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Determination of Phenylalanine Metabolites in Rat Tissues by Gas-Liquid Chromatography

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A rapid and accurate gas-liquid chromatographic method for the quantitative determination of phenylalanine metabolites is described. All the steps for their extraction from blood, liver and brain of the rat and their conversion into volatile derivatives are detailed. The following acids have been determinad: benzoic, phenylacetic, mandelic, o-hydroxyphenylacetic, phenyllactic, p-hydroxyphenylacetic and phenylpyruvic.

Gas-liquid chromatographic (GLC) methods for the determination of aromatic acids have been widely employed in the study of their urinary excretion in phenylketonuria (1, 5, 8, 10) and some other inborn errors of metabolism (3-6,9). Recent studies on experimental phenlyketonuria have made necessary to apply the available methods for the analysis of the aromatic acids derived from phenylalanine and tyrosine in animal tissues (2, 7).

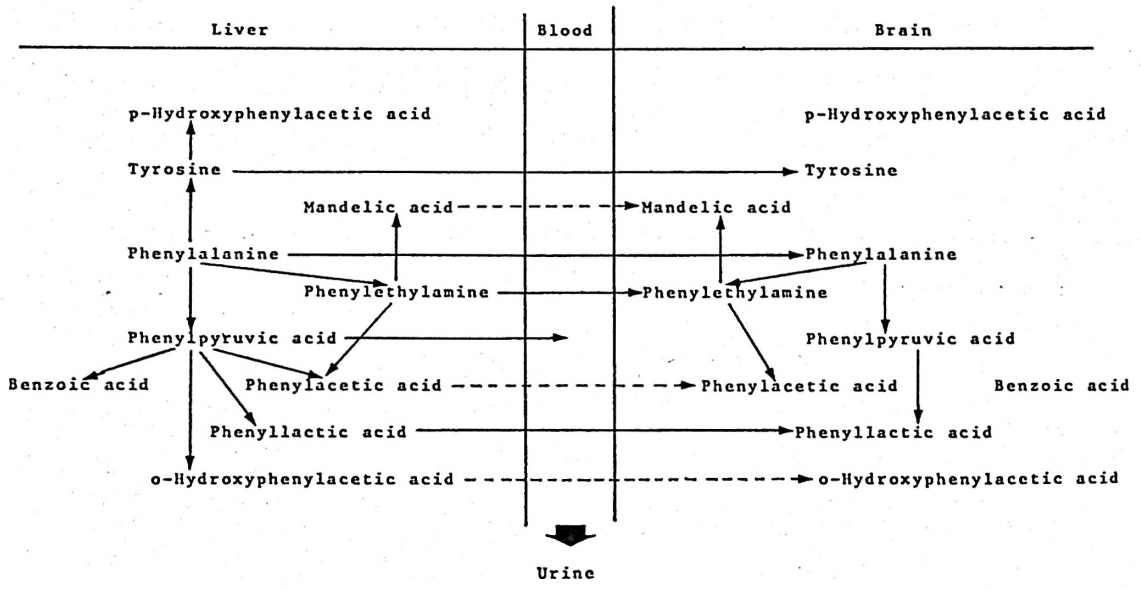
In the present paper a GLC method for the quantitative determination of aromatic acids in rat tissues is reported. Scheme 1 gives a schematic representation of the biochemical pathways through which aromatic acids are derived from phenylalanine.

Materials and Methods

Animals. Adult female rats of the Wistar strain weighing 150-200 g were used. All animals had free access to food and water.

Chemicals. All common laboratory chemicals were of reagent grade and were used without further purification. Bis-(trimethylsilyl)-acetamide (BSA) was obtained from Pierce Chemical Co. (Rockford, Ill.). Authentic aromatic acids were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Apparatus. A Perkin Elmer Model F-7 chromatograph equiped with a flame



Scheme 1. *Phenylalanine metabolism.*
Dashed arrows indicate pathways that have not been described yet.

ionization detector was used. A stainless steel column, 2 m × 1/8 inch. was packed with 10 % GE-SE 52 on Chromosorb W acid washed and treated with dimethyl clorosilane, 80-100 mesh.

