Biochemical Pharmacology, Vol. 30, No. 13, pp. 1879–1882, 1981.
Printed in Great Britain.

0006-2952/81/131879-04$02.00/0
Pergamon Press Ltd.

EVIDENCE FOR THE OCCURRENCE OF A NOVEL PATHWAY OF BENZOIC ACID METABOLISM INVOLVING THE ADDITION OF A TWO CARBON FRAGMENT

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The metabolism of benzoic acid has been investigated in the horse as part of a study on the fate of carboxylic acids in this species (1). The greater part of an orally administered dose of the acid is excreted in the urine in the form of the glycine conjugate, hippuric acid, together with small quantities of benzoyl glucuronide and the free acid. In addition to these the urine samples were found to contain a further metabolite, accounting for some 2% of the dose which exhibited a chromatographic mobility intermediate between that of the parent acid and hippuric acid. This communication provides evidence that this unknown metabolite arises from the addition of a two carbon fragment to the carboxyl group of benzoic acid, leading to the excretion of β-hydroxyphenylpropionic acid and the corresponding β-keto acid in the urine.

MATERIALS AND METHODS

Compounds [^{14}C-carboxyl]-Benzoic acid, sp. act 56mCi/mmol, radiochemical purity > 99% was purchased from the Radiochemical Centre, Amersham, U.K., (ring-D_5)-Benzoic acid and ethyl benzoylacetate were purchased from Aldrich Chemical Co., Gillingham, Dorset, U.K. Benzoylacetic acid (β-ketophenylpropionic acid), which readily decomposes in air to acetophenone, was prepared by the alkaline hydrolysis of ethyl benzoylacetate. β-Hydroxyphenylpropionic acid was prepared from ethyl benzoylacetate by $NaBH_4$ reduction followed by alkaline hydrolysis. The product was recrystallized from ethyl acetate, m.p. 142-3°, and gave the expected proton magnetic resonance and mass spectra.

Thin layer chromatography (tlc) This employed Merck Silica gel F_{254} plates (0.2mm thick on aluminium support), solvent benzene: acetone: glacial acetic acid (6:2:1 v/v). R_F values in this system were benzoic acid 0.70, β-hydroxyphenylpropionic acid 0.57, benzoylacetic acid 0.55 and acetophenone 0.80.

High pressure liquid chromatography (hplc) This used a Waters Associates U6K valve loop injector and Model 6000A pump and a Cecil 2012 u.v. detector set at 235 nm. The column was 250 x 5 mm packed with 5μ ODS-Hypersil and the mobile phase was 40% aqueous methanol flow rate 2 ml/min. Retention times were benzoic acid 7.2 min and β-hydroxyphenylpropionic acid 3.5 min. 1 ml Fractions of eluant were collected with a fraction collector and counted for ^{14}C.

Gas chromatography-mass spectrometry (gc - ms) This was performed with a Finnigan Series 4000 instrument with a Finnigan 6100 data system. The gc column was glass, 1.8 m x 3.2 mm i.d. packed with 2QE30, carrier gas He at 20ml/min and the oven temperature was initially 200° temperature programmed at 10°/min to 280°, injection port temperature 240°. The ms ionizing voltage was 70 eV, source temperature 200°.

Radiochemical techniques ^{14}C radioactivity was determined by liquid scintillation spectrometry (Packard TriCarb Model 3385) using a toluene-Triton X-100 scintillant, and quench correction was by reference to an external standard. ^{14}C on chromatograms was located by scanning (Packard Model 7201).

Dosing The horse, a gelded male weighing 374 kg was given a mixture of (ring-D_5)-benzoic acid and [^{14}C]-benzoic acid by stomach tube, dose 5.35 mg/kg containing 150μCi, and urine collected for 24h.

Isolation of unknown metabolite The urine of the horse was subjected to hplc, which showed the presence of benzoyl glucuronide, hippuric acid and benzoic acid (1). In addition, radioactivity corresponding to 2% of the administered dose eluted from the column with a retention time of 3.5 mins. This fraction was collected, the solvents removed on the rotary evaporator and the residue taken up in 0.1 ml methanol. Tlc followed by scanning for ^{14}C showed a single peak of R_F 0.56. Portions of this material were treated with ethereal diazomethane and examined by gc-ms.

