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URINARY PHENYLKETO ACIDS IN PHENYLKETONURIA. OBSERVATION BY MEANS OF GAS CHROMATOGRAPHY AND GAS-MASS SPECTROMETRY.

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Abstract

It is necessary to estimate urinary excretion of phenylheto acids (PKA's) for the differential diagnosis of classical phenylketonuria (PKU) from common phenylalaninemia of various origins as well as for the observation of therapeutic effect of diets on PKU. Urine specimens were collected from six patients of PKU and eight heterozygotes of PKU belonging to 3 independent families.

PKA's were extracted from urine specimens into ethylacetate and silylated so that it might be subjected to gas chromatographic (adsorbent OV-1) and gas-mass spectrometric studies. PKA's produced by the abnormal metabolic pathway of phenylalanine (Phe) in PKU were identified gas chromatographically with phenylacetic acid, mandelic acid, o-hydroxyphenylacetic acid, phenyllactic acid and phenylpyruvic acid. Their elution peaks appeared in the order listed above, with millimolar ratio of 1:1:3.1:25.7:46.5 in the untreated and stationary PKU patients. This ratio remained in a satisfactorily narrow range to the extent that its calculation is thought to be helpful for differential diagnosis of PKU from hyperphenylalaninemias of etiologically varived types.

No significant peaks of these five PKA's were seen gaschromatographically with the urines of PKU heterozygotes as well as with those of the normal subjects. However, one point worthy of special mentioning is a transitory appearance of extremely flattened (or equivocal) PKA peaks with the urines collected from the PKU heterozygotes for 6 hours after Phe-loading. This is suggestive of a compensatory reaction of heterozygotes in response to administration of large amount of Phe.

In the authors experience gas chromatography provided the most sensitive and reliable measure of the various diagnostic tests for PKU, and mass spectrometer proved to be a powerful tool for identification of PKA's.

INTRODUCTION

Phenylketonuria (PKU) discovered and reported in 1934 is a genetic disease which is characterized by hyperphenylalaninemia, phenylketo aciduria, mental retardation and low pigmentation.

Later studies revealed that the disease arised from defective structural genes for production of enzyme, phenylalanine 4-hydroxylase in the liver, and it became well known as the first instance which supports Garrod's hypothesis: one gene one enzyme theory on the causation of clinical symptoms.[1-3]

However, recent remarkable progress in the screening survey of PKU has demonstrated the presence of atypical PKU and hyperphenylalaninemia which are not always related to so called classical PKU.

Therefore, precise study based on qualitative and quantitative determination of the classical PKU metabolites has been thought to be necessary for the subtypes of this disease.

The present paper aims to report the results of our trial of the identification and quantitation of urinary phenylketo acids in patients with classical PKU and subjects heterozygous for PKU gene by means of gas mass spectrometry and gas chromatography.

MATERIALS AND METHOD

Urine specimens were collected from six cases of classical PKU and eight individuals heterozygous for PKU (Fig. 1).

At the time of steady state and/or before and after phenylalanine loading. The specimens were subjected to analyses as quickly as possible or kept in a deep freezer (−20°C) before use.

Reagents: a) *Standard solution:* Commercially available analytically pure grade reagents or the reagent purified by recrystallization was used as standard substance, namely they include phenylpyruvic acid (PPA), phenyllactic acid (PLA), o-hydroxyphenylacetic acid (OHPAA), and mandelic acid (MA). Each of them was dissolved individually in ethylacetate, adjusting the concentration to 100 mg % to use for standard solution.

Another standard solution dissolving 1 mg of phenylbutylic acid (PBA) and 1 mg of dicyclohexyl phthalate (DCHP) in 1 ml of ethylacetate was prepared so that it might be used as the markers and internal standard for gas chromatography. This standard solution is equivalent to 6.1 mM PBA, and 3.0 mM DCHP.