Preparation of tissue extracts. The rats were killed by decapitation. The blood was collected in centrifuge tubes containing 6 % (w/v) perchloric acid from the severed neck vessels of each animal. The brains and livers were rapidly removed, weighed, minced finely with scissors and transferred to a Potter-Elvehjem all glass homogenizer. Then, 4 vol. of ice-cold 6 % perchloric acid was added and the mixture was homogenized with a glass pestle driven by a low-speed motor. The homogenates were centrifuged at 15.000 × *g* for 20 min. The supernatant fluid were neutralized with 10 % (w/v) KOH. After the solutions had stood for 1 h at 0° C the $KClO_4$ precipitate was sedimented by centrifugation. The supernatant solutions were adjusted to pH 1-2 with concentrated hydrochloric acid. Then, the addition of 1 vol. of saturated sodium chloride solution was followed by extraction twice with 4 vol. of ethyl acetate. When necessary, interface emulsion was broken by centrifugation. The combined ethyl acetate solutions were dried over anhydric Na_2SO_4, filtered and the solvent was evapored to dryness in vacuum below 30° C, after adding the internal standard (0.5 mg phenylbutyric acid in dry pyridine).

Preparation of derivatives for gas-liquid chromatography. Volatile derivatives of the extracted aromatic acids were prepared by addition of 0.5 ml of bis-(trimethylsilyl)-acetamide in chloroform (1:3, v/v). The reaction left at 30° C for 20 min. The vials were kept closed until samples were withdrawn for analysis.

Gas-liquid chromatography. One-five μl of the supernatant were injected into the gas chromatograph, analyses being carried out with the column operated isothermally at 165° C, and the inlet and the detector at 180° C. Gas flow at the inlet for N_2, H_2

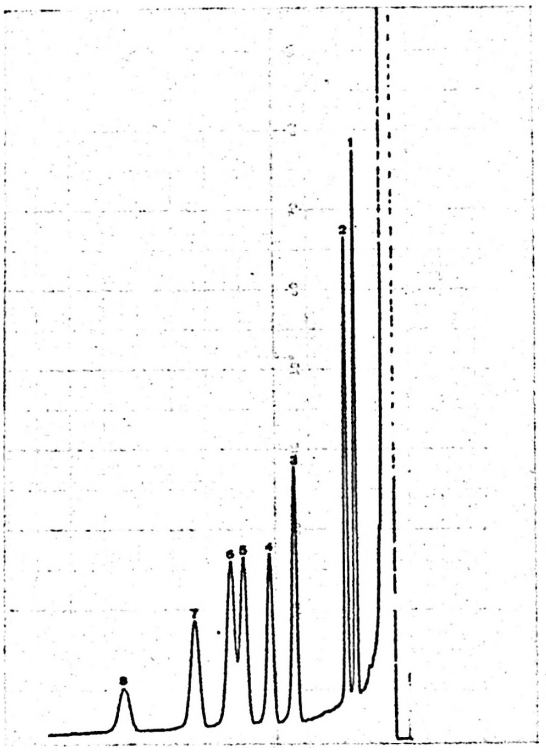


Fig. 1. *Chromatogram of trimethylsilyl-derivatives of a mixture of authentic aromatic acids derived from phenylalanine.*
Peak identification. 1: benzoic acid; 2: phenylacetic acid; 3: mandelic acid; 4: phenylbutyric acid (used as internal standard); 5: o-hydroxyphenylacetic acid; 6: phenyllacetic acid; 7: p-hydroxyphenylacetic acid; 8: phenylypruvic acid. For methodical details see the material and methods section.

and air was 55, 70 and 490 ml/min, respectively. Attenuation was varied according to the concentration of the aromatic acids in the extract.

Calculation. Identification of each aromatic acid in the tissue extracts was achieved by comparison with the chromatogram of the derivatives of authentic compounds (fig. 1). Mean retention times (n = 10) in seconds were as follows: benzoic acid: 238; phenylacetic acid: 282; mandelic acid: 556; phenylbutyric acid: 654; p-hydroxyphenylacetic acid: 815; phenyllactic acid: 881; p-hydroxyphenyllactic acid: 1077; phenylpyruvic acid: 1182.

For calculations calibration curve for each aromatic acid was used. The acids were dissolved together and aliquots were evaporated to dryness before derivatives were prepared. The peak area ratio of each acid to internal standard was plotted against their corresponding weight ratio (fig. 2).

Results and discussion

The chromatogram obtained for trimethylsilyl ether/ester derivatives of seven authentic aromatic acids is shown in figure 1. As can be seen the separation of the peaks permits a safe identification and an accurate measurement of each acid.