RESULTS

Gc-ms analysis of the isolated unknown after treatment with diazomethane showed the presence of two benzoic acid related peaks in the total ion chromatogram. The major peak gave the ms shown in Fig. 1, which shows the presence of pairs of diagnostic ions 5 amu apart at m/e 180/185 (molecular ions), 162/167, 120/125, 107/112 (base peaks) and 105/110. These pairs of ions, in which the M/M+5 ratio is constant at 2.5:1, show that this compound is an endogenous constituent of urine as well as arising from the (ring-D_5)-benzoic acid administered. This mass spectrum was assigned to methyl β-hydroxylphenylpropionate and similar analysis of the methyl ester of an authentic sample of β-hydroxyphenylpropionic acid yielded a spectrum containing all of the appropriate fragment ions, at the same gc retention time. A second minor gc peak gave the ms of acetophenone, again containing pairs of ions 5 amu apart with an M/M+5 ratio of 2.5:1. Authentic acetophenone afforded an identical fragmentation pattern with similar gc retention time.

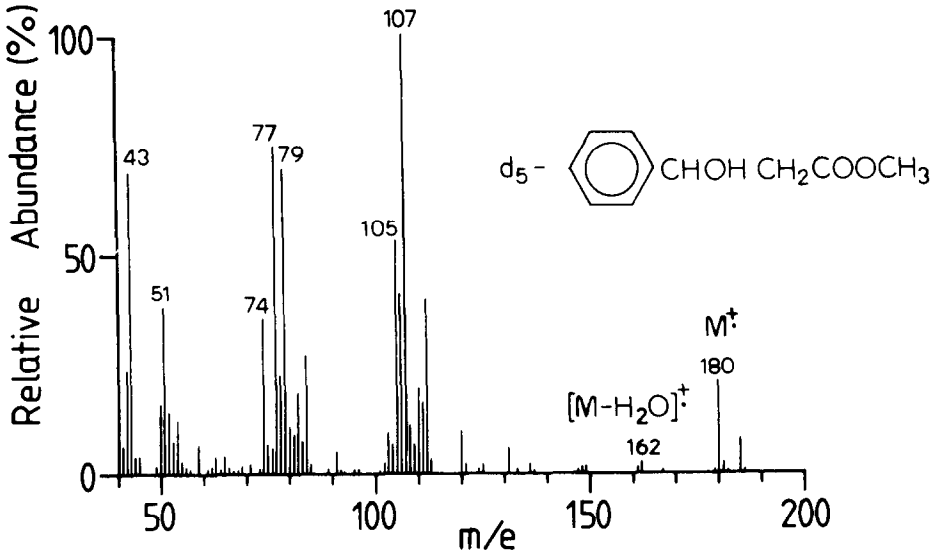

Fig. 1. Mass spectrum obtained by gc-ms, of the methyl ester of β-hydroxyphenylpropionic acid and β-hydroxy-(ring-D_5)-phenylpropionic acid isolated from the urine of a horse dosed with (ring-D_5)-benzoic acid.

DISCUSSION

This communication gives details of the isolation and identification of a novel product of benzoic acid metabolism, namely β-hydroxyphenylpropionic acid, in horse urine. It is proposed that this arises by a novel synthetic pathway, involving the addition of acetic acid to benzoic acid, at the carboxyl group, producing first the β-keto acid, benzoylacetic acid, which is then reduced to β-hydroxyphenylpropionic acid. The latter was characterized by comparison of its chromatographic properties and mass spectrum with those of an authentic sample. In addition to this a minor gc peak was found to give the mass spectrum of acetophenone. The origin of this is at present uncertain but it is very likely to arise from the decarboxylation of benzoylacetic acid, already referred to above. Benzoylacetic acid is known to be unstable and readily affords acetophenone by decarboxylation which could have occurred during isolation and work-up.

Addition of a two carbon acid fragment to a xenobiotic has been reported previously in the formation of furylacrylic acid from furfural (2) and in the metabolism of 5-(4'-chloro-n-butyl)-picolinic acid (3). The mechanism of this novel reaction is unknown, and may involve the reduction of the carboxyl group to an aldehyde (viz. furfural (2)) or the condensation of the xenobiotic acyl CoA with acetyl CoA, analogous with events involved in fatty acid biosynthesis.

The present findings raise the possibility that there may occur important links between the reactions of fatty acid biosynthesis and the metabolism of xenobiotic carboxylic acids.

Acknowledgements Mary Varwell Marsh is an SRC-CASE student. Andrew J. Hutt is supported by Sir Halley Stewart Trust.

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