Journal of Chromatography, 275 (1983) 237—243
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1682

HAWKINSINURIA — IDENTIFICATION OF QUINOLACETIC ACID AND PYROGLUTAMIC ACID DURING AN ACIDOTIC PHASE

CHARLES H. HOCART*,*, BERTHOLD HALPERN**, LARRY A. HICK and CHOON O. WONG

Department of Chemistry, The University of Wollongong, Wollongong, N.S.W. 2500 (Australia)

and

JUDITH W. HAMMOND and BRIDGET WILCKEN

The Macquarie Hospital, North Ryde, N.S.W. 2113 (Australia)

(First received November 22nd, 1982; revised manuscript received February 17th, 1983)

SUMMARY

A second Australian family with the genetic disease Hawkinsinuria has been identified. Affected members excrete hawkinsin and *cis*- and *trans*-4-hydroxycyclohexylacetic acid. An infant in this family presented with metabolic acidosis and excreted quinolacetic acid and pyroglutamic acid in the urine together with the tyrosine derived phenolic acids reported in the original index case. It is thought that quinolacetic acid is accumulated as a by-product of the partially defective enzyme, *4-hydroxyphenylpyruvate dioxygenase* (EC 1.13.11.27) and that pyroglutamic acid indicated lowered glutathione levels.

INTRODUCTION

Niederwieser and co-workers [1—3] have described the excretion of hawkinsin, 2-(2-L-cystein-S-yl-1,4-dihydroxy-5-cyclohexen-1-yl)acetic acid, and *cis*- and *trans*-4-hydroxycyclohexylacetic acid (4-HCHAA) in the urine of a child (L.H.), suffering from transient tyrosinaemia, and in the urine of her mother. When the child was weaned from breast feeding to artificial feeding with a concomitant higher intake of phenylalanine and tyrosine, a serious metabolic acidosis characterised by high levels of 4-hydroxyphenylpyruvic acid (4-HPPA), 4-hydroxyphenyllactic acid (4-HPLA) and 4-hydroxyphenyl-

*Present address: Developmental Biology, R.S.B.S., Australian National University, P.O. Box 475, Canberra, A.C.T. 2601, Australia.
**Deceased November 15th, 1980.

0378-4347/83/$03.00

acetic acid (4-HPAA) was induced. It was postulated that a partial defect in 4-hydroxyphenylpyruvate dioxygenase (EC 1.13.11.27), normally responsible for the oxidation, decarboxylation and rearrangement of 4-HPPA to homogentisic acid, prevented the final rearrangement of an intermediate. They proposed that either quinolacetic acid [2-(1-hydroxy-4-oxo-2,5-cyclohexadien-1-yl)acetic acid] or the epoxide [2-(1,2-epoxy-4-oxo-5-cyclohexen-1-yl)-acetic acid] accumulated and was disposed of by either conjugation with cysteine through the intervention of glutathione to produce hawkinsin, or by a series of reductions to produce the *cis*- and *trans*-4-HCHAA [1].

A second infant (K.G.) with Hawkinsinuria has been described [4]. In this paper we report on the urinary excretion of pyroglutamic and quinolacetic acid in addition to the 4-hydroxyphenolic acids while this child was acidotic.

MATERIALS AND METHODS

Solvent extraction of organic acids

Sodium chloride (0.5 g) was added to 1 ml of urine, followed by sufficient hydrochloric acid to bring the pH to 1. The solution was then extracted twice with ethyl acetate (2 × 2 ml) and once with diethyl ether (2 ml) and the pooled organic phases evaporated to dryness under a gentle stream of nitrogen at room temperature. The dried urinary extract was then silylated with 125 μl of bis-(trimethylsilyl)trifluoracetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) (60 °C, 30 min).

Identification of organic acids

Urinary metabolites were identified using gas chromatography—mass spectrometry (GC—MS) in which a Varian Aerograph (2700) gas chromatograph was interfaced, via a jet separator, to a DuPont 491B mass spectrometer fitted with a dual electron impact (EI)/chemical ionisation (CI) source. The gas chromatograph was fitted with a 1.8 m × 6 mm glass column packed with 3% SE-30 on 80—100 mesh dimethyldichlorosilane-treated Chromosorb W. A helium flow-rate of 25 ml/min was used, and during the analyses the temperature was programmed from 80°C to 250°C at 6°C/min.

Nuclear magnetic resonance spectra

Nuclear magnetic resonance (NMR) spectra were recorded with a Hitachi Perkin-Elmer R-24 spectrometer operating at 60 MHz using tetramethylsilane (TMS) as an internal standard. Chemical shifts are quoted on the δ scale and the signals described in terms of the number of protons, multiplicity, coupling constants and assignment.