b) *Extraction and silyl agents:* All the reagents necessary for the

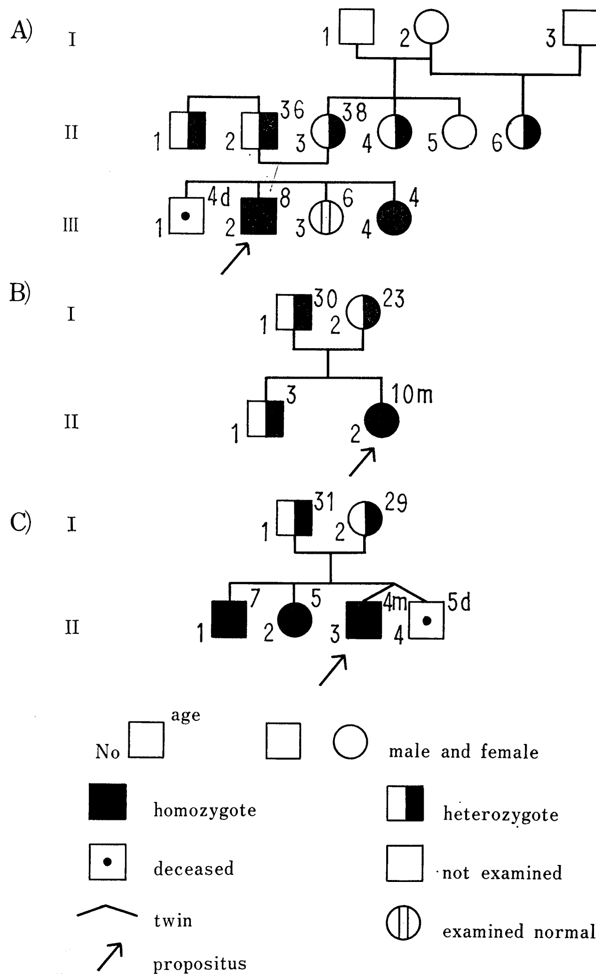

Figure 1. Family tree of three phenylketonuria (Genoytpe).

extraction and silylation including ethylacetate, anhydrous sodium sulfate, saturated sodium chloride solution, acetonitrile, and bis-(trimethylsilyl) acetamide (BSA) were prepared with commercially available analytical pure grade reagents or the reagents purified by distillation.

Apparatus: Gas chromatographic determination of phenylketo acids were performed with the Shimazu Gas Chromatographic Apparatus Model GC-5APTF with integrator, and gas-mass spectrometric identification of the acids were carried out with Hitachi Model RMU-6A which is the combined assembly of gas chromatography and mass spectrometer.

Procedure: Five ml of urine specimen was adjusted to pH 2.0 with concentrated hydrochloric acid, followed by addition of 5.0 ml of saturated NaCl solution and 1.0 ml of internal standard solution. The mixture was extracted two times with 20.0 ml of ethylacetate. The extracted layer (totaling 40 ml) was dried by shaking with 3.0 grams of anhydrous Na_2SO_4 and allowed to stand for about one hour at room temperature.

Ethylacetate extract deprived of water was filtered and it was evaporated off with the aid of rotary evaporater at temperature below 40°C. The residue was dissolved with minimum amount of ethylacetate and transferred into a small glass stoppered test tube and evaporated to dryness by blowing pure nitrogen gas. The dry residue was then dissolved in one ml of acetonitrile, and silylation process was carried out by addition of 0.2 ml of BSA and kept at 60°C for 15 minutes.

One or two μl of the sililated solution was applied to either gas chromatograph or gas-mass spectrometer, which had been set preliminarly in good condition.

RESULT

Gas chromatographic detection and identification of the urinary phenylketo acids of the patient with phenylketonuria were carried out in both ways of checking mass number and of comparing retention time with those of the standard substances.

Mass spectrogram of PLA is shown in Fig. 2 as a representative example of the phenylketo acids.

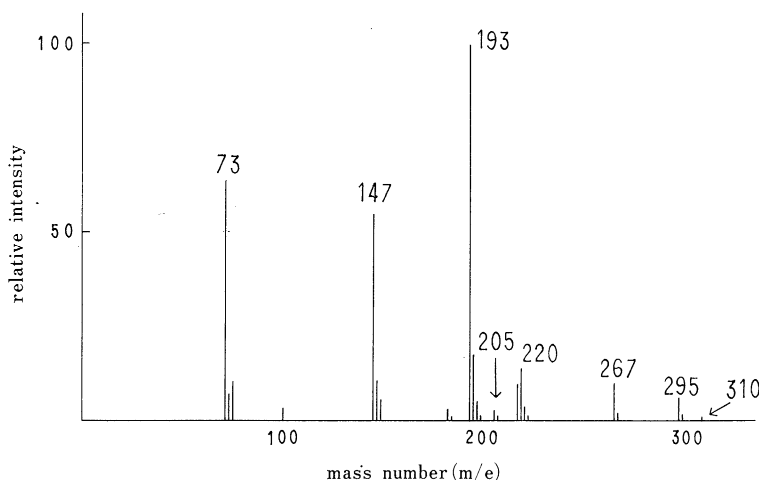

Figure 2. Mass spectrum of trimethylsilylated phenyllactic acid. Mass peak of 310, 295, 267 and 193 are corresponding to those of parent $(M)^+$, $(M-15)^+$, $(M-43)^+$, $(M-117)^+$, respectively. See the text and Table 1 about mass peak of 220^+, 205^+, 147^+ and 73^+. Mass spectrometry conditions were as follows: Ionization energy, 20 eV; Ionization chamber, 210~220°C; Mass number range, 1~1000 m/e.

