Measurements of Homogentisic Acid levels in Alkaptonuria Patients Using an Optimized and Validated Gas Chromatography Method/Mass Spectrometry

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Abstract

Alkaptonuria is a very rare genetic disorder, characterized by a lack of homogentisate dioxygenase and causes accumulation of homogentisic acid. Clinical manifestations include dark urine, dark-black pigmentation of connective tissues (ochronosis), and arthritis of large joints and spine. The disease is usually diagnosed in adulthood by assessment of signs and symptoms of ochronosis. Confirmation of suspected diagnosis can be achieved by quantitative measurements of homogentisic acid levels. In this study, homogentisic acid was analyzed in 17 alkaptonuria patients. After liquid-liquid extraction, the analyte was determined by Gas Chromatography/ Mass Spectrometry (GC-MS) method. For quantitation purposes, external calibration was applied first, regression coefficient of ≥ 0.995 indicated the linearity in the concentration range of 1 -100 ng/µl. The instrumental detection limit (IDL) and lower limit of quantitation were 3.82 and 12.7 µg/L, respectively. Recovery rate was ≥ 89%. Precision given as relative standard deviation (RSD) ranged from 3 – 10 %. The results showed that the concentration of homogentisic acid ranged from 0.46 to 1.5 g/24 hours.

Keywords: Homogentisic acid, Black urine, Ochronosis, GC-MS, Jordan.

1. Introduction

Alkaptonuria (AKU) was designated by Sir Garrod as the first inherited metabolic disease (Garrod, 1908). AKU is a rare autosomal recessive disease caused by a deficiency of a specific enzyme, homogentisate 1,2 dioxygenase (HGD), leading to accumulation of homogentisic acid (HGA) (La Du, 1958). Most of the HGA is excreted in urine, and some is deposited in connective tissues as a melanine-like polymer in a process known as ochronosis (Zannoni et al., 1969). The pathophysiological mechanism of AKU is still unclear. Oxidative stress and amyloid formation may play a fundamental role in AKU. Indeed, recent studies have shown the presence of serum amyloid A (SAA) and serum amyloid P (SAP) in vitro and ex vivo AKU models and highlighted AKU as a secondary amyloidosis (Millucci et al., 2012; Braconi et al., 2013). The clinical features of AKU are characterized by homogentisic aciduria, blush-black discoloration of connective tissues and arthropathy of weight-bearing joints such as hips and knees and spondyloarthropathy (O’Brien et al., 1963). Complications of the disease include stones formation in kidneys, prostate, gall bladder and salivary glands, rupture of tendons, ligaments, muscles and cardiovascular manifestations (cardiac arrhythmias, aortic valve disease) (Phornphutkul et al., 2002). AKU severity score index (AKUSSI) have been developed for the first time as an assessment tool to quantitate disease severity, to compare the severity between AKU patients and to measure the progression of the disease (Ranganath, 2011; Cox, 2011).

AKU can be diagnosed at birth. The earliest clinical signs are dark urine and discoloration of nappies. However, some AKU patients are asymptomatic, and the majority of patients are diagnosed late in the third decade of life when they are affected by the ochronotic arthropathy (Ranganath et al., 2013). The urine of the
AKU patient turns dark-black when exposed to air. This reaction can be accelerated by alkalining urine (Castagna et al., 2006). Other screening tests include darkening of urine after adding ferric chloride (FeCl₃). Confirmation of diagnosis is established by the identification and quantification of urinary HGA using chromatographic techniques, such as gas chromatography mass spectrometry (GC-MS) and high-performance liquid chromatography (HPLC) (Abdulrazzaq et al., 2009; Bory et al., 1989).

The incidence of AKU around the world is as low as 1 in 250,000; however, high incidences have been reported in Slovakia and the Dominican Republic (1 in 19,000) (Srsen et al., 1978; Milch, 1960). The incidence of AKU in Jordan is unknown; however, recent studies have identified 60 cases with AKU. Most of these cases were identified in a small village in south Jordan, where nine AKU patients were diagnosed in the same family. The high rate of this rare genetic disease in Jordan is believed to be due to high rates of consanguineous marriages (Al-sbou et al., 2012a; Al-sbou et al., 2012b). Several methods have been used to measure HGA in subjects with AKU include chromatographic techniques such as spectrophotometric methods, high-performance liquid chromatography (HPLC), and gas chromatography mass spectrometry (GC-MS) (Seegmiller et al., 1961; Borry et al., 1989 Markus et al., 2001). The aim of this study is to describe the determination of urinary HGA using a sensitive and specific GC-MS method.