Synthesis of 2-(1-hydroxy-4-oxo-2,5-cyclohexadien-1-yl)acetic acid

The synthesis was achieved using a modification of the original procedure described by Siegel and Keckeis [5]. *tert*-Butyl bromoacetate was prepared by the method of McCloskey et al. [6] and purified by distillation at reduced pressure in the presence of magnesium oxide using alkali washed glassware and the fraction boiling at 40°C/4 mm Hg was collected as a colourless oil, yield 74%, GC—MS (isothermal at 40°C) *m/e* 181 ($M^{+81}Br - 15$), 179 ($M^{+79}Br - 15$).

tert.-Butyl bromoacetate (3.5 g) was dissolved in anhydrous diethyl ether (100 ml) and activated zinc powder (2.3 g) added. The zinc powder had been washed with dilute hydrochloric acid, water, alcohol and anhydrous diethyl ether then dried in vacuo before activating with a single crystal of iodine by heating in vacuo. The reaction was initiated, while being vigorously stirred under reflux, by the addition of a few drops of an ethereal solution of freshly prepared methyl magnesium iodide. Refluxing continued for 90 min whereupon the solution was cooled and decanted from the excess zinc. An ethereal solution of 1,4-benzoquinone (1 g) was added slowly to the cooled solution of the organozinc compound. The precipitate was filtered and washed with anhydrous diethyl ether followed by addition to hot water (200 ml). The aqueous solution was then filtered to remove the zinc hydroxide and extracted with diethyl ether (3 × 70 ml). The ether extracts were dried over anhydrous magnesium sulphate and the ether removed by distillation to give a brown residue which was chromatographed on Merck alumina (activity II). Elution with 20% chloroform—light petroleum yielded *tert.*-butyl 2-(1-hydroxy-

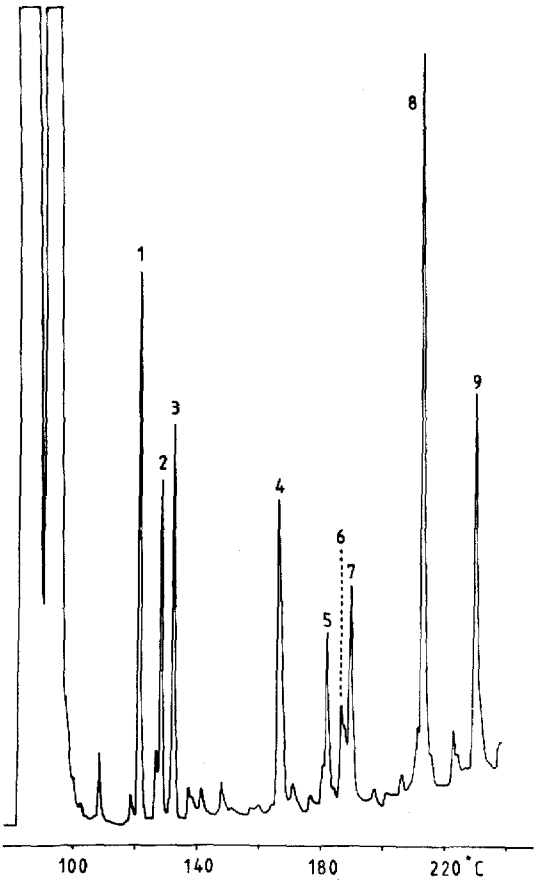

Fig. 1. GC of urinary organic acids as per-TMS derivatives excreted by K.G. while acidotic. Peaks: 1, 3-hydroxybutyric acid; 2, urea; 3, acetoacetic acid; 4, pyroglutamic acid and adipic acid; 5, 4-HPAA; 6, quinolacetic acid; 7, suberic acid; 8, 4-HPLA; 9, unknown, molecular weight 434.

4-oxo-2,5-cyclohexadien-1-yl)acetate (0.69 g, 33%) which was crystallised from chloroform—pentane; m.p. 63—65°C; MS (50°C, 70 eV) *m/e* (%) 168 (63), 150 (17), 126 (31), 124 (31), 123 (38), 122 (17), 109 (base peak); NMR (60 MHz, C^2HCl_3) δ 1.35 (9-H, s, *tert.*-butyl methyls), 2.52 (2-H, s, methylene α to acid), 4.42 (1-H, s, hydroxyl), 6.07 (2-H, d, J = 10 Hz, C-3 and C-5 protons), 6.90 (2-H, d, J = 10 Hz, C-2 and C-6 protons).

A sample of *tert.*-butyl 2-(1-hydroxy-4-oxo-2,5-cyclohexadien-1-yl)acetate was hydrolysed by dissolution in 0.1 *M* hydrochloric acid in formic acid and allowed to stand at room temperature for an hour. The solvent was removed by freeze-drying and the brown oily residue washed twice with water. NMR analysis of the crude hydrolysate indicated the production of the lactone [7]. This was then silylated with BSTFA—1% TMCS yielding di-TMS quinolacetic acid.