Identified phenylketo acids are summarized in Table 1 together with their parent and fragment mass number and their relative intensities to the base peaks of respective components.

TABLE 1.
Mass number (m/e) and relative intensity of trimethylsilyl (TMS) derivatives of urinary phenylketo acids in patients with phenylketonuria.

TMS-phenylketo acid	molecular weight	relative intensity (%)										
		common fragment peak								specific fragment peak (relative intensity %) m/e		
		M+	M+ -15	M+ -43	M+ -117	147	77	75	73 (m/e)			
Phenylacetic acid	208	1	47	21	11		22	100	25	164 (71)	117 (11)	
Mandelic acid	296		1	7	100	24	7	4	52	201 (1)	100 (34)	
o-Hydroxyphenyl acetic acid	296	8	6	11	11	51	37	96	100	206 (21)	164 (7)	100 (13)
Phenyllactic acid	310	1	7	11	100	56	10	7	62	220 (15)	205 (6)	
Phenylpyruvic acid	308	3	37	1		100	6	2	13	237 (2)	221 (1)	118 (2)

The calibration curves for the determination of phenylketo acids had different sensitivity to flame ionization detector but were proportional to the concentration of individual acid passing on the origin of the chart.

Recovery tests by adding 1.0 mg of PPA, PLA, OHPAA, PAA and MA into a 5.0 ml urine specimen were 75.8, 92.5, 93.5, 109.7 and 90.8 per cent, respectively. Recovery value of PPA seemed to be fairly low as compared with those of other components.

The levels of excretion of phenylketo acids in patients with PKU were PPA $>$ PLA $>$ OHPAA $>$ MA $=$ PAA in the order listed as shown in Table 2 and Fig. 3. MA and PAA was almost equal in excretion level in all the urine specimens tested.

In heterozygote, none of the urinary phenylketo acids were detected by the method employed in this study, making a sharp contrast to homozygotes. It is clearly seen from Fig. 3 that only PBA and DCHP which were preliminary added as internal standard were demonstrable. However, when the specimen was increased to 10 times as much and the sensitivity of the instrument was elevated the presence of PPA, PLA and OHPAA was noticeable, but it was too small in amount for reliable estimation. The same tendency of urinary excretion of phenylketo acids

TABLE 2.
Urinary excretion of phenylketo acids (mM/g creatinine), and serum phenylalanine (Phe) concentration (mg %).

		PAA	MA	OHPAA	PLA	PPA	Phe
A	II 1	U	U	U	U	U	2.03
	II 2	U	U	U	U	U	2.41
	III 1	0.28	0.26	0.70	4.93	11.4	21.9
	III 2	U	U	U	U	U	2.31
	III 3	0.07	0.10	0.40	2.67	5.01	28.2
B	I 1	U	U	U	U	U	1.15
	I 2	U	U	U	U	U	1.20
	II 1	U	U	U	U	U	1.67
	II 2	0.37	0.59	1.91	16.6	30.4	20.2
C	I 1	U	U	U	U	U	1.14
	I 2	U	U	U	U	U	1.53
	II 1	0.28	0.40	1.31	10.1	19.1	22.8
	II 2	0.38	0.36	1.13	10.9	21.4	26.8
	II 3	0.38	0.33	0.86	8.55	10.4	28.3
control		U	U	U	U	U	0.09

