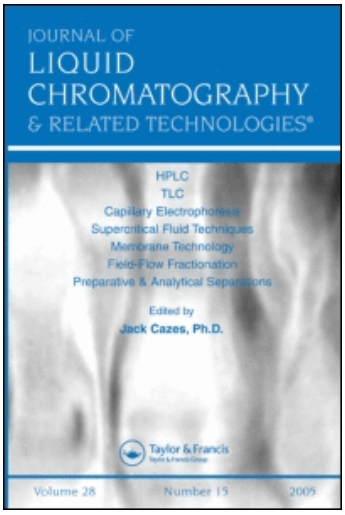

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Analysis of Fluorescent Compounds in Urine by Liquid Chromatography

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ANALYSIS OF FLUORESCENT COMPOUNDS IN URINE BY LIQUID CHROMATOGRAPHY

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ABSTRACT

High speed separation of fluorescent compounds was examined. The retention time of 21 compounds was measured in two reversed phase modes and an ion-exchange mode liquid chromatography. Furthermore, urine samples of new-born babies, cancer patients and normal subjects were analyzed by the above systems. Several peaks were positively identified from the retention time, however there were many unknown fluorescent compounds. Among them, two peaks were found on the chromatograms in the reversed phase modes. These compounds were very polar and could not be identified, however the ratio of these peak height was used for classification of urine samples. Furthermore, indole-3-acetic acid and 5-hydroxyindole-3-acetic acid in urine were selectively analyzed on an ion-exchange resin with isocratic eluent after filtration.

INTRODUCTION

The liquid chromatography separation of ultraviolet absorbing constituents in urine becomes faster and will be a practical technique in a

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clinical research due to development of new packings. In an ion-exchange liquid chromatography, the separation time was drastically reduced by using a small particle macro-porous anion-exchange resin [1,2]. Nucleosides and polar compounds were analyzed by reversed phase liquid chromatography [3,4,5].

In a previous report, we have demonstrated the possibility of high-speed separation of ultraviolet absorbing constituents in urine with 50 standard compounds in both hydrophobic and ion-exchange modes on chemically bonded silica gels [6]. Further improvements of the separation and the selectivity of the detector simplifies the chromatograms. Urine samples of new-born babies and patients with cancer were analyzed with the new systems. The possibility of fingerprint analysis of the chromatograms and the simple determination of the amount of 5-hydroxyindole-3-acetic acid and indole-3-acetic acid were demonstrated.

MATERIALS AND METHODS

Equipment

A liquid chromatograph was assembled with two Waters model 6000A pumps, Waters model 660 solvent programmer (Waters Associates, Milford, MA 01757), Rheodyne model 7125 injector (Rheodyne, Berkeley, CA 94710), Altex model 153 ultraviolet absorbance monitor (UV 254 nm) (Altex Scientific Inc., Berkeley, CA 94710) and Perkin-Elmer model MPF-4 fluorescence spectrophotometer (Perkin-Elmer Corp., Normalk, CT 06856) with an Aminco model B18-63019, 20 µL flow cell (American Instrument Com., Silver Spring, MD 20910). The recorder was Linear Instrument model 915 (Linear Instrument Corp., Reno, NV 70044).

A 5 µm chemically bonded ion-exchanger, TSK 540 DEAE and 5 µm octadecyl bonded silica gel, TSK LS 410, were kindly gifted by Dr. T. Hashimoto (TOYO SODA Mtg. Co. Ltd., Tokyo, Japan) and a 5 µm anion-exchange resin, Hitachi 3013N, was kindly gifted by Dr. N. Takai (Tokyo University, Tokyo Japan).

Chemicals

Chemicals were mainly supplied from Sigma Chem. Com., St.Louis, MO 63178, and Chem. Service Inc., West Chester, PA 19380. UV grade acetonitrile

was from Burdick & Jackson Lab. Inc., Muskegon, MI 49442. Distilled water was further treated through MilliQ system (Millipore Corp., Bedford, MA 01730).

The urine samples from babies were directly injected and the other samples analyzed were after filtration through a 0.45 μm membrane filter (Gelman Sci., Ann Arbor, MI 48106).

RESULT AND DISCUSSIONS

The retention times of fluorescent compounds in different chromatographic modes are listed in Table I and some typical chromatograms of urine samples are shown in Figs. 1-5.

The urine samples of new-born babies were very pure and clear. One example of chromatograms in different separation modes of a four days old boy's urine is shown in Figs. 1-a and b. The compounds surmounted by a symbol were positively identified from the results of ion-exchange and reversed-phase chromatographies. Two unidentified interesting compounds were found with the reversed-phase system and are numbered 1 and 2. The same compounds were found several times in different samples.

The chromatograms of one and half year old boy's urine sample showed the influence of food, and the obtained fingerprint was close to that observed for adult samples. The chromatograms are shown in Figs. 2 and 3.

Examples of chromatograms of the urine of a patient with cancer are shown in Figs. 4 and 5. The patient was 79 years old, and it contained large amounts of protein related to the intake of food.

The chromatograms observed for 38 samples obtained at different times, from 26 persons, among them 5 new-born babies, 12 healthy persons whose age ranges from 1 to 50 years, 9 patients with cancer, one was 5 years old and the others were over 50 years old, revealed an interesting feature. The peak height ratio of the well resolved unknown compounds labelled Nos. 1 and 2 indicates the possibility for a classification of these urines in three

TABLE I. Retention Index of Urinary Fluorescent Compounds (Unit: mL)

Compound	Symbol	RP[1]	RP[2]	Ion-exchange[3]
Noradrenalin	NA	1.40	1.44	-
Adrenalin	AD	1.80	1.80	-
Dopamine	DA	2.60	3.08	1.34
Dopa	DP	-	4.40	-
4-Hydroxy-3-methoxymandelic acid	4H3MeOMA	6.62	8.00	24.9
5-Hydroxytryptophan	5HT	8.62	12.0	3.60
2,5-Dihydroxyphenylacetic acid	25DHPhA	9.90	11.0	25.9
bis(4-Hydroxy-3-methoxyphenylglycol)	4H3MeOPG	10.0	10.