RESULTS AND DISCUSSION

The GC—MS analysis of the silylated urinary organic acids from the child (K.G.) showed that the urine contained three compounds (pyroglutamic acid and two unknowns) not reported in the original index case (Fig. 1). The molecular weights of the two unknowns were determined by GC—CIMS, to be 312 a.m.u. and 434 a.m.u. The mass spectrum of the first unknown was shown to be very similar to that of the di-TMS derivative of quinolacetic acid as prepared by us (Fig. 2). The di-TMS derivative gave signals at *m/e* 312 (M^+), 297 ($M-CH_3$), 269, 255, 253, 222 (M—HOTMS), 204, 194 (M—HCOOTMS), 181 ($M-CH_2COOTMS$), 147, 75, 73. The identity of the

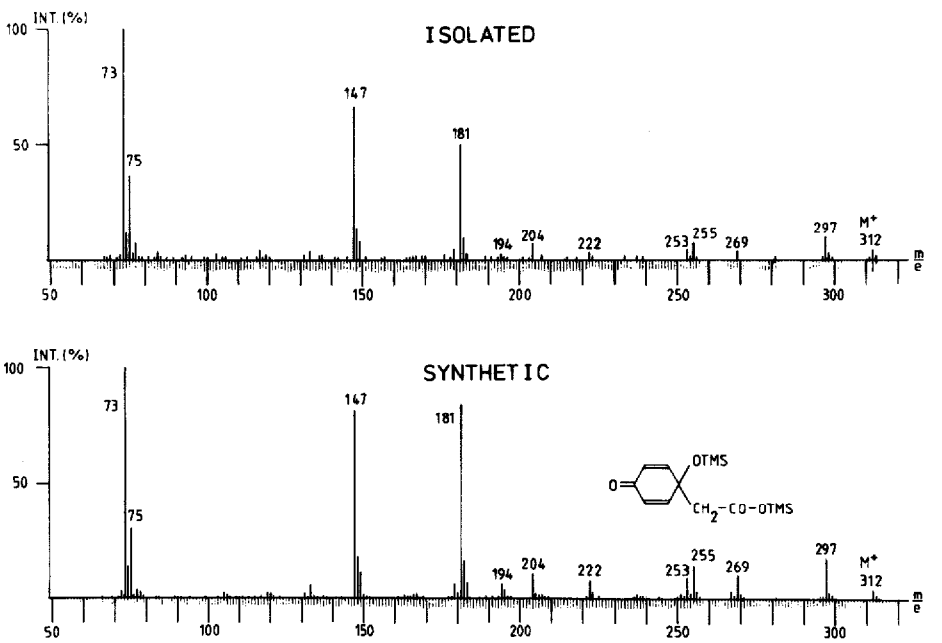

Fig. 2. EI mass spectra obtained from GC—MS of urinary quinolacetic acid and synthetic quinolacetic acid as di-TMS derivatives.

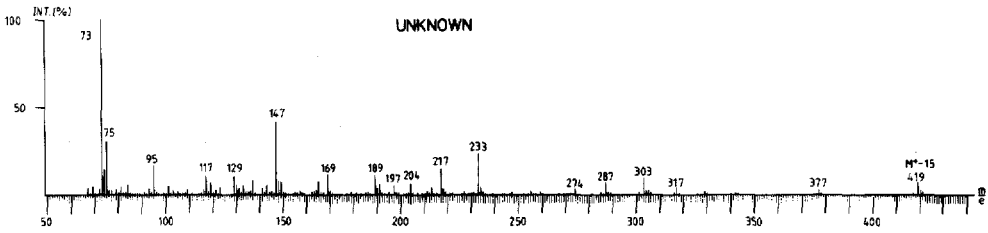

Fig. 3. EI mass spectra obtained from GC—MS of the unknown compound (molecular weight 434) excreted by K.G.

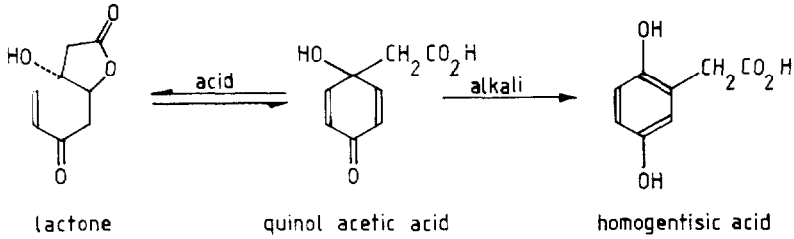

Fig. 4. Under acidic conditions quinolacetic acid may undergo an intramolecular Michael addition to form a lactone. Under alkaline conditions both the lactone and the free acid rearrange to homogentisic acid.