U=undetected

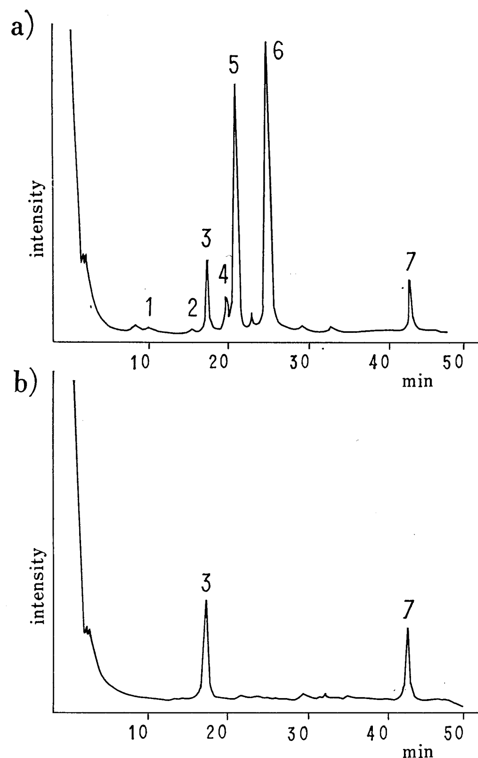

Figure 3. Gas chromatogram of phenylketo acids extracted from urine of a) patient (B–II–2) and b) normal control. 1, PAA; 2, MA; 3, PBA; 4, OHPAA; 5, PLA; 6, PPA; 7, DCHP. Gas chromatographic conditions were as follows: glass column and packing, 2 m×0.4 mm, OV–1, 1.5 per cent (80~100 mesh). Temperature range, 70~250°C, 4°C/min.; Nitrogen carrier, FID detector.

was more evident when the specimens were collected 0-6 hrs after phenylalanine loading (Phenylalanine 0.1 g/Kg) but still the level was not so high as enabled us to perform exact estimation.

Similar aspect was seen in the normal control. Therefore, no discrimination was possible between the heterozygotes and the normal subjects.

DISCUSSION

There are two characteristic features in the cleavage of the phenylketo acids by ionization energy (20 eV) of the mass spectrometer.; one is the common fragmentation into all the possible phenylketo acids, and another the specific fragmentation into individual phenylketo acids.

In general, there is appearance of fragment peaks having the mass numbers corresponding to 1) parent molecule (M^+), 2) $(M-15)^+$ produced by elimination of methyl radical, 3) $(M-43)^+$ arising from removal of CH_3 and CO radicals, 4) $(M-117)^+$ formed by removal of $COOSi(CH_3)_3$ radical, 5) 73^+ indicating $Si(CH_3)_3^+$, 6) 147^+ referring to the structure $(CH_3)_2=Si=0^+-Si(CH_3)_3$ which might be produced by recombination of $Si(CH_3)_2$ and $OSi(CH_3)_3$, and 7) 75^+ representing $HO^+Si(CH_3)_2$.

As to the specific character, the specific mass numbers are mentioned as follows: in MA, 207^+ showing $(M-SiO(CH_3)_3)^+$; in OHPAA, 206^+ indicating $(M-HOSi(CH_3)_3)^+$; in PLA, 220^+ and 205^+ both of which might have the construction of $(M-(CH_3)_3SiOH)^+$ and $(M-(CH_3)_3SiOH-CH_3)^+$, respectively, and in PPA, 237^+, 221^+, and 118^+ indicating uninterpretable mass number in usual way.

The appearance of uninterpretable specific peaks for PPA was, however, not an obstacle against its identification since it demonstrated common nature and standard PPA showed the same phenomena in the mass spectrometry.

In the gas chromatographic determination of PPA the average recovery rate (75.8%) was low. This is thought to be based on the instability of PPA. Therefore, in order to increase the recovery, preliminary addition of ascorbic acid or hydroxylamine to convert PPA into more stable oxime form had been devised[6,7]. In our method without taking such a devices a satisfactory recovery level was obtained by processing quickly the produce from extraction to silylation at temperature below 40°C.

When the average millimolar proportions of PPA, PLA, OHPAA, MA, and PAA were calculated, the values were 46.5, 25.7, 3.1, 1, 0.95 in the order described. The ratios remained almost constant in different individual patients of PKU.

The existence of MA in the urine of the patients with PKU had been questionable, because it was so small in amount that it escaped from detection by paper chromatographies.[8] However, in the present investigation MA was identified unequivocally both by gas-mass spectrometry and by gas chromatography. Moreover, since it varied in amount in parallel with other phenylketo acids, MA is thought to be placed in a concrete step in the metabolism of phenylalanine in patients with PKU.

Since the renal threshold of OHPAA and PLA is so low as compared with the relatively high threshold for PPA which is excreted in abundance, the detection of OHPAA and PLA is more recommendable than that of PPA for the diagnosis and follow-up of the effect of treatment of this disease[9].

However, checking all the component peaks in gas chromatographic chart is desirable since chromatography can be processed rapidly with ease and with small amount of specimens.

So far as the simple gas chromatographic and gas-mass spectrographic studies on PKU urine are concerned no discriminative criteria are obtainable. This will be an evidence for the conception that the heterozygotes possess a PKU gene and a normal gene for phenylalanine 4-hydroxylase and this normal gene compensates completely for the defective activity of the PKU gene. The difference between the normal subjects and the PKU heterozyotes can be established only by the analysis of blood specimens after phenylalanine loading test or by measurement of phenylalanine/tyrosine ratio.[10-12]

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