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Note

Gas chromatographic—mass spectrometric determination of aromatization of cyclohexanecarboxylic acid in guinea pig liver

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Shikimic acid (3α,4α,5β-trihydroxy-1-cyclohexene-1-carboxylic acid) is an ubiquitous plant compound and an intermediate in the biosynthesis of essential amino acids [1, 2]. Interestingly, it has been claimed to be mutagenic and possibly carcinogenic to mice [3]. The anaerobic metabolism of shikimic acid by rat gastrointestinal microorganisms results mainly in the formation of cyclohexanecarboxylic acid [4].

Studies on the mammalian biotransformation of shikimic acid and cyclohexanecarboxylic acid have revealed that the latter is probably an essential intermediate in the metabolism of the former and that both undergo extensive aromatization [5]. That cyclohexanecarboxylic acid undergoes aromatization in mammals has long been known and efforts have been made to elucidate the properties of the enzyme(s) which catalyze the reaction(s) [6, 7]. A cell-free system from guinea pig liver was prepared which was able to convert cyclohexanecarboxylic acid to hippuric acid [8]. Later, soluble enzymes for the conversion of cyclohexanecarboxyl-coenzyme A to benzoyl-coenzyme A were isolated and some aspects of the mechanism of aromatization partially clarified [7]. Nevertheless, the information available on this subject is relatively meagre.

One of the limitations in the investigation of these enzymes has been the lack of sensitivity and specificity of the methods for quantitatively following the reaction. These include a colorimetric method [8] and a gas chromatography—radioactivity procedure [7]. The present report describes a specific and more sensitive gas chromatographic—mass spectrometric (GC—MS) method for following the enzymatic formation of hippuric acid from cyclohexanecarboxylic acid.

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EXPERIMENTAL

Materials

Adenosine triphosphate (ATP), N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) and bovine serum albumin (fraction IV) were purchased from Sigma (St. Louis, MO, U.S.A.); α-ketoglutaric acid (αKGA) and hippuric acid from Fluka (Buchs, Switzerland); glycine and sucrose from Koch-Light Labs. (Colnbrook, Great Britain); ethylenediaminetetraacetic acid (EDTA) and cyclohexanecarboxylic acid from E. Merck (Darmstadt, G.F.R.).

p-Methoxybenzoylglycine was prepared from *p*-methoxybenzoic acid by the method described by Sheehan and Hess [9] for the synthesis of peptides and had a m.p. of 168—170°C (literature value 171°C).

Cyclohexanecarboxylic acid was dissolved in methanol—water (1:3, v/v).

Animals

In all experiments described here, livers from Dunkin Hartly guinea pigs (Olac 1976 LTD) weighing 300—350 g were used. The animals were fed ad libitum on a standard guinea pig pelleted feed.

Preparation of whole homogenate of guinea pig liver

Animals were stunned and exsanguinated and the livers were immediately removed and chilled in 0.25 *M* sucrose containing 10 m*M* HEPES (pH 7.4) and 1 m*M* EDTA. All further procedures were carried out at 0—5°C. The livers were minced and homogenized in 4 volumes of the same buffer as described above using a Potter-Elvehjem homogenizer at 720 rpm and with two strokes of a loose-fitting PTFE pestle. This homogenized tissue mixture was used as a homogenate fraction.

Determination of protein

Protein was determined employing a Bio-Rad Protein Assay Kit (Bio-Rad Labs., Richmond, CA, U.S.A.) using bovine serum albumin as the standard.

Procedure

The incubation mixture contained 10 m*M* glycine, 5 m*M* αKGA, 5 m*M* Mg^{2+}, 0.1 m*M* ATP and 1 m*M* EDTA in 0.03 *M* phosphate buffer, pH 7.4. To this was added whole homogenate in amounts described in the text; final volume 2.9 ml. The mixture was preincubated at 37°C for 10 min in a Citenco shaker at 80 oscillations per min; then 3 μmol (100 μl solvent) of cyclohexanecarboxylic acid were added and the mixture further incubated at 37°C for periods described in the text. The reaction was terminated by the addition of 6 ml of 0.2 *M* hydrochloric acid and the flasks were placed on ice.

p-Methoxybenzoylglycine (internal standard) (1 μmol, 200 μl solvent) was then added and diethyl ether extraction was carried out using a total of five 25-ml portions. The combined ether extracts were dried over anhydrous sodium sulphate and evaporated to ca. 2 ml. The samples were then transferred to small tubes and further evaporated to ca. 0.5 ml. Following conversion of the carboxylic acids to their methyl esters by adding an ether solution of

diazomethane the samples were evaporated to dryness and finally redissolved in 200 μl methanol.

For the blank, the same procedure was carried out except that hydrochloric acid was added just before the commencement of incubation.

