COMPARISON OF URINARY ORGANIC ACID BETWEEN NEONATE AND ADULT BY GAS CHROMATOGRAPHY- MASS SPECTROMETRY

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Urinary organic acid of neonate and adult were analyzed by the use of gas chromatography (GLC) and gas chromatography-mass spectrometry (GC-MS) after extraction with diethyl ether at pH 1. Similar chromatograms were obtained from urines of both neonate and adult. Standard deviations of all peaks were relatively low in neonates as compared with those of the adults. None of specific compound was found in neonatal urine, that is, almost the same kinds of compounds were found to be excreted in nearly the same quantity in urine of neonate and adult. Although fatty acids of palmitic acid and stearic acid are excreted in fairly large amounts into neonatal urine, this may be due to milk fat intake. And excretion of large amounts of fumaric acid and succinic acid in neonate suggests more synthesis of ATP due to neonatal development. A relatively less amounts of 4-hydroxyphenylacetic acid and homovanillic acid was excreted in urine of the neonate, because of altered tyrosine metabolism due to enhancement of protein synthesis.

INTRODUCTION

Urinary acids are of interest for many reasons. Amino acids and biogenic amines were metabolized to corresponding keto acids and organic acids by transaminase or oxidative deaminase reactions. These metabolites were excreted into urine either directly or after further metabolized in the free or conjugated form. Carbohydrates and lipids are also major source of urinary organic acids. The excretion of organic acids in normal healthy human is unexpectedly constant in quantity. However, in the several diseases, some

specific organic acids accumulate in blood and are excreted in urine because of an inherited enzyme defect. For example, in phenylketonuria, organic acids of phenylpyruvic acids, phenyl-lactic acid and o-hydroxyphenylacetic acids as well as phenylalanine are excreted in large amounts in urine. And alkaptonurics excrete several grams of homogentisic acid daily.

Gas-liquid chromatography is the most versatile technique available for the simultaneous and quantitative study, in minute amounts, of a wide range of compounds on different physical properties. The use of gas chromatography

for qualitative and quantitative determinations of urinary acids is, therefore, the most suitable for the study of human metabolic disease and has been used by the majority of workers in this field (Mamer et al., 1971; Horning and Horning, 1971; Thompson and Markey, 1975). Combination of mass spectrometry to gas chromatography facilitated the direct identification of compounds, which were effectively separated by gas chromatography (Jellum et al., 1973). The usefulness of this technique in research on inherited diseases has been generally recognized (Jellum et al., 1971; Gan et al., 1974; Horning et al., 1975).

Based on the same reason, the author applied this technique to the comparative studies on urinary organic acids in neonate and adult in order to have an idea concerning the changes on enzyme activities. This may be related with age, that is, it has been known that the enzyme activity of phenylalanine hydroxylase increases during the first 4 to 12 days after birth (Hermann and Tootle, 1964) and that of tryptophan pyrrolase also increases within a short time after birth (Barnett, 19__). The results obtained are reported and discussed.

EXPERIMENTATION

Reagents and materials

Bis (trimethylsilyl) acetamide (BSA) and trimethylchlorosilane (TMCS) were obtained from Tokyo Kasai Co. Ltd.. A column packing, 3% OV-17, was purchased from Nihon Chromato Co. Ltd.. n-Eicosane, glutaric acid and other reagents were purchased from Wako Pure Chemical Industries, Ltd..

Gas chromatography (GLC) and gas chromatography-mass spectrometry (GC-MS)

A JEOL JGC-1100 gas chromatograph with dual flame ionization detectors was used. A glass coiled column (2m × 3 mm i.d.) were packed with 3% OV-17 on Gas Chrom Q (80-100 mesh). Nitrogen was used as a carrier gas with a flow rate of 30 ml/min and oven temperature was programmed from 80°C to 280°C at a rate of 6°C/min.

A JEOL JMS D-100 gas chromatography-mass spectrometer equipped with a glass column (2m × 2 mm i.d.) packed with 3% OV-17 on Gas Chrom Q (80-100 mesh) was used. Operating conditions were as follows; column temperature was the same as GLC, ion source temperature was maintained at 290°C and separator temperature was kept at 280°C. Ionizing energy was 75 eV and ionizing current was 300 μ A.

