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Short communication

Quantitation of homogentisic acid in normal human plasma

John C. Deutsch*, Cheruppolil R. Santhosh-Kumar

Divisions of Gastroenterology and Hematology, University of Colorado Health Sciences Center, 4200 East Ninth Avenue, Campus Box B-170, Denver, CO 80220, USA

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Abstract

A new stable isotope dilution gas chromatograph–mass spectrometric method of analysis of homogentisic acid is described. Using this method, homogentisic acid is measured for the first time in normal human plasma. The assay of sera from nine normal individuals yielded a range of values from 2.4 to 12 ng/ml. The method appears to be very sensitive and may be useful in the characterization of heterozygotes for alkaptonuria and other disorders of tyrosine degradation.

Keywords: Homogentisic acid

1. Introduction

Homogentisic acid is a normal intermediate in the metabolism of tyrosine. Alkaptonuria is a rare autosomal recessive disease in which there is an absence of homogentisic acid oxidase [1,2]. This condition leads to a massive increase in plasma levels of homogentisic acid which then can directly deposit or oxidize and deposit in tissues resulting primarily in arthropathies, but also with other manifestations including cardiovascular and renal disease [1–4]. Although the abnormal gene has been mapped to chromosome 3q [5] it is not currently possible to identify heterozygotes with this condition and it is unclear if heterozygotes have diseases related to the genetic abnormality. Homogentisic acid oxidase is very efficient, and it is likely that heterozygotes do not have a marked increase in homogentisic acid levels, making identification of these subjects difficult with current clinical parameters. Furthermore, homogentisic acid is unstable, and oxidizes readily [6]. Because of that, it has not been possible to measure homogentisic acid content in the blood of normals or obligate heterozygotes [7,8].

This report describes the determination of homogentisic acid in normal human plasma using a sensitive and specific GC–MS isotope dilution assay. This method has the potential to identify heterozygotes and to better define the potential health consequences of mild increases in homogentisic acid levels.

2. Experimental

2.1. Chemicals

[1,2-$^{13}C_2$]Homogentisic acid (99% [^{13}C]) was custom-synthesized by MSD Isotopes (Montreal, Canada). AGMP-1 resin was purchased from BioRad

*Corresponding author.

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Laboratories (Hercules, CA, USA). Homogentisic acid and other reagents of of the highest grade available were obtained from Sigma Chemicals (St. Louis, MO, USA), Aldrich Chemicals (Milwaukee, WI, USA) and Fisher Chemicals (Pittsburgh, PA, USA). N-Methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide (TBDMS) was obtained from Regis Chemicals (Morton Grove, IL, USA). [1,2-$^{13}C_2$]-Homogentisic acid was made up as a 50 ng/ml solution in water and stored at −20°C until use. The quantity of [1,2-$^{13}C_2$]homogentisic acid added to samples was back-quantitated with freshly prepared solutions of homogentisic acid to account for potential degradation of stock solutions. However, stock solutions of [1,2-$^{13}C_2$]homogentisic acid had less than 20% degradation with repeated freeze–thaw cycles over a four-month period.

2.2. Sample derivatization

Samples were prepared by drying in a Savant (Farmingdale, NY, USA) vacuum centrifuge at 60°C, following which the dried aliquots were derivatized by adding 15 μl of TBDMS and 35 μl of acetonitrile and incubating the samples for 1 h at 60°C.

2.3. Instrumentation

Samples (2 μl) were applied to a Hewlett-Packard (Avondale, PA, USA) 5890 gas chromatograph. Gas chromatography was carried out through a Supelco (Bellefonte, PA, USA) 10-m fused-silica [poly(dimethylsiloxane)] capillary column (0.25 mm I.D.) with a film thickness of 0.25 μm using a temperature ramp of 30°C/min from 80 to 300°C with helium as a carrier, and mass spectrometry was performed on a Hewlett-Packard 5971 A mass spectrometer. The scan mode was used to obtain full spectra (including the $[M-57]^+$ ion) and appropriate retention times. Analysis was carried out by scanning and by selected ion monitoring at the respective $[M-57]^+$ ions with the electron multiplier set at 1800 V. Isotopic spillover was corrected for mathematically. Quantation was determined based on peak area of *m/z* 453 derived from the endogenous homogentisic acid relative to the peak area of *m/z* 455 derived from the [$^{13}C_2$]homogentisic acid internal standard.