2. Materials and Methods

2.1. Chemicals and Standard Solutions

m-Methoxy-acetophenone (internal standard-I.S.) and N, O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) used for derivatization were purchased from Riedel-de Haën (Germany): n-Hexane, ethyl acetate, methanol and pyridine. Aldrich/ Germany. The following solvents of GC-grade were purchased from Riedel-de Haën (Germany): n-Hexane, ethyl acetate, methanol and pyridine.

2.2. Sampling and Sample Preparation

Twenty-four hour urine samples were collected from 17 AKU patients. Those patients were registered in the Jordanian Society of Alkaptonuria and were diagnosed having the disease based on results of laboratory investigations and clinical assessment. First, urine samples were left standing at room temperature for 48 h and were observed for changing the color. Urine samples of AKU patients turned dark-black upon standing as shown in Figure 1. Second, ferric chloride test was performed by adding one drop of ferric chloride solution and was positive if a transient blue color was observed. Clinical assessment and radiological examinations of patients were conducted to confirm the presence of signs and symptoms of ochronosis.

N,O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) is mostly used as derivatizing agent and it has a high reactivity. The derivatization of homogentisic acid compound for GC-MS analysis was performed according to (Zafra et al., 2006) with slight modification, in which a mixture of 20:5:25 (v/v/v) BSTFA-pyridine-ethyl acetate (containing homogentisic acid) is allowed to stand for 2 min at room temperature. The procedure is enough to get adequate derivatization. 1.0 mL of liquid urine sample was transferred into polypropylene test tube and 1g NaCl, 200μL of 5M HCl and 6mL ethyl acetate were added to the sample. The last mixture was shaken well for 10 min and centrifuged at 4000 rpm for 3min, and then the upper layer (ethyl acetate) transferred into vacuum test tube. The extracts were evaporated to dryness using a gentle stream of nitrogen. The residues were reconstituted in750 μL of 6.65 mg/L internal standard dissolved in ethyl acetate using. 250μL of the previous solution were mixed with50μL pyridine and 200μL BSTFA. The mixture was shaken well for two minutes in order to derivatize the phenolic compounds. Finally, a volume of 1μL of the prepared solution was injected into GC-MS instrument.

Figure 1. Urine samples from AKU patient, fresh (left) and after 48 hours (right).

2.3. GC-MS Analysis

The gas chromatographic analysis was performed using an Agilrent 6890 Series II. A gas chromatograph fitted with an auto sampler injector. A capillary column HP-5 fused silica column (30m × 0.25mm, film thickness 0.25μm,(5%)–biphenyl-(95%)–dimethylsiloxane copolymer) was used. A silanized injector liner split/splitless (2mm I.D.) was used. Detection was carried out with a 7683 mass-selective single quadrupole detector (Agilent Technologies). The injector temperature was 250°C. The oven temperature was held at 80°C for 3 min, and then increased to 240°C at a heating rate of 13°C min⁻¹, and the temperature was held for 20 min. The detector temperature was 280°C. The carrier gas used was helium (purity 99.9999%) at a flow rate of 1.0mL min⁻¹. The samples were injected in the splitless mode and the splitter was opened after 7 min (delay time). The sample volume in the direct injection mode was 1μL. The ion energy used for the electron impact ionization (EI) mode was 70 eV. The mass range was scanned from 150–550m/z. Single ion monitoring (SIM) acquisition mode was used (Deeb et al., 2012). The mass spectrum showed the molecular ions at m/z 384, 341 at 14.2 min and 150, 135 at 9.5 min which corresponds to the correct molecular formula C17H32O4Si3 (homogentisic acid) and C9H9O2 (I.S.), respectively as shown in Figure 2.
2.4. Analytical Quality Assurance

Standard mixture solutions of 1.0, 5.0, 20, 50, 100 mg/L of the derivatized homogentisic acid with 6.65mg/L IS were prepared in order to define the linear working range. Internal standard method was preferred in order to correct any loss of phenolic compound (homogentisic acid) during sample preparation. Regression coefficient ($r^2$) was in all cases $> 0.995$, indicating the linearity of the calibration function in this concentration ranges.