The data was processed with a Chromatopac 4-B on lined to GLC and a JEC 980-B computer on lined to GC-MS.

Internal standard

n-Eicosane and glutaric acid were used as internal standards. 40 μ g of n-eicosane and 40 μ g of glutaric acid corresponding to 1 mg of creatinine were added to 2 ml of fresh morning urine sample.

Extraction procedure

40 μ g of n-eicosane and glutaric acid were added to about 2 ml portions of fresh morning urine as described by Jellum (1971). The urine was then acidified by dropwise addition of 2N HCl to pH 1. The acidified urine specimens were extracted 3 times with equal volume of diethyl ether. The organic phase were combined and dried for 1-2 hours over anhydrous sodium sulphate. Then the volume was reduced to about 0.2-0.5 ml by vacuum evaporator. After

transferred the extracts into a small vial, the rest of solvent was dried by the aid of nitrogen stream.

Preparation of trimethylsilylation

150 μ l of BSA and 38 μ l of TMCS were

added to the sample under dry condition. The vials were sealed and heated for 30 min with a heating block kept at 60°C (Mamer et al, 1971). After cooling at room temperature, an aliquot of the reaction mixture was injected into GLC.

Fig. 1 Gas chromatographic separation of the TMS derivatives of the extract obtained from normal neonatal urine. BSA/TMCS ratio 4 : 1. Column : mixed liquid phase of 3 (w/w) % OV-17 on 80/100 mesh, Gas Chrom Q, 2m $\times$ 3 mm I. D., glass. Initial temperature 80°C than 6°C/min, up to 280°C. The peaks are identified as : 1) 2-hydroxybutyric acid, 2) 3-hydroxyisobutyric acid, 3) 3-hydroxyisovaleric acid, 5) 2-hydroxyisovaleric acid, 6) urea, 7) succinic acid + fumaric acid, 9) glutaric acid (internal standard), 10) 2-hydroxyglutaric acid, 11) adipic acid, 12) tartaric acid, 13) 5-hydroxymethyl 2-furoic acid, 14) 4-hydroxyphenylacetic acid, 15) suberic acid, 16) azelaic acid, 17) homovanillic acid, 18) n-eicosane (internal standard), 19) hippuric acid + palmitic acid, 20) 3-indolepyruvic acid, 22) stearic acid.

Fig. 2 Gas chromatographic analysis of the TMS derivatives of urinary organic acid obtained from healthy adult urine. Temperature ; programmed at a rate of 6°C/min. up to 280°C, initial temperature 80°C. Column : 3 (w/w) % OV-17 on 80/100 mesh, Gas Chrom Q, 2m $\times$ 3mm I. D., glass. Nitrogen flow rate 30 ml/min. Volume injected 2 μ l. The peaks are identified as : 1) 3-hydroxyisobutyric acid, 2) 3-hydroxyisovaleric acid, 3) 2-hydroxyisovaleric acid, 6) urea, 7) succinic acid + fumaric acid, 9) glutaric acid (internal standard), 11) adipic acid, 13) 3-methyladipic acid, 15) 5-hydroxymethyl 2-furoic acid, 16) 4-hydroxybenzoic acid, 17) 4-hydroxyphenylacetic acid, 18) suberic acid, 21) azelaic acid, 22) homovanillic acid, 23) n-eicosane (internal standard), 25) hippuric acid + palmitic acid, 27) stearic acid.

RESULTS

Twenty urine samples of neonate aged from 1 to 7 days old were collected by a pediatric urine collector from three hospitals and 60 urine samples of normal healthy young adults aged from 19 to 23 were collected. The ratio of male and female was almost 1:1. Fig. 1 and 2 are gas chromatograms on TMS derivatives of organic acids of the neonates and the adults. About 40 peaks were observed in both gas chromatograms. It has been reported that 500 to 1,000 small and large peaks of urinary organic acids could be separated by capillary column. Since the number of plate is not satisfactorily high as compared with that of capillary column, the number of gas chromatographic peaks obtained was less than 1/10 of

capillary column.

