Diagnosis of Alkaptonuria by NMR Urinalysis: Rapid Qualitative and Quantitative Analysis of Homogentisic Acid

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Alkaptonuria is an inborn error of metabolism characterized by ochronosis as dark deposits in the sclerae, early and severe osteoarthritis, and high incidence of heart disease. They excrete dark urine because of homogentisic aciduria that results from homogentisic acid oxidase deficiency. Biochemically, homogentisic acid has been identified by paper chromatography (Knox and LeMay-Knox 1951) or thin layer chromatography (Sankoff and Sourkes 1963). Quantitative analysis is only performed by enzymatic method, which needs a cumbersome procedure (Seegmiller et al. 1961).

Currently proton nuclear magnetic resonance spectroscopy (\(^1\)H-NMR) is reported as a useful method for the detection of metabolites in urine, especially in case of inborn error of metabolism (Yamaguchi et al. 1984, 1985; Iles et al. 1985). \(^1\)H-NMR urinalysis has several advantages; 1) the quick procedure with untreated urine samples, 2) simultaneous analysis of metabolites such as amino acids, organic acids, carbohydrates and others.

Qualitative and quantitative analysis of homogentisic acid with NMR urinalysis was studied in the patients with alkaptonuria. The diagnosis of alkaptonuria using this technique is easier than the methods ever established.

Method and Materials

Control urine specimens were collected from children without any known metabolic or neurologic disorders. Urine samples from two patients with alkaptonuria were provided by Dr. William L Nyhan, University of California and Dr. Hatae Maesaka, Kanagawa Children’s Medical Center. Aliquots of untreated urine, 0.45 mL, were placed with 0.05 mL of 10 mM trimethylsilylpropionate-2, 2, 3, 3, -d\(_4\) (TSP-d\(_4\), MSD Isotopes, Montreal, Quebec, Canada) in D\(_2\)O as an internal standard in 5 mm i.d. NMR tubes. \(^1\)H-NMR was recorded at 89.55 MHz with JEOL FX-90Q as previously described (Yamaguchi et al. 1984).

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Chemical shifts (ppm) were referred to TSP-d$_4$. Authentic standard of homogentisic acid was dissolved in 10 mM phosphate buffered saline, pH 7.4.

**RESULTS AND DISCUSSION**

$^1$H-NMR spectra in the urine of normal control subjects (Fig. 1a) showed signals for water (3.5 to 5.3 ppm) and creatine plus creatinine (3.0 ppm). Since the signals of water not necessary for our purposes, they were not recorded. $^1$H-NMR spectra of authentic homogentisic acid were shown as two separated signals, 6.76 ppm and 6.78 ppm. In the urine of a patient with alkaptonuria, two signals of homogentisic acid were observed as chemical shift 6.76 ppm and 6.78 ppm (Fig. 1b). The dependency of homogentisic acid to the intensity of the signals, 6.76 ppm, was studied. Linearity was observed between 5 mM to 125 mM of homogentisic acid and when applied to the signal of 6.78 ppm, a similar linearity was obtained. The amount of homogentisic acid in calculated from the division of the intensities of signals of homogentisic acid and TSP-d$_4$. Relative or absolute concentration in calculated with creatinine or with daily urine volume.

This procedure needed only 15 minutes, which is far rapider than that of the enzymatic ones. It is advantages to require very small amount of urine, not to need pretreatment and to be able to make a qualitative and quantitative determination simultaneously.

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**References**