Extraction recovery of homogentisic acid was determined for urine samples at 3 spiking levels, 1, 20, 100µg/L, where the average recovery was found between 89 – 105%. Additionally, blanks were analyzed for background concentration. Precision of the method for homogentisic acid, calculated as relative standard deviation (RSD) was ranged between 2-9 %. The instrumental detection (IDL) was estimated form injections of standard solutions successively diluted until reaching a concentration level corresponding to a signal-to-noise ratio of 3. The method quantification limits (MQL) was determined form spiking urine sample, as the minimum detectable analyte concentration, which give signal-to-noise ratio of 10.

3. Results and Discussion

AKU patients in this study were 12 males and 5 females with a mean age of 29 years (age range 5-53 years). All patients had a history of dark urine since birth. Ferric Chloride test was positive for all patients. Quantitative measurements of urinary HGA showed that all patients excreted large quantities of HGA, the mean was (0.95 g/24 h). A GC/MS representative chromatogram for a real sample of AKU patient is shown in Figure 3.

There was a marked variation in the concentration of HGA excreted between different AKU patients (a 3-fold variation) and the range was (0.46 - 1.5 g/24 h) (Table 1). This variation could be due to different dietary protein content between different individuals.

<table>
<thead>
<tr>
<th>Case No</th>
<th>Age (Years)</th>
<th>Sex</th>
<th>Urinary HGA g/24 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>M</td>
<td>0.46</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>M</td>
<td>0.53</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>M</td>
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</tr>
<tr>
<td>4</td>
<td>8</td>
<td>F</td>
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</tr>
<tr>
<td>5</td>
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<td>M</td>
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</tr>
<tr>
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<td>24</td>
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<td>7</td>
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</tr>
<tr>
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</table>

M=male, F=female

AKU is a very rare genetic condition and in most cases it is misdiagnosed at childhood. Few signs and symptoms of this disease appear before the 3rd decade of life, apart from passage of dark urine.
The reason for the delay appearance of ochronotic manifestations is unknown. Therefore, the majority of cases with AKU are recognized in adulthood (Ranganath et al., 2013). Homogentisic acid is a normal intermediate in the metabolism of tyrosine and normal individuals do not excrete HGA because it is converted into maleylacetoacetic acid by the homogentisate 1,2 dioxygenase. AKU patients excrete high concentrations of HGA in urine (range 4-8 g/day) (Castagna et al., 2006). Therefore, testing urine samples for the presence of HGA is crucial for establishing the diagnosis of AKU. Some screening tests can provide help in diagnosis of AKU such as darkening of urine after addition of sodium hydroxide, and ferric chloride test. This test can be used to detect metabolites in urine samples of patients with inborn error of metabolism diseases such as AKU (Frohlich et al., 1973). The basis of this test is that phenols of HGA form a violet complex with Fe (III), which is intensely colored, therefore, the urine containing HGA yields a transient blue color after adding few drops of ferric chloride solution. In this study, this test was positive for all tested AKU patients, thus it can be used to help in screening for AKU. However, confirmation of diagnosis of AKU can be achieved by specific identification and quantification of HGA in urine and blood samples.

The present Gas Chromatography/ Mass Spectrometry method was developed for the determination of homogentisic acid in AKU patient’s urine; in addition the derivatization of homogentisic acid compound was performed with a slight modification to Zafra’s method (Zafra et al., 2006). The main advantage of this method includes its high sensitivity, with MQL for HGA in urine as low as 12.7 µg/ L, also this method showed a very good separation of analyte (homogentisic acid) in less than 15 min.

4. Conclusion

The described method represents a useful and suitable analytical tool that can be used for diagnosing AKU and for the monitoring treatment effect on HGA levels in AKU patients.

References