Majority of the peaks of organic acids are 3-hydroxyisobutyric acid, 3-hydroxyisovaleric acid, urea, adipic acid, 4-hydroxyphenylacetic acid, suberic acid, homovanillic acid, hippuric acid, palmitic acid and stearic acid. Each peak was identified by comparison of the retention time and the mass spectra with those of authentic sample.

Some of typical mass spectra are shown in the followings. In the lower column of Fig. 3, mass spectrum of authentic 3-hydroxyisobutyric acid di-TMS is shown. Molecular ion, m/e 248 is not detected, whereas $M-15$, m/e 233 is clearly detected. M/e 147 corresponding to pentamethyldisiloxane, which is characteristic to polytrimethylsilylate compound, is a base peak. The mass spectrum of the peak No. 2 (in Fig. 1)

Fig. 3 Mass spectrum of 3-hydroxyisobutyric acid di-TMS. Temperature of ion source 290°C and of separator 280°C. Energy of electron 75 eV.

from urine specimen in upper column is quite similar to that of authentic sample. In Fig. 4, mass spectrum of urea di-TMS is shown. Molecular ion is seen at m/e 204 at a relative intensity of 10%. $M-15$ peak of m/e 189 is clearly observed. Again m/e 147 corresponding to pentamethyldisiloxane is a base peak in this mass spectrum. The mass spectrum of urine sample in the upper column and of authentic sample in the lower column are quite similar. As urea was a major nitrogen-containing compound in urine, it always appeared in the minor peak on gas chromatogram, though theoretically it may not be present in acidic extract of urine. Mass spectrum of hippuric acid mono-TMS is shown in Fig. 5. The molecular ion, m/e 251 is not intense

Fig. 4 Mass spectrum of urea di-TMS. Temperature of ion source 290°C and of separator 280°C . Energy of electron 75 eV.

Fig. 5 Mass spectrum of hippuric acid mono-TMS. Temperature of ion source 290°C and of separator 280°C . Energy of electron 75 eV.

but distinctly identified. The ion of m/e 236 is due to a loss of methyl radical. M/e 105 is a base peak formed by cleavage of peptide bond. The mass spectrum of urine sample in the upper column is almost identical to that of authentic sample in the lower column. An elaborate mass spectrometric analysis of peak No. 19 demonstrates that the peak consists of hippuric acid and palmitic acid and that the peak sometimes contains either hippuric acid or palmitic acid only depending on physiological conditions. Similarly, peak No. 7 consists of succinic acid and fumaric acid.

Statistical study revealed that the percentage of standard deviation to average ($\bar{X}$) of each peak on gas chromatogram of normal neonatal urine was between 30.4 % and 80.0 %, which is good enough because of less than 100 %, except urea. The result is shown in Table 1. On the contrary, the percentage of standard deviation to average was relatively high in adult than in neonatal and is between 53 % and 224 % as seen in Table 2. Average value of % (indicated in Table 1 and 2) on all the peaks in neonatal was 50.5 % and 90.6 % in adult. The result clearly showed that the average of all the peaks

TABLE 1

Peak No.	Identified compounds	$\bar{X}$	S. D.	n	%
1	2-hydroxybutyric acid	0.07	0.02	18	28.6
2	3-hydroxyisobutyric acid	0.12	0.08	20	66.7
3	3-hydroxyisovaleric acid	0.20	0.16	20	80.0
5	2-hydroxyisovaleric acid	0.02	0.01	19	50.0
6	Urea	0.39	0.35	20	89.7
7	Succinic acid + Fumarate	0.48	0.19	20	39.6
8		0.82	0.29	19	35.4
9	Internal standard (Glutaric acid)	2.26	0.41	17	18.1
10	2-hydroxyglutaric acid	0.15	0.08	20	53.3
11	Adipic acid	0.06	0.02	17	33.3
12	Tartaric acid	0.09	0.05	17	55.6
13	5-hydroxymethyl 2-furoic acid	0.30	0.11	20	36.7
14	4-hydroxyphenylacetic acid	0.39	0.16	18	41.0
15	Suberic acid	0.18	0.09	19	50.0
16	Azelaic acid	0.23	0.07	18	30.4
17	Homovanillic acid	0.22	0.08	19	36.4
18	Internal standard (n-eicosane)	1.00		20	
19	Hippuric acid + Palmitic acid	0.91	0.32	20	35.2
20	3-indolepyruvic acid	0.10	0.06	18	60.0
21		0.09	0.06	20	66.7
22	Stearic acid	1.06	0.59	19	55.7
25		0.35	0.22	20	62.9
26		0.43	0.23	20	53.5

$\bar{X}$: Mean of relative ratio of each peak to internal standard which is regarded as 1.