For calibration curves, the 3 μmol of cyclohexanecarboxylic acid was replaced by 0.05—1.4 μmol of a standard solution of hippuric acid.

Instrumentation

Analysis was performed on a Hewlett-Packard 5992A GC—MS system.

Separations were made on a OV-1 wall-coated open tubular capillary column (18 m × 0.29 mm I.D.). The chromatographic conditions were as follows: oven temperature, 160°C for 2 min then programmed 14°C/min to 260°C; injection port temperature, 245°C; and helium flow-rate, 3 ml/min. Quantitative selected ion monitoring was performed at 70 eV focusing the instrument on the ion at *m/z* 105 for methyl hippurate and *m/z* 135 for methyl *p*-methoxybenzoylglycinate.

Calculations

The concentration of hippuric acid was determined from the ratio of peak areas of methyl hippurate derived from the assay solution to that of methyl *p*-methoxybenzoylglycinate from the internal standard. Using the standard curve the mole ratio of methyl hippurate to methyl *p*-methoxybenzoylglycinate could be estimated and multiplying this number with the amount of internal standard added gives the total amount of hippuric acid in that specimen.

RESULTS AND DISCUSSION

The mass spectra of the methyl esters of hippuric acid and *p*-methoxybenzoylglycine (internal standard) are shown in Fig. 1. The molecular and base peak ions for methyl hippurate are *m/z* 193 and *m/z* 105, respectively, and for methyl *p*-methoxybenzoylglycinate, *m/z* 223 and *m/z* 135. The mass spectra indicate that the choice of molecular ions would allow for the unambiguous determination of the two compounds when using selected ion monitoring (SIM). However, these ions have relatively low intensity and the base peak ions at *m/z* 105 and *m/z* 135 were therefore chosen in order to maximize sensitivity. Fig. 2 shows the ion current profiles of a SIM chromatogram of an incubated sample containing 1 μmol of the internal standard. The retention time was 5.4 min for methyl hippurate and 7.8 min for the corresponding *p*-methoxy derivative.

The above results therefore show that only an insignificant amount of the SIM response at the chosen *m/z* values was due to the second component, and that this was eliminated by the chromatographic separation achieved. A further positive feature of the analysis is that the physical and chemical properties, including reactivity for derivatization and sensitivity for SIM, would be expected to be very similar for the two compounds. No interference from endogenous compounds was observed in our experiments.

A standard curve was constructed in which known amounts of hippuric

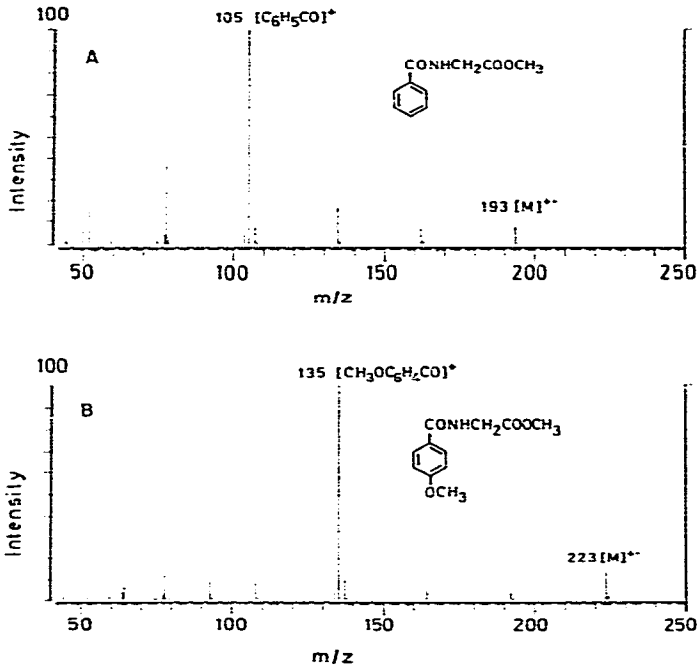

Fig. 1. Mass spectra of the methyl esters of (A) hippuric acid and (B) *p*-methoxybenzoylglycine.

acid (0.05—1.4 μmol) were added to flasks containing liver homogenate, hydrochloric acid and a fixed amount of *p*-methoxybenzoylglycine (1 μmol) and the mixture carried through the extraction and derivatizing procedure. A least-squares fit [r=0.997 ± 0.002 (S.D.)] gave a linear relationship between peak area ratios (*m/z* 105:135) and mole ratios. The intercept on the y axis (peak area ratio) was $0 \pm 1.37 \cdot 10^{-2}$ and the slope $0.96 \pm 2 \cdot 10^{-2}$ (n=5). This standard curve encompasses the range of values of current interest.

It is possible to measure smaller quantities of hippuric acid by constructing a standard curve using only one fifth of the given amount of internal standard. In this way we obtained lower measurement limits in the range of 10—25 pmol.