Table I shows the yield of the aromatic acids on extraction from tissues to which known amounts of all compounds were added. According to WADMAN *et al.* (9) phenylpyruvic acid and p-hydroxyphenylacetic acid were more susceptible to losses than the other acids.

The values of aromatic acids obtained from pooled tissues of rats are shown in table II. These results are in agreement with those obtained by EDWARDS *et al.* (2) except for phenylacetic acid, which was found in sensibly higher concentration when our method was applied. This disagreement may be due to our practice of

Table I. *Recovery of the aromatic acids on extraction from tissue homogenates.*

Acids	Mean recovery (N = 4) %
Benzoic	95.8
Phenylacetic	86.1
Mandelic	59.5
o-Hydroxyphenylacetic	72.8
Phenyllactic	82.6
p-Hydroxyphenylacetic	71.0
Phenylpyruvic	73.7

Table I. *Aromatic acids derived from phenylalanine in rat tissues.*
The values are expressed in nmol/g wet tissue. n.d. = not detected (very small quantities or absent).

	Blood*	Brain*	Liver**
Benzoic	5.16	22.8	15.6±2
Phenylacetic acid	5.62	198.7	165.0±19
Mandelic acid	0.49	13.2	23.0±2
o-Hydroxyphenylacetic acid	n.d.	1.02	17.7±7
Phenyllactic acid	2.17	0.88	1.4±0.1
p-Hydroxyphenylacetic acid	0.44	n.d.	37.0±5
Phenylpyruvic acid	0.74	n.d.	n.d.

* Average of triplicate determinations from two pools of ten samples each.
** Average (± S.E.M.) of single determinations from three pools of three liver each.

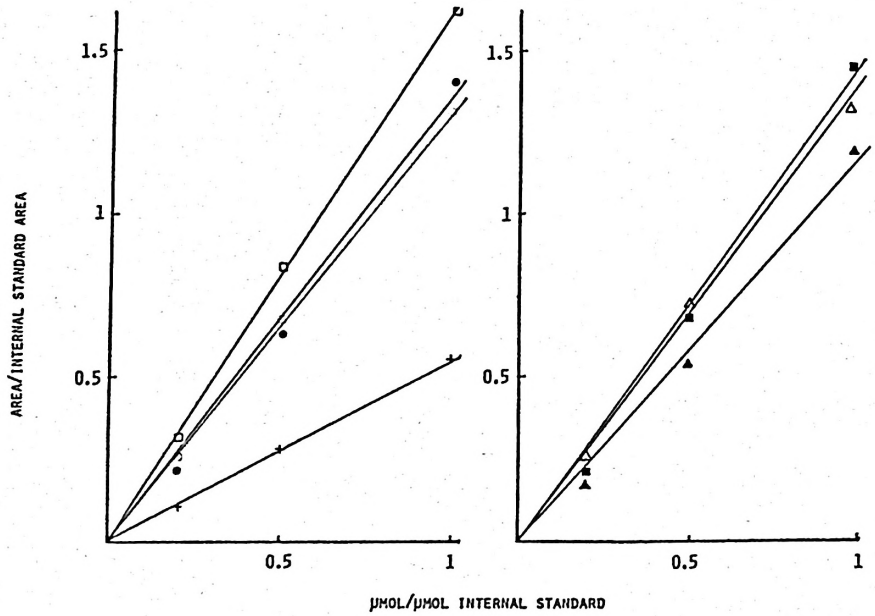


Fig. 2. *Calibration curves of aromatic acids derived from phenylalanine.*
Each point represents average of three determinations. Conditions of analysis in Material and Methods section. ○ = benzoic acid; ● = phenylacetic acid; □ = mandelic acid; ■ = o-hydroxyphenylacetic acid; △ = phenyllactic acid; ▲ = p-hydroxyphenylacetic acid; + = phenylpyruvic acid.

using a large amount of tissues extract in the measurement and to the different procedures of extraction.

It can be concluded that the method developed is useful, highly specific and time-saving in the determination of the different aromatic acids from phenylalanine.

Resumen

Se describe un método rápido y exacto para la determinación cuantitativa de ácidos aromáticos por cromatografía en fase gaseosa. Asimismo, se detallan todos los pasos necesarios para la extracción de estos compuestos en tejidos de rata y para su conversión en derivados volátiles. Se han determinado en sangre, hígado

y cerebro de rata los siguientes ácidos: benzoico, fenilacético, mandélico, o-hidroxifenilacético, fenil-láctico, p-hidroxifenilacético y fenilpirúvico.

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