second unknown (Fig. 3) remains undetermined. Saito et al. [7] have noted that quinolacetic acid can undergo an internal Michael addition to form a lactone and that both the lactone and the free acid may, under alkaline conditions, be irreversibly rearranged to homogentisic acid (Fig. 4). Therefore it is likely that under the conditions of the urine extraction any free quinolacetic acid will be, at least partially, converted to the lactone form. The silylation conditions used are such that the lactone is in equilibrium with the acid form which is then, for the purposes of our work, irreversibly silylated to produce the di-TMS derivative of quinolacetic acid. This was demonstrated when the lactone obtained from the acid hydrolysis of the *tert.*-butyl ester was silylated to yield quantitatively the di-TMS derivative of quinolacetic acid. Finally, the silylated derivatives of crystalline samples of authentic quinolacetic acid and the lactone received from Professor Saito proved to have mass spectra identical with samples synthesised by us and found in the urine.

A small sample of urine excreted by the original index case (L.H.) during an acute phase of her illness was obtained from Professor Danks. The GC trace of the urinary organic acids (Fig. 5) contained a large peak which we now identify as pyroglutamic acid. The excretion of pyroglutamic acid by both children while acidotic probably indicates lowered glutathione levels. Glutathione normally exerts feedback inhibition on γ-glutamylcysteine synthetase [8] and lowered levels, as are seen for example in glutathione synthetase deficiency, may lead to a modified γ-glutamyl cycle, with increased formation of pyroglutamic acid (5-oxoproline), which exceeds the capacity of 5-oxoprolinase [8]. This supports Niederwieser's supposition that hawkinsin results from the conjugation of glutathione with the accumulated en-

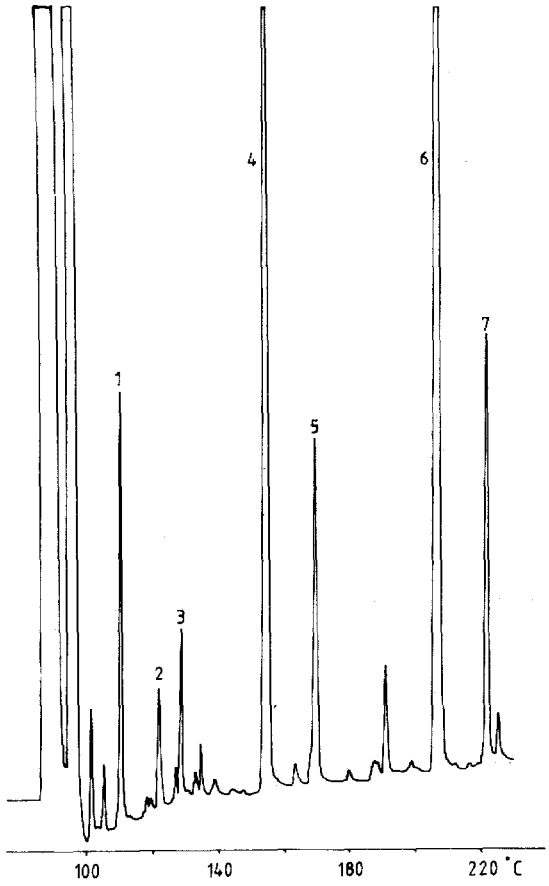

Fig. 5. GC of urinary organic acids as per-TMS derivatives excreted by L.H. while acidotic. Peaks: 1, lactic acid; 2, 3-hydroxybutyric acid; 3, urea; 4, pyroglutamic acid; 5, 4-HPAA; 6, 4-HPLA; 7, 4-HPPA.

zyme intermediate [1]. An alternative explanation might be that the accumulated intermediate metabolite inhibits glutathione synthetase, with the same result. Neither quinolacetic acid nor the unknown were detected during re-examination of urine from the original case (L.H.). This failure may perhaps be due to extensive storage and handling of the urine sample.

The finding of quinolacetic acid in the acute phase of Hawkinsinuria does not necessarily imply that this is an intermediate in the conversion of 4-HPPA to homogentisic acid. It is possible that an epoxide intermediate had been converted to quinolacetic acid in the acidic conditions of extraction and derivatisation.

ACKNOWLEDGEMENTS

We would like to express our thanks to Professor Saito, Department of Synthetic Chemistry, Kyoto University, Kyoto, Japan for his very generous gift of samples of quinolacetic acid and the lactone. Also to Professor Danks,

Genetics Research Unit, Royal Children's Hospital Research Foundation, Melbourne, Australia for allowing us to examine urine excreted by L.H.

Acknowledgement is also made of financial support of C.H.H. by Ampol Petroleum Ltd. and of O.C.W. by the National Health and Medical Research Council.

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