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Interferences of homogentisic acid (HGA) on routine clinical chemistry assays in serum and urine and the implications for biochemical monitoring of patients with alkaptonuria

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ABSTRACT

Objectives: We have assessed the effect of elevated concentrations of homogentisic acid (HGA) as in alkaptonuria (AKU), on a range of routine chemistry tests in serum and urine.

Design and methods: HGA was added to pooled serum and a range of assays was analysed with Roche Modular chemistries. Effects on urine were assessed by diluting normal urine with urine from a patient with AKU, adding HGA to urine and after lowering output of urinary HGA with nitisinone treatment.

Results: Serum enzymatic creatinine showed 30% negative interference with 100 μmol/L HGA and >50% at 400 μmol/L. Serum urate 100 to 480 μmol/L was reduced up to 20% at 100 and to 50% with 400 μmol/L HGA. Serum cholesterol between 3 and 11 mmol/L was reduced by 0.5 mmol/L with 400 μmol/L HGA. Urine enzymatic creatinine and urate with >2 mmol/L HGA showed concentration dependent negative interference up to 80%. A positive interference in urine total protein by benzethonium turbidometric assay was observed, with 10 mmol/L HGA equivalent to 1 g/L protein. Jaffe creatinine, Na, K, Cl, Mg, Ca, phosphate, ALT, GGT, ALP activities and urea in serum and or urine were not affected by increases in HGA.

Conclusions: To avoid interferences by HGA in alkaptonuria concentration of HGA should be established before samples are assayed with peroxidase assays and benzethonium urine protein.

Introduction

Alkaptonuria (AKU) is an inborn error of metabolism caused by homogentisate-1,2-dioxygenase deficiency [1]. The block in phenylalanine metabolism results in the accumulation of homogentisic acid (HGA) and other hydroxyphenyl acid intermediates. The metabolic consequence is elevated HGA in plasma, 39 μmol/L ± 10 mean (±1 SD), in normal plasma it is undetectable (<1 μmol/L) [2] and in urine there is increased HGA excretion up to 50 mmol/L [3]. In an animal model of AKU plasma HGA concentrations may be as high 200 μmol/L [2].

Interference effects from such elevated concentrations of HGA in urine have been well documented in particular in the measurement of urate, oxalate and enzymatic creatinine [4–6]. Other dihydroxy phenol type reducing agents e.g. dopamine and dobutamine have been shown to affect hydrogen peroxide based reactions for serum assays of urate, enzymatic creatinine, total cholesterol and glucose using glucose oxidase [7]. These effects have been described because of sampling of blood from drip arms where infusions of such ionotropes can be very high. Interference from coloured substances is also possible e.g. bilirubin (yellow) interference with the Jaffe creatinine assay (orange) [8].

It is important therefore to know the chemistry of the assays involved and to evaluate possible interference with as many routine biochemical investigations as possible. Thus other assays which involve reduction may be affected e.g. the detection of protein by colour development at high pH with reduction of cupric ions Cu^{2+} to cuprous ions Cu^{1+} (the biuret reaction) coupled with the highly sensitive and selective colourimetric detection of the cuprous cation (Cu^{1+}) using bicinchoninic acid [9]. Interference from reducing substances with strip assays for urine testing is well described e.g. high levels of ascorbic acid (Vitamin C) inhibit reagent strip reactions, such as glucose, blood, bilirubin, nitrate and leukocyte esterase [10]. The dipstick's package insert provides information about a range of potential interfering substances, including ascorbic acid and salicylate.

In AKU the urine darkens on standing as HGA a reducing agent with 2 phenolic groups oxidises to benzoquinone acetic acid (oxidising agent) which forms a melanin-like pigment [1]. This intensely coloured urine makes it difficult to correctly interpret colour reactions on the dipstick. Indeed it is recommended that tests on such urines should not be reported using dipstick technology and to use an alternative method of testing if available.