The rate of formation of hippuric acid by the homogenate of guinea pig liver was linear with time for at least 60 min (Fig. 3A). The mean activity in four different guinea pig liver homogenates was 11.5 ± 0.8 (S.D.) μmol/h/g of liver (69.4 ± 6.6 nmol/h/mg protein). This activity is somewhat higher than that observed earlier using guinea pig liver mitochondria [8]. This difference is partly due to the use of whole homogenate in the present experiments and partly due to the fact that the conditions for the enzyme reaction were optimized.

The most effective change we noted was the decrease in phosphate concentration from the earlier value of 83 m*M* [8] to 30 m*M* which resulted in a considerably greater enzyme activity (results not shown).

The formation of hippuric acid was linear with respect to protein concentration up to 8.4 mg/ml (Fig. 3B). The amount of protein used in the rate versus time studies described above was 5.6 mg/ml.

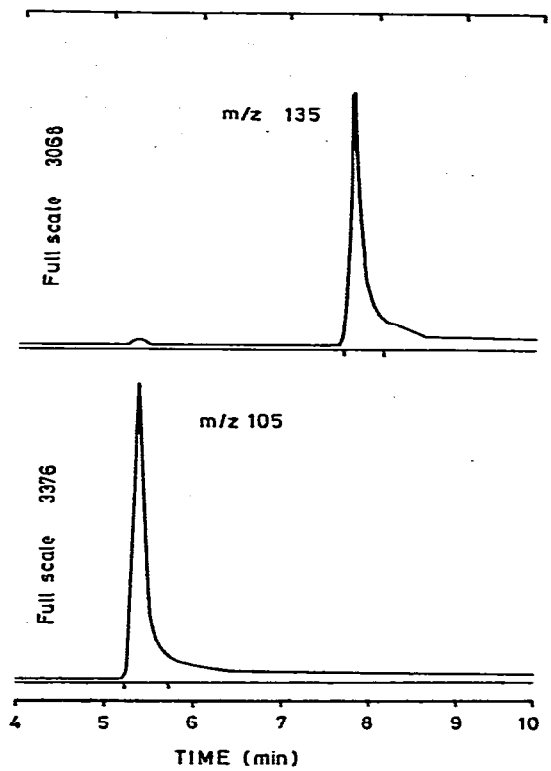

Fig. 2. Selected ion current profiles of a whole homogenate sample after incubation with cyclohexanecarboxylic acid and addition of *p*-methoxybenzoylglycine (*m/z* 135) as internal standard (1 μmol). The measured hippuric acid (*m/z* 105) concentration was 1.35 μmol.

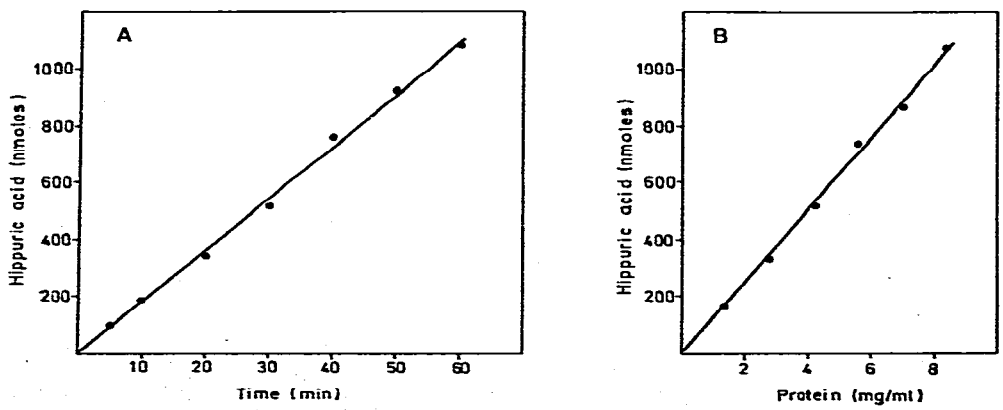

Fig. 3. Formation of hippuric acid in guinea pig homogenate as a function of time (A) and protein (B). In (A) 5.6 mg protein per ml was used and in (B) the incubation time was 40 min. Conditions otherwise were as stated in Experimental.

In conclusion, we believe that the GC—MS method described here for following the aromatization of cyclohexanecarboxylic acid has advantages over the methods reported previously [7, 8]. It shows high specificity and sensitiv-

ity and may be easily adapted to homologous substrates in order to study substrate specificity of the enzyme system. Furthermore, the method is suitable for investigation of the aromatizing activity in animal species other than the guinea pig, in tissues other than the liver and, significantly, in subcellular fractions. Such studies are of considerable interest with respect to assessing the biochemical significance of the aromatization reaction.

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