Profiles in Altered Metabolism
II—Accumulation of Homogentisic Acid in Serum and Urine Following Acetylsalicylic Acid Ingestion†

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Homogentisic acid elevations have been found in urine and serum of patients on long term heavy salicylate therapy and those acutely intoxicated with acetylsalicylic acid. Loading of two normal volunteers with single oral acetylsalicylate doses produced transient elevations in urinary homogentisic acid excretion. These findings suggest that heavy salicylate use results in the partial inhibition of homogentisic acid oxidase in vivo.

INTRODUCTION

The serum of a patient recently diagnosed with acute acetylsalicylic acid (ASA) intoxication was found to contain not only the expected high concentrations of salicylic acid (SA) and its metabolite gentisic acid (GA) but also an abnormally high concentration of homogentisic acid (HGA) corresponding to 1.3 µg ml⁻¹ serum. A subsequent review of the urinary and serum organic acid gas chromatographic profiles accumulated over several years for this type of patient has revealed variable but significantly elevated HGA concentrations in these fluids. To our knowledge HGA accumulation has not been reported following ASA use or misuse, and since HGA is one methylene unit larger than the SA and GA molecules and therefore not a direct metabolite, it seemed of interest to determine the circumstances of its elevation in the otherwise healthy human.

To ascertain whether the accumulation of HGA was a normal consequence of ASA use, we followed the time course of urinary HGA excretion in two normal volunteers after a large oral ASA load. In addition, sera of patients currently presenting with ASA intoxication along with sera and urine of patients receiving high therapeutic doses of ASA for arthritic conditions were analysed for HGA.

The quantities of HGA were measured by an isotope dilution technique using α,α-[²H₂] homogentisic acid as the internal standard and selected ion monitoring to determine the amounts of endogenous HGA present relative to the deuterium labelled analogue.

We wish to report briefly the results of these early studies.


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EXPERIMENTAL

Preparation of α,α-[²H₂]homogentisic acid

Homogentisic acid (100 mg) was dissolved in 4 ml of 1 N sodium deuterioxide in deuterium oxide containing approximately 5 mg of sodium borohydride to maintain a reducing medium. To prevent air oxidation of the acid it was essential to purge the exchanging medium of traces of oxygen with a nitrogen bubbler prior to the addition of the HGA and to rigorously exclude oxygen during the reaction by maintaining the mixture under a nitrogen atmosphere. Failure to carry out this procedure leads to rapid blackening of the mixture and the destruction of the HGA. The reaction mixture was heated in a boiling water bath for 15 h. The resulting straw-coloured mixture was then cooled in an ice–methanol slurry, rapidly acidified with dilute hydrochloric acid in protium oxide and extracted with ether. The ether extract was evaporated to dryness under a stream of nitrogen.

Recrystallization of the crude solid from an ethyl acetate–petroleum ether solvent pair yielded a light buff powder that was analysed as the trimethylsilyl (TMS) derivative by GCMS ([M]⁺ = 386). It was confirmed to be HGA approximately 98% labelled with two deuterium atoms on the carbon adjacent to the carboxyl group. The remaining 2% appeared to be α-[²H₂]HGA, and there was no detectable unlabelled HGA.

The ease of isotope re-exchange was tested by dissolving the labelled acid in distilled water and allowing it to stand at 25 °C for 15 h. An ether extract was made and analysed as described above and there was no detectable loss of labelling.

Patient studies

Two volunteers ingested ASA early after rising (A, 41.0 mg kg⁻¹; B, 36.0 mg kg⁻¹). Urine samples were collected frequently during the day and were stored frozen.
Four patients receiving ASA in the range of 2–2.5 g per day in divided doses for arthritic conditions, but otherwise healthy and receiving a normal diet were also selected. Random urine and serum samples were collected and frozen for later analysis.

Eight patients admitted to the emergency department of Royal Victoria Hospital with acute ASA intoxication were also included in this study. Residues of serum samples submitted for emergency salicylate determinations (a routine ferric chloride–colourimetric method) were kept that had high levels of salicylate detected. Urine samples taken during the first 12 h following admission were not available for analysis.

Analysis of urine and serum samples

To measured volumes of serum or urine (1–2 ml, depending on quantity available) was added α,α-[2H₂]HGA in ethyl acetate (conc. 2 mg ml⁻¹) in the proportion of 10 µg ml⁻¹ of sample volume. The serum samples were then deproteinized by the addition of a few drops of saturated aqueous sulphosalicylic acid. The supernatant fractions were then treated in a manner identical to that described here for the urine samples.

The samples were saturated with sodium chloride and acidified with 10% hydrochloric acid. Following ether extraction, the organic layer was dried over anhydrous magnesium sulphate and evaporated to dryness in a nitrogen stream. The samples were then deproteinized by the addition of a few drops of saturated aqueous sulphosalicylic acid. The supernatant fractions were then treated in a manner identical to that described here for the urine samples.