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With the development of new instrumentation using improved technology and optimised assays the impact of metabolic derangements requires constant re-assessment on a wide range of routine chemical measurements in both serum and urine. We have therefore studied the effect of increasing HGA in serum and urine on a range of biochemical assays. The effects in serum assays were assessed by adding in known amounts of HGA into pooled sera from patients without AKU. The HGA concentrations for the serum experiments covered a wide range to cover the possibility that concentrations may be higher than thought due to data obtained on samples after long term storage (months/years) with subsequent deterioration of HGA [2].

The effects in urine were assessed by three procedures; 1) mixing various urines from non-AKU patients with urine from a patient with alkaptonuria; 2) adding in known amounts of HGA into non-AKU urines; and 3) analysis of urine collected after treatment with nitisinone to reduce the output of HGA.

Methods

The chemicals were obtained as the Aristar grade from Sigma Chemical Co. (Poole, Dorset, UK). Solutions were prepared using high grade pure double deionised water (Ultra High Q, Elga Products, High Wycombe, UK). Stock solutions of HGA from 0 to 100 mmol/L, were prepared by dissolving in water and prepared freshly before use.

Blood and urine samples were taken from patients without AKU and those with AKU (as diagnosed by LR) in accordance with local ethical guidelines as part of routine clinical management request profiles. Nitisinone (NTBC) was provided by Swedish Orphan Biovitrum International (Stockholm, Sweden) and given to AKU patients at the National Alkaptonuria Centre, Liverpool as 2 mg tablets once daily according to information provided by the supplier specifically for the treatment of alkaptonuria. Blood samples were taken into 2.7 mL fluoride-EDTA (yellow top tubes) for plasma glucose and lactate and serum (brown top tubes, Sarstedt Ltd., Leicester, UK) for the other analytes. The 24 h urine samples were collected into plain plastic 2.5 L volume bottles with added preservative (30 mL of 2 M H_2SO4) and taken pre, 2 days and 3 months post nitisinone treatment in 10 patients.

Interference of HGA in routine serum/plasma biochemistry measurements was investigated by taking aliquots (9 parts) of pooled samples from several non-AKU patients to give varying concentrations of analytes and spiking with freshly prepared solution of HGA (1 part) to give final concentrations of 0, 50, 100 and 400 μmol/L. The samples were assayed for the electrolytes sodium (Na) and potassium (K), calcium (Ca), magnesium (Mg), urea, creatinine, alkaline phosphatase (ALP), alanine amino transferase (ALT), gamma glutamyl transferase (GGT), total protein and albumin, total and HDL cholesterol, glucose and lactate. The effect of elevated HGA on urinary measurements of urea, creatinine (enzymatic and Jaffe), protein, electrolytes, microalbumin, phosphate, magnesium and urate was investigated by three separate procedures; 1) by dilution of normal urine samples ($n = 7$ to $n = 12$ depending on analyte) with an AKU urine containing approximately 20 mmol/L HGA (to give 0%, 33%, 67% and 100% AKU); 2) HGA was supplemented from 0.5 to 10 mmol/L (final concentration) into a series of 11 urines from normal (non-AKU) individuals (in the ratio of 1 part HGA solution to 9 parts urine) for assay of urate, creatinine and total protein using two separate methods as indicated below and 3) urine samples collected from AKU patients ($n = 6$) after commencement with nitisinone (NTBC) pre, 1, 2 days and 3 months of treatment.

Assays were established routine procedures on Roche modular platforms (Cobas-Roche Diagnostics, West Sussex, England) unless stated. A summary of each method is given below to indicate the chemistry involved. These assays usually employ readings at two wavelengths (biochromatic) first as a monitor of any fluctuation in lamp intensity and a second wavelength for measurement of the analyte and indicated as 700/540 nm. Assays of a specific analyte were by endpoint measurement and those of enzyme activity were by rate monitoring.