S.D. : Standard deviation.

n : Sample number.

% : Percentage of S.D. to $\bar{X}$.

in neonatal was lower than in adult.

Table 1 and 2 also showed a relative ratio ($\bar{X}$) of every peak to internal standard of n-eicosane which was re-

garded as 1.0. One may say in the tables that the majority of the compounds in urine of neonate and adult were excreted nearly equally, however

TABLE 2

Peak No.	Identified compounds	$\bar{X}$	S. D.	n	%
1	3-hydroxyisobutyric acid	0.22	0.15	55	70
2	3-hydroxyisovaleric acid	0.18	0.15	54	83
3	2-hydroxyisovaleric acid	0.04	0.03	43	83
4		0.02	0.015	14	75
5		0.015	0.008	6	53
6	Urea	1.405	0.895	56	64
7	Succinic acid Fumarate	0.14	0.17	53	121
8		0.286	0.185	55	65
9	Internal standard (Glutaric acid)	1.625	0.32	54	20
10		0.06	0.056	42	93
11	Adipic acid	0.064	0.052	32	81
12		0.12	0.14	40	117
13	3-methyladipic acid	0.143	0.116	52	81
14		0.065	0.055	21	85
15	5-hydroxymethyl 2-furoic acid	0.22	0.18	51	82
16	4-hydroxybenzoic acid	0.13	0.13	48	100
17	4-hydroxyphenylacetic acid	0.60	0.45	56	75
18	Suberic acid	0.135	0.10	51	74
19		0.14	0.155	47	111
20	4-hydroxymandelic acid	0.22	0.26	25	118
21	Azelaic acid	0.245	0.216	53	88
22	Homovanillic acid	0.49	0.42	56	86
23	Internal standard (n-eicosane)	1.00		56	
24		0.09	0.097	49	108
25	Hippuric acid Palmatic acid	2.24	2.29	56	102
26		0.32	0.40	56	133
27	Stearic acid	0.13	0.11	55	85
28		0.17	0.15	28	88
29		0.08	0.05	19	63
30		0.06	0.057	28	95
31		0.04	0.02	11	50
32		0.04	0.04	10	100
33		0.045	0.035	2	78
34		0.37	0.22	55	59
35		0.17	0.38	46	224

$\bar{X}$: Mean of relative ratio of each peak to internal standard which is regarded as 1.

S.D. : Standard deviation.

n : Sample number.

% : Percentage of S.D. to $\bar{X}$.

the average of relative ratios of peak No. 7, 14, 17, 19 and 22 of neonate were remarkably different from those of corresponding peaks of adult.

DISCUSSION

Comparison of gas chromatographic patterns of urinary organic acids of neonates and adults was performed by the use of gas chromatography and gas chromatography-mass spectrometry.

About 40 small and large peaks were separated with a 2 m 3 % OV-17 glass column. More than 500 peaks of urinary organic acids are separated by capillary column. Since the author used a multi-screening system (Haraguchi et al, 1976) for in born errors of metabolism which prefers simplicity and rapidity to accuracy, some peaks consisted of two or more components and not separated well one another (Matsumoto, 1976). For example, peak No. 19 consists of hippuric acid and palmitic acid and peak No. 7 also consists of succinic acid and fumaric acid. Such a disadvantage will be overcome by the use of GC-MS, since mass spectrometric analysis may provide an identification of mixed compounds. It is, however, desirable to perform capillary column equipped with GC-MS for more detailed experiment to extend my research further.

For the analysis of urinary organic acids, trimethylsilylation (TMS) was chosen for derivatization (Horning and Horning, 1970). The derivatization gives great advantage of simplicity, stability and reproducibility. However, in some compounds, derivatization is not always complete and gives two peaks, such as mono- and di-TMS derivatives of hippuric acid depending on the condition of derivatization. It is, therefore, necessary to keep the conditions strict-

ly, for example, at 50°C for overnight, (Mrochek et al, 1971) or at 120°C for 5 min (Hutterer et al, 1971).

