pencil. Label as patient's urine and normal control urine. Add 100microlit of fresh urine samples into the respective circles. Allow to dry, add 100microlit of freshly prepared

5% Ammoniacal silver nitrate solution and observe for any colour change.

Metallic Black spot develops immediately on addition of Ammoniacal silver nitrate solution due to reduction of silver by HGA.

2.2.6.5. Caution. High level of uric acid may give a false positive test but the colour produce is brownish black.

2.2.7. *N-butanol extraction test for HGA:*²¹

2.2.7.1. Principle. HGA can be extracted in aqueous organic phase which reacts with copper sulphate in alkaline medium to produce pink colour complex.

2.2.7.2. Reagents required. A] 01% CuSO₄ Solution: Dissolve 10mg CuSO₄ salt in distilled water.

B] 0.1N NaOH solution: Dissolve 4gm NaOH in 100ml distilled water. Reagent is stable can be stored at RT. [Caution: Keep the flask in cold water bath as dissolution of NaOH in water is exothermic, produce heat].

2.2.7.3. A] Concentrating and extraction of Homogentisic acid. Take 10 – 15 ml freshly voided urine sample in 50ml beaker. Keep the beaker in boiling water bath for evaporation, wait till 1/3 sample remains. To this concentrated urine add 3ml N- butanol and shake vigorously on vortex. Add 5ml of distilled water to the butanol extract and mix properly. This helps in the extraction of pigment in aqueous phase. Allow the content to settle for some time at room temperature. Two phases will separate out. Now collect aqueous fraction which contains most of the urinary pigment. Use aqueous fraction for colour reaction. Same extract can be used for spotting for Paper or Thin Layer chromatography.

2.2.7.4. B] Colour development. Test procedure: In a fresh tube take 2ml of aqueous extract; add 2 drops of (0.01%) CuSO₄, mix and add 5ml of distilled water followed by 2 drops of 0.1N NaOH. The tube is agitated after addition of alkali and kept aside for 20 min for colour development.

A pinkish brown colour developed indicate a positive result. (Intensity of colour is proportional to the concentration of Alkapton polymer present in urine). Aqueous solution of Hydroquinone or Benzoquinone can be used as Positive control test.

2.2.8. *Chromatography for HGA:*²²

2.2.8.1. Principle. HGA get partitioned based on its solubility in aqueous organic phase, which than can be visualized as black spot on spraying with Ammoniacal Silver nitrate solution.

2.2.8.2. Test protocol. Take Whitman filter paper no 1 used for chromatography. Prepare the chromatography paper for sample loading. Along with patient's urine sample, one spot

of the aqueous phase of N-butanol extraction, one normal urine (as control) and a quinone sample (as standard) are spotted at a distance of 1cm from bottom. Suspend the chromatography paper in chromatography jar containing a mixture of Butanol : Acetic acid : water in the ratio 4:1:5 as mobile phase. After allowing the mobile phase to run up to $\frac{3}{4}$ of paper, remove from jar. Air dry the chromatography paper and spray freshly prepared 5% solution of ammoniacal silver nitrate for colour development. HGA from the patient's urine, n-butanol extract and quinone gives instant black spot due to reduction with Silver, where as normal urine will not show any spot. Instead Whatman filter paper one can use Thin Layer chromatography plate to run chromatography.

Advancement in instrumentation have added few more methods like, Spectrophotometer, HPLC, Capillary electrophoresis,²³ Microbiological Guthrie test and Mass spectroscopy but, they are good for research but technically demanding.

3. Conclusion

Present technical review explains precise, time tested, simple and effective chemical methods for diagnosis of rare inborn error AKU at any primary centre, even this can be practised at bed side clinics. There are other advanced modern methods available now but looking into patients benefit and economy they are not adding any extra value for patients. Though symptoms and ochronotic complication appear at early adult age, detection of this rare disease by such simple tests at paediatric age will help to delay the complication by providing proper clinical help.

4. Source of Funding

None.

5. Conflict of Interest

None.

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Cite this article: Trivedi DJ. Detection of Alkaptonuria by simple, effective and precise chemical methods: A technical review. *Int J Clin Biochem Res* 2020;7(3):297-301.