Alkaline phosphatase (ALP) activity used the hydrolysis of nitrophenyl phosphate to p-nitro phenol measured at 480/450 nm. Alanine amino transferase (ALT) by the production of pyruvate and reduction to lactate was measured by increased NAD at 700/340 nm. Gamma glutamyl transferase (GGT) colourimetric assay used the increase in absorbance at 700/415 nm of 5-amino-2-nitro benzoate by transference of L-glutamyl to glycylglycine from L-glutamyl-3-carboxy-4-nitroanilide.

Cholesterol using cholesterol oxidase and the production of H_2O_2 were measured by reaction of aminophenazone and phenol with measurement at 700/505 nm. HDL cholesterol was based on the production of H_2O_2 and subsequent increase in absorbance at 700/600 nm of a purple blue pigment by reaction with 4-amino-antipyrine and sodium N(2-hydroxy-3-sulphopropyl) 3,-5 dimethoxyaniline. Triglyceride was based on the liberation of glycerol from digestion with lipoprotein lipase and subsequent reaction of H_2O_2 with 4-aminophenazone and 4-chlorophenol was measured at 700/505 nm.

Glucose using glucose oxidase (GOD) and reaction of H_2O_2 (generated by peroxidase POD) with amino phenazone and phenol were measured at 700/505 nm. Glucose by hexokinase (HK) mediated phosphorylation to glucose-6-phosphate linked with glucose-6-phosphate dehydrogenase (G6PDH) to form NADH determined by increase in absorbance at 700/340 nm.

Lactate by the oxidation to pyruvate with lactic acid oxidase (LOD) and reaction of H_2O_2 (generated by peroxidase POD), with 4-aminoantipryrine was measured at 700/660 nm.

Bicarbonate from the production of oxalo acetate with phosphoenolpyruvate carboxylase and conversion to malate by malate dehydrogenase was measured by absorbance of NAD at 505/415 nm. Phosphate through the formation of the yellow ammonium phosphomolybdate in the presence of sulphuric acid was measured at 700/340 nm.

Serum creatinine by the enzymatic procedure with creatininase and final reaction of hydrogen peroxide (H_2O_2) with 4-amino phenazone and 2,4,6 triiodo-3-hydroxy benzoic acid was measured at 700/546 nm.

Uric acid using uric acid oxidase and reaction of generated $H_2O_2$with 4-amino phenazone and Nethyl-N-(2 hydroxy-3-sulphopropyl-3-methyl aniline) to form a red coloured quinine diimine dye was measured at 700/546 nm.

Urea based on the production of NH_4 ions and reaction with α-ketoglutarate was measured by increased NAD at 700/340 nm. Bilirubin by reaction with a diazonium dye at pH 1.2 to form azobilirubin was measured at 600/546 nm.

Calcium based on the reaction of Ca^{2+} ions with o-cresolphthalein complexone at pH 10.6 was measured at 629/552 nm. Magnesium by reaction with xylidyl blue a diazonium salt at pH 11.25 and the subsequent decrease in the blue absorbance were measured at 505/600 nm. Albumin was by reaction with bromocresol green at pH 4.1 measured at 505/570 nm. Total protein in serum by reaction with cupric ions in 0.4 M NaOH to form a blue–protein complex in the presence of bicinchininoic acid was measured at 700/546.

Urine micro albumin was by immuno-turbidometry with measurement at 659/340 nm. Urine creatinine by alkaline picrate generation of a red/orange complex was measured at 570/505 nm. Total protein in urine was measured by two separate methods: 1) based on the denaturation by benzethonium chloride and the subsequent fine suspension quantified turbidometrically at 700/505 nm; and 2) reaction with pyrogallol red and subsequent absorbance at 700 nm (Randox Laboratories, Belfast, UK).

Statistical analysis

The statistical significance of varying concentrations of HGA on biochemical analysis was determined using Wilcoxon signed rank tests (paired non-parametric analysis). The analysis was carried out by