There is a particular importance in this project concerned with the sampling. Quite reasonably, it is used 24 hr urine specimen for GC-MS analysis, however it is quite difficult to collect many samples of 24 hr urine from normal adults. As Jellum (1971) mentioned in his earlier work, the morning fresh urine contains almost the same constituents as that of 24 hr urine in quality and quantity, and it is more characteristic of the endogeneous metabolism. This was confirmed in the preliminary experiment (Haraguchi et al, 1976) and, therefore, fresh morning urine specimen were used throughout the experiment.

Gas chromatographic profiles of urinary organic acids in neonates and adults were quite similar except a few peaks. Horning, E. C. (1971) also analyzed urinary aromatic acids of neonatal infant and he obtained the result of simpler profile of neonate than that of adult. No particular compound was detected either neonatal urine or in adult urine. The remarkable difference in the chromatographic profiles of urinary organic acids in between the neonates and the adults is the standard deviations of each peak, that is, the standard deviation of the neonates is always smaller than that of adults. This suggests that the excretion of organic acids into urine is a fairly constant level and not different in all the neonates. To the contrary, excretion of organic acids into urine varies remarkably from person to person in the adults (Jellum et al, 1973). The reason why the variation is not so big in the neonates and big in the adults, is not fully understandable. However one may point out that the diet intake in the neonate during first 7 days is almost

the same. This is reflected on gas chromatographic profiles constantly and vice versa in the adults. In other words, there is no significant difference upon the excretion of organic acids into urine between the neonates and the adults, if the diet intake is the same. If this is true, the major metabolism in the neonate and the adults should be the same, as Jellum (1973) mentioned the difference may be due to different food habit and partly to genetic variations in adult.

The more careful statistic investigation indicates that the excretion of succinic acid plus fumaric acid and stearic acid are significantly high and, on the contrary, the excretion of 4-hydroxyphenylacetic acid and homovanillic acid are low in the neonate. The remarkable increase of peak No. 19 (Table 1), which corresponds to hippuric acid plus palmitic acid in the neonate, is due to the increase of palmitic acid, which was confirmed by a mass chromatographic technique (unpublished). However it was not confirmed yet which compound was responsible for the increment of peak No. 7. Most reasonably, it may be anticipated that both succinic acid and fumaric acid are increased simultaneously. Since stearic acid and palmitic acid are found comparatively high in urine of the neonate and both fatty acids are known to be major components of milk fat, it is quite reasonable to assume that these two acids were originated from milk. Relatively large amounts of succinic acid and fumaric acid in the urine of the neonates suggest highly activated tricarboxylic acid cycle and result in forming more ATP. Of course the more ATP is required for the growth of neonates.

The lower levels of both 4-hydroxyphenylacetic acid and homovanillic acid are quite difficult to be understood.

The former is an endoproduct of tyrosine metabolism via tyramine and 4-hydroxyphenylacetaldehyde, and the latter is another endoproduct of tyrosine and is metabolized either via DOPA, 3-methoxytyrosine, 3-methoxy-4-hydroxyphenylpyruvate, or via DOPA, Dopamine, homoprotocatechuate and, in some cases, via DOPA, Dopamine, 3-methoxytyramine. These two compounds are not closely related each other. This observation, however, suggests that tyrosine metabolism is greatly altered in the neonates, probably due to great enhancement of protein synthesis. Anyway further investigation will give a development in this field.

As a result of fact, the deviation of urinary organic acids on gas chromatogram of neonate was quite low and the neonatal urine rather than adult can be basically useful for screening of metabolic disorders by GC-MS-COM system. An abnormal compound is, however, detected as a very large peak 10 times or sometimes 100 times more than that of normal (Thompson and King, 1964). Derivatization of trimethylsilylation is very simplified and separation by GLC is quite well in comparison with liquid chromatography being time-consuming, such as 48 hours (Scott et al., 1967) and less suited when one requires increased capacity to analyze many samples for screening.

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