Analysis of urine and serum samples

The mass spectra of the tris-TMS derivatives of HGA and m-HPHA very nearly co-eluting with the HGA peak. The TMS derivative of m-HPHA had an [M⁺-CH₃]⁺ ion at m/e 383 and because of natural heavy isotope substitution, the attendant ion at m/e 384 interfered with the measurement of HGA. A 15% OV-101 column of 2 mm length effected sufficient resolution on GC of the two acids, however, to eliminate this problem altogether. To ensure that m-HPHA did not interfere as the analyses progressed, m/e 383 was monitored in each analysis together with m/e 384 and 386.

Known quantities of unlabelled HGA added to normal serum samples showed the extraction efficiency to be approximately 95%.

The patients with ASA overdose showed a wide range of serum HGA levels (µg ml⁻¹: 0.05, 0.02, 0.08, 0.10, 0.19, 1.30, 2.86 and 5.40, 5.80). Clear that heavy ASA use results in the

| Table 1. Homogentisic acid concentrations in serum and urine of patients taking acetylsalicylic acid in arthritis therapy and in overdose quantities |
|------------------|------------------|---------------------|
|                   | Homogentisic acid concentration (µg ml⁻¹) | Homogentisic acid concentration (µg g⁻¹ creatinine) |
| Patients          | Serum                  | Urine                     |
| Arthritis therapy | 0.11 ± 0.08            | 2.65 ± 0.96               |
| n = 4             |                        |                             |
| Overdose          | 1.3 ± 1.93            | NA                        |
| n = 8             |                        |                             |
| Controls          | 0.12 ± 0.11            | 0.74 ± 0.59               |
| n = 10            |                        |                             |

* Patients were receiving between 2 and 2.5 g ASA per day, NA. Early urines from overdose patients were not available.
accumulation of HGA in serum. The HGA levels did not show any correspondence with the measured levels of serum GA or SA.

The arthritic patients on continuous high ASA therapy exhibited a nearly four-fold increase in mean urinary HGA excretion but failed to show a corresponding significant increase in serum concentrations when compared with the controls. This may simply be the result of altered excretion of HGA under the mild acidosis usually precipitated by heavy ASA use. Alternatively, when the ASA is ingested in divided doses over the period of a day, SA may not reach the levels in serum required to produce a transient rate of accumulation of HGA greater than its rate of excretion.

The analysis of the serial urines collected following single oral ASA loads in the two normal volunteers revealed the expected large concentrations of SA and GA which peaked simultaneously approximately 3 h after the loadings (Fig. 2). HGA was seen to pass through its maximum urinary concentration 2 h later and then fall to near normal levels by the time of the next urine sample. This suggests that the time of sampling is very important if large concentrations of HGA are to be found. In the overdose group the time elapsing between the taking of the ASA and the taking of the serum, as well as the quantity of ASA remaining following gastric lavage are not known accurately. This is probably the reason for the wide range of HGA concentrations and their lack of correspondence with SA or GA levels found in this group.

Although GA is not metabolized by HGA oxidase, it may through its structural similarity to HGA act as an inhibitor by competing for the binding site on the enzyme. HGA oxidase is the sole enzyme responsible for the oxidation of HGA to maleylacetoacetic acid and is a very specific enzyme requiring oxygen, ferrous ion, sulfhydryl groups and pH 7 to be effective. Whether the inhibition of the enzyme is direct or through an alteration of one of its requirements is not known.

Our findings do not eliminate the possibility that other salicylate metabolites may be responsible for the inhibition of HGA oxidase. On occasion small gas chromatographic peaks having retention times and mass spectra identical to those for 2,3- and 2,4-dihydroxybenzoic acids were noted in the urines of the arthritic patients. These could be potent inhibitors of HGA oxidase even though SA and GA are present in overwhelmingly greater concentration.

Alcaptonuria, an hereditary metabolic disorder, results in the excretion of large amounts of HGA into the urine (up to 8 g per day) and is the result of a single autosomal recessive gene leading to the constitutive lack of active HGA oxidase. Residual activity in homozygous alcaptonuric patients may be inhibited completely by ASA therapy prescribed for the arthritis that commonly develops in this disorder in middle life. ASA and other salicylates should possibly be avoided therefore in favour of other analgesics.

Acknowledgements

The authors wish to thank Dr Paul Ryan of the Department of Rheumatology and Miss Joan Ohashi of the Department of Clinical Biochemistry, all of Royal Victoria Hospital who kindly provided the urine and serum samples of patients with arthritis on ASA therapy and the serum samples of patients with acute ASA intoxication respectively. This work was supported by the Medical Research Council of Canada.

REFERENCES


Received 3 June 1977
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