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NEW TYROSINE METABOLITES IN HUMANS: HAWKINSIN AND *CIS*- AND *TRANS*-4-HYDROXYCYCLOHEXYLACETIC ACIDS

UNUSUAL ADSORPTION OF DEUTERATED AND NON-DEUTERATED HAWKINSIN DURING GAS CHROMATOGRAPHY

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SUMMARY

In a new inborn error of metabolism, where obviously a defect of 4-hydroxyphenylpyruvate dioxygenase (EC 1.13.11.27) exists, hawkinsin [(2-cystein-S-yl-1,4-dihydroxycyclohex-5-en-1-yl) acetic acid] and *cis*- and *trans*-hydroxycyclohexylacetic acids were found in the urine. A partially reversible adsorption of deuterated and non-deuterated hawkinsin (as the penta-trimethylsilyl derivative) in gas chromatography—mass spectrometry has inhibited a mass fragmentographic quantitation of this compound to date. However, quantitation seems to be possible using mass fragmentography of 1,4-dihydroxycyclohexylacetic acid, formed by desulfuration of the sample with active nickel.

INTRODUCTION

We have recently been studying the unusual urinary excretion products of an Australian woman, and her daughter who suffered from transient tyrosinemia during her first year of life. One of these compounds revealed a new sulfur amino acid, namely (2-cystein-S-yl-1,4-dihydroxycyclohex-5-en-1-yl) acetic acid, which we named hawkinsin (Haw) [1]. The others were *cis*- and *trans*-4-hydroxycyclohexylacetic acids [2]. All of these unusual compounds were proved to be tyrosine metabolites by a loading test with 50 mg/kg of 3,5-bis-deutero-L-tyrosine, orally, and selected ion-monitoring of the non-deuterated and bis-deuterated molecule species in urine [2].

In this paper we report on the unusual adsorption effect of Haw on the columns used for gas chromatography—mass spectrometry (GC—MS), an effect

which has inhibited quantitation of this compound by mass fragmentography to date.

MATERIALS AND METHODS

Haw and 1,4-dihydroxycyclohexylacetic acid were prepared as previously described [1]. Hexa-deutero-Haw was prepared similarly. 4-Hydroxyphenylacetic acid was deuterated in D_2O-H_2SO_4 to give 2,3,5,6,α,α hexa-deutero-4-hydroxyphenylacetic acid (contaminated with the penta- and tetra-deutero derivatives) and the product was transformed into the hexa-deutero-4-quinolacetic acid by photo-oxidation using Rose—Bengal as catalyst. The quinolacetic acid was then reacted with cysteine to give a mixture of the mono- and bis-addition products. These were separated by ion-exchange chromatography and precipitated from the methanolic solution in diethyl ether.

Active nickel for desulfuration was prepared from nickel chloride and sodium borohydride [3]. Trimethylsilylation was performed using bis-trimethylsilyltrifluoracetamide (BSTFA)—acetonitrile (1:1, v/v) for 1—4 h at 135° (hawkinsin) or 60° (1,4-dihydroxycyclohexylacetic acid). GC of penta-trimethylsilyl (TMS)-Haw was performed on 15—60 cm × 2 mm columns of 1% SE 30 or 1% Dexsil 300 on Chromosorb W AW DCS at 210—220° and on 1.8-m long OV-17 Pyrex glass capillary columns at 180—200°.

GC—MS was performed on a Micromass F-16 mass spectrometer (Vacuum Generators Micromass, Winsford, Great Britain) combined with a Carlo Erba Model 2101 AC gas chromatograph over a jet separator (for packed columns) and a further Model 2101 AC gas chromatograph equipped with a glass capillary column coupled directly with the ion source.

RESULTS AND DISCUSSION

In order to be able to quantitate, and also to detect traces of Haw with selected ion monitoring, we prepared a hexa-deutero-Haw (Haw-d_6) in a similar manner to which we prepared non-deuterated Haw (Haw-d_0) [1]. The mass spectrum of the corresponding penta-TMS derivative is shown in Fig. 1. As can be seen from the fragment at 540, the product was a mixture of hexa-, penta- and tetra-deutero-Haw. The base peak at *m/e* 218 is a typical α-amino acid fragment. The molecule ions are below 1% and just visible. The fragments at *m/e* 444 and 450 (non-deuterated and hexa-deuterated, respectively) result from the loss of COOTMS and TMSOH. The fragments at *m/e* 360 and 364, respectively, result from a retro-Diels—Alder reaction and characterize the position of the double bond. Also of note is the presence of the tropylium ion at *m/e* 179 and 184; this is an ion which intrigued us during our efforts to identify this compound. Two residues, namely trimethylsilanol and cysteine, must be split off in order to obatin a phenolic intermediate from this aliphatic compound. At least one or even two deuterium ions are lost during the tropylium ion formation (Fig. 1).