2.4. Sample preparation

Approval for collecting plasma from human subjects was obtained from the Combined Multi-Institutional Internal Review Board located at the University of Colorado Health Sciences Center. Blood was collected in purple-top (K_3EDTA) Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA). Plasma was separated from cells by centrifugation at 3000 *g* for 10 min, and plasma was stored at 4°C for use within 12 h, or stored at −70°C. Plasma was separated into 1.00 ml aliquots in 12 × 75 mm borosilicate glass tubes to which was added 250 ng of [1,2-$^{13}C_2$]homogentisic acid. When indicated, 5–20 ng of homogentisic acid was also added. Samples were gently mixed, and 2 ml of a buffer (consisting of 50% (v/v) methanol, 770 m*M* ammonium acetate, 100 m*M* 2-mercaptoethanol, pH 7.7) were added. Samples were passed over 50 mg of AGMP-1 resin (100–200 mesh chloride form), placed in 4-ml columns that had a frit installed (Alltech Associates, Deerfield, IL, USA) and were washed with 9 ml of water. Samples were then eluted with 1.0 ml of 4 *M* acetic acid, 0.1 *M* HCl into borosilicate tubes and were processed as described above except that derivatization was performed at 55°C. GC–MS analysis was performed with the electron multiplier set at 2100 V. Experiments were done in triplicate. The mean values and standard deviations were determined on relative ion abundances where indicated.

3. Results

Based on the structure of homogentisic acid, a predicted mass of the TBDMS-derivatized compound is 510 [9,10], and the $[M-57]^+$ ion is *m/z* 453. Fig. 1 shows the spectra obtained from TBDMS-derivatized homogentisic acid and [1,2-$^{13}C_2$]homogentisic acid, both of which show a good proportionate abundance of their predicted $[M-57]^+$ ions. To ensure that gentisic acid would not impede homogentisic acid analysis [11], a standard of gentisic acid was analyzed by GC–MS. Gentisic acid eluted 0.12 min ahead of homogentisic acid and had an $[M-57]^+$ ion of *m/z* 439. There was no interference or overlap in either the chromatogram or spectrum between homogentisic acid and gentisic acid.

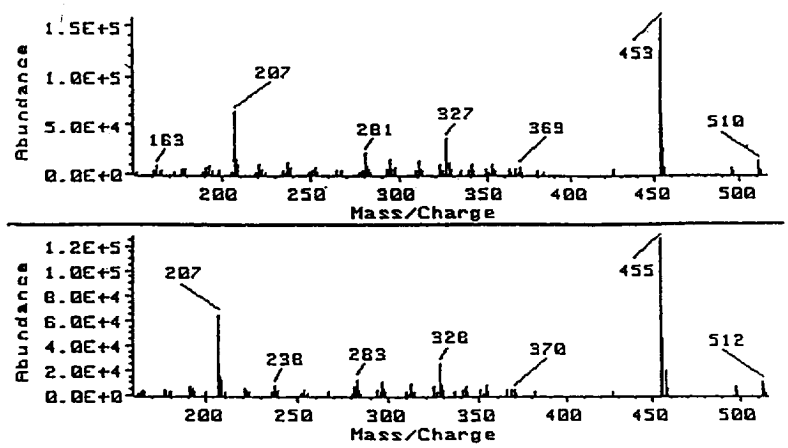

Fig. 1. The spectra derived from TBDMS-derivatized homogentisic acid (top) and [1,2-$^{13}C_2$]homogentisic acid (bottom).

Selected ion monitoring (SIM) analysis (*m/z* 453, 455) of a mixture of homogentisic acid and [1,2-$^{13}C_2$]homogentisic acid is shown in Fig. 2A and Fig. 2B while an example of a chromatogram of plasma homogentisic acid (*m/z* 453) is shown in Fig. 2C. There was a substance that eluted just after homogentisic acid. This extra peak did not appear to interfere with the analysis at the levels found in normal human plasma, as shown in Table 1. In addition, this substance did not contain the ions *m/z*

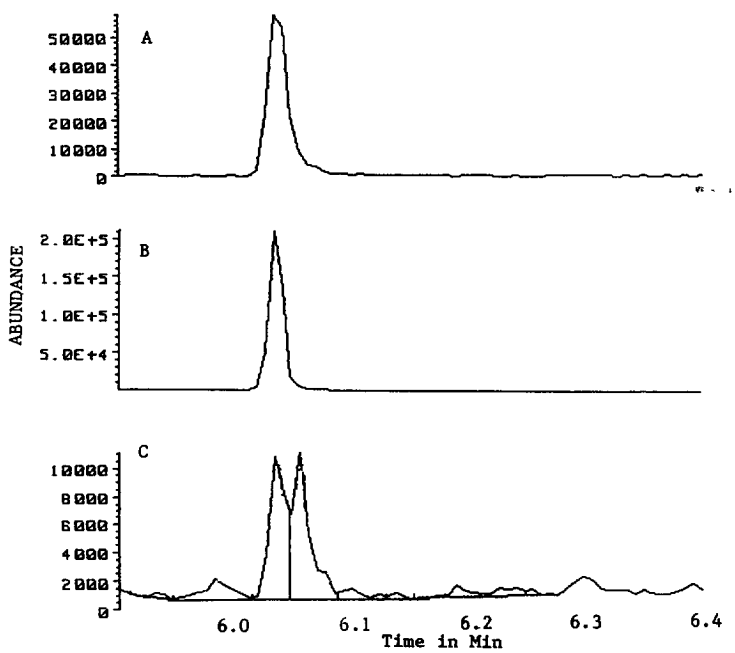

Fig. 2. (A) A chromatogram that arises during selected ion monitoring of *m/z* 453 from TBDMS-derivatized homogentisic acid standard. (B) A chromatogram that arises during selected ion monitoring of *m/z* 455 from TBDMS-derivatized [1,2-$^{13}C_2$]homogentisic acid standard. (C) A chromatogram that arises during selected ion monitoring of *m/z* 453 from TBDMS-derivatized serum. The first peak is from endogenous homogentisic acid. The second, later eluting, peak is from an unknown substance. This second peak is not seen when monitoring for *m/z* 327 (homogentisic acid, Fig. 1) and *m/z* 328 ([1,2-$^{13}C_2$]homogentisic acid, Fig. 1).

Table 1
Increment in measured homogentisic acid values when known quantities of homogentisic acid are added to plasma

Added (ng/ml)	Measured (ng/ml)
0	12.0 ± 0.35
5	16.1 ± 0.67
10	22.1 ± 1.4
15	28.3 ± 1.5
20	34.0 ± 2.2

327/328 (another ion derived from homogentisic acid).

A 15-ml sample of plasma was collected from a single individual and was subsequently divided into fifteen separate 1-ml aliquots. Either 0, 5, 10, 15 or 20 ng of homogentisic acid were added to three of the 1-ml aliquots, and all fifteen samples had their homogentisic acid content determined. As shown in Table 1, the endogenous level of homogentisic acid was 12 ng/ml. As also shown in Table 1, with the addition of homogentisic acid, there was a linear increase in the measured amount of homogentisic acid as would be predicted based on the added quantities.

Finally, plasma stored at −70° for less than 30 days from nine normal subjects had homogentisic acid values determined. These values ranged from 2.4 to 12.0 ng/ml.

4. Discussion

The measurement of homogentisic acid in normal individuals has been difficult in the past due to a variety of factors, including the instability of homogentisic acid and the low levels found in plasma. Although several methods are available to measure homogentisic acid in subjects with alkaptonuria [7,8,11–14], these methods do not appear to be sensitive enough to detect homogentisic acid in normal plasma or in the plasma of obligate heterozygotes [8,13,14]. GC–MS is a useful method in these circumstances, particularly when a suitable internal standard can be found [15]. When care is taken during the purification procedure, it is now possible to measure homogentisic acid in a specific and accurate fashion in normal individuals. Plasma levels of 5–10 ng/ml (approximately 0.05 μmol/l) were found in the current study, which is 1/1000th of the level reported in homozygote individuals [8] and is well below the limit of detection reported (320 ng/ml, 1.9 μmol/l) for HPLC methods [13].

Although a peak was noted at m/z 453 which eluted just after homogentisic acid, this peak did not appear to interfere with our analysis, based on the data shown in Table 1. Although a longer column could resolve this peak better, we found that by using other ions derived from homogentisic acid/[$^{13}C_2$]homogentisic acid (m/z 327/328) we could avoid the substance that eluted later.

Although alkaptonuria is a rare condition, the absolute frequency of heterozygous individuals and clinical conditions associated with heterozygosity are not currently defined. Furthermore, the degradation of tyrosine is altered in other genetic and acquired diseases including cirrhosis from any cause [16]. It is also important to note that tyrosine degradation involves several ascorbic acid dependent steps [16]. It is possible that determination of tyrosine-related degradative products will provide information on nutritional status.

The method described in this manuscript should be useful to better define the clinical spectrum of conditions in which there is a modest elevation of homogentisic acid. We are currently investigating how homogentisic metabolism is related to tyrosine degradation in liver disease and nutritional deficiency states.

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