GC of Haw was possible only as the penta-TMS derivative. The best results were obtained using very short columns of 15—60 cm in length. It was necessary to treat the columns repeatedly with BSTFA reagent at a high temperature

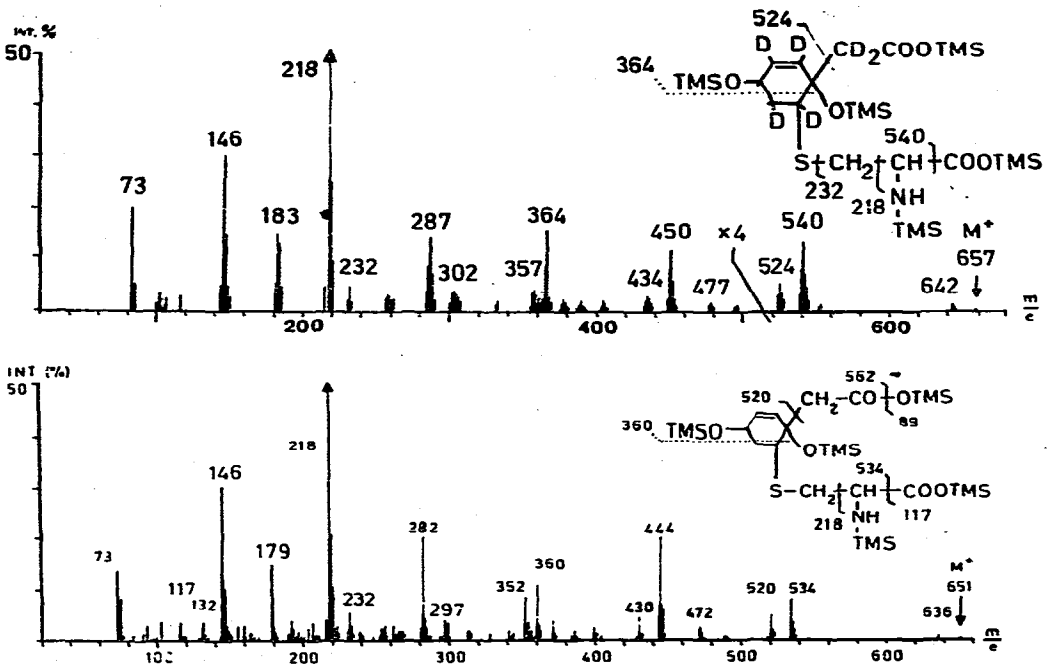


Fig. 1. Electron impact (EI) mass spectrum of deuterated Haw (above) and non-deuterated (below) as the penta-TMS derivative at 20 eV. GC—MS on a 60 cm × 2 mm column of 1% SE-30 on Chromosorb W AW DCS at 210°. VG Micromass F-16 mass spectrometer.

before use. The possible temperature range of the column was narrow: no peak at all was obtained if the compound eluted slowly because of lower temperature, lower carrier gas flow-rate or longer columns. Experiments with selected ion monitoring of non-deuterated and deuterated derivatives gave quite unexpected results; these are summarized in Fig. 2. The fragments at *m/e* 444 and 450 were monitored for Haw-d_0 and -d_6, respectively. At first non-deuterated Haw was injected several times. Then, pure deuterated Haw was injected. Unexpectedly, a high signal from the previously-injected Haw-d_0 was observed at *m/e* 444, simultaneously with the expected signal at *m/e* 450. The signal of the unlabeled compound decreased only slowly during further injections of pure Haw-d_6. This phenomenon can only be explained by a partially reversible adsorption of Haw on the column; a newly injected sample equilibrates partially with the adsorbed one. As can be seen in Fig. 2, the amount of Haw-d_0 which was desorbed by the first injection of Haw-d_6 even increased in the second and third series. Under such conditions, no quantitative analysis and no trace analysis was possible.

Attempts to diminish the adsorption effects by using glass capillary columns have not been successful to date. No peak at all could be obtained on 20-m OV-17 glass capillaries. Only on very short capillary pieces of less than 2 m was a flame-ionization detector signal obtained. In order to demonstrate the adsorption effect, a dilution series of the same mixture of Haw · $(TMS)_5$ and an alkane as an internal standard was analyzed. Because the same mixture was

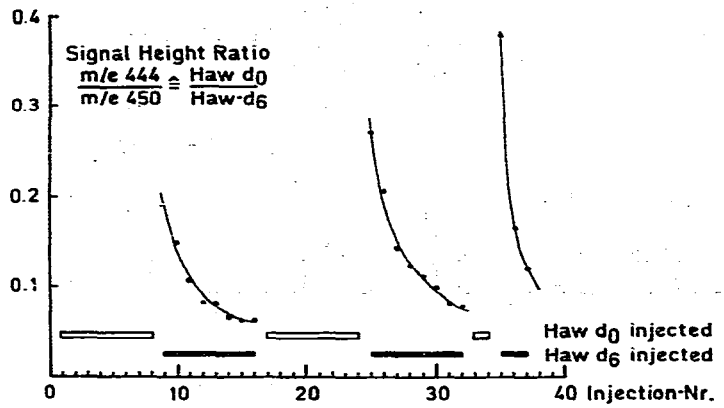


Fig. 2. Desorption of Haw-d_0 · $(TMS)_5$ from a packed GC column by repeated injections of hawkinsin-d_6—$(TMS)_5$. A 300 × 2 mm column of 1% SE-30 on Chromosorb G AW DMCS was operated at 215°. Selected ion monitoring of Haw-d_0 and Haw-d_6 at *m/e* 444 and 450, respectively.

analyzed, a constant peak height ratio could be expected if no adsorption ocurred. However, a dramatic decrease of this ratio with decreasing amounts of sample injected was observed (Fig. 3). Adsorption was even higher on a Pyrex capillary which had been pretreated several times with Carbowax 20M before it was coated with OV-17 (Fig. 3).

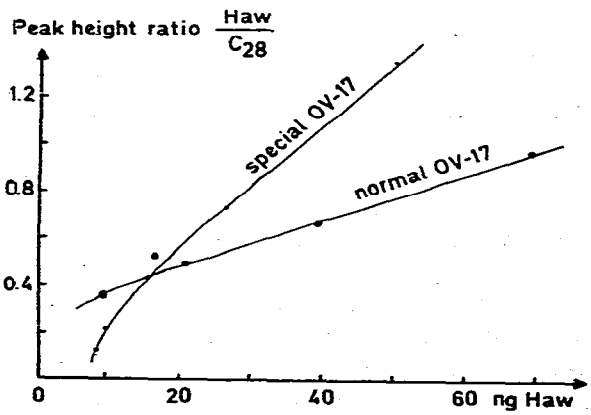
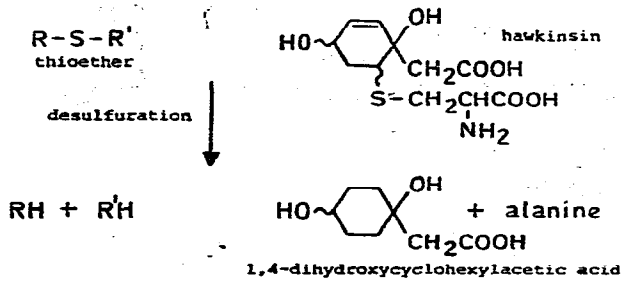


Fig. 3. Adsorption of Haw on 1.8-m long OV-17 glass capillary columns. The sample consisted of a mixture of 500 ng Haw and 200 ng of alkane C_{28}. The mixture was reacted with BSTFA—acetonitrile (1:1, v/v) at 135° for 1 h and then diluted with the reagent. The actual amount separated on the capillary column was estimated, assuming an inlet split ratio of 1:10. A constant peak height ratio would be expected if no adsorption occurred. Temperatures: 265°, injector, 195°, normal OV-17 and 188°, special OV-17 column. The special column was treated 4 times with Carbowax before it was coated.

Hence, we had to look for another possibility for quantitation. Haw is a thio-ether which can be split by desulfuration with active nickel into 1,4-dihydroxycyclohexylacetic acid and alanine under reduction of the double bond [1]:



It has been shown recently [3, 4] that desulfuration can be used as a general method for the quantitation of N-acetylcysteine conjugates (mercapturic acids), which are products of the glutathione detoxification pathway of many drugs and other xenobiotics. In the case of the mercapturic acids, N-acetylalanine is split off and measured with selected ion monitoring, and a trideuterated N-acetylcysteine derivative is used as an internal standard [3, 4].

Hence, we tried to apply the desulfuration technique to the quantitation of Haw. Measurement of the resulting 1,4-dihydroxycyclohexylacetic acid could be a specific means for quantitation. After several trials we found conditions were such that the 1,4-dihydroxyhexylacetic acid could be gas chromatographed practically without adsorption as the per-TMS derivative on 1 m × 2 mm columns of 1% Dexsil on Chromosorb W AW DCS. (Methylation with diazomethane followed by silylation gave incomplete derivatisation of the hydroxyl group at C-1). On the other hand, Haw-d_6 could be desulfurated practically without loss of the isotope labels. This is demonstrated by the mass spectrum in Fig. 4. The intensity at *m/e* 381 is a little lower than one would expect from the Haw-d_6 spectrum, but this is certainly no contra-indication to the use of the desulfuration technique. Thus, even our imperfect hexadeuterated hawkinsin preparation can be used as an internal standard for mass fragmentography of Haw-d_0 and Haw-d_2. Nevertheless, we hope to be able to synthesize an isotopically pure Haw-d_6 in the near future and we intend to search for the presence of traces of Haw in the urine of patients with liver diseases and tyrosinemia.

However, it could well be that the intermediate, from which Haw and *cis*- and *trans*-4-hydroxycyclohexylacetic acids are derived, is not normally in a free state but is immediately rearranged by the enzyme into homogentisic acid. From our observations we postulate a defective 4-hydroxypyruvate dioxygenase which is still able to oxidize and decarboxylate the substrate, but is unable to rearrange the intermediate into homogentisic acid. The occurrence of the fully reduced 4-hydroxycyclohexylacetic acid and of Haw strongly indicates an epoxide intermediate, namely (1,2-epoxy-4-hydroxycyclohexa-3,5-dien-1-yl) acetic acid, which can lose its potentially aromatic character by

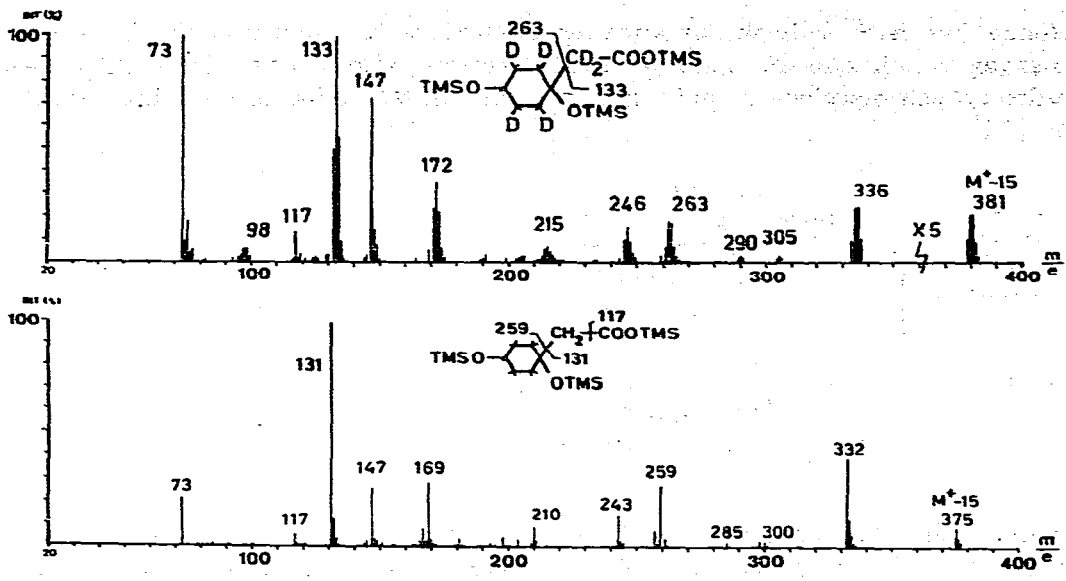


Fig. 4. EI mass spectra of deuterated and non-deuterated 1,4-dihydroxycyclohexylacetic acid—$(TMS)_3$ at 20 eV. The acids were obtained by desulfuration of Haw-d_6 and Haw-d_0 with active nickel, and analyzed on a Vacuum Generators Micromass F-16 mass spectrometer.

a simple tautomerization into (1,2-epoxycyclohex-5-en-4-on-1-yl) acetic acid. An attack at C-2 by a hydride ion followed by further reductions will lead to 4-hydroxycyclohexylacetic acid (predominately *trans*), and an attack at C-2 by cysteine (or glutathione) followed by reduction will lead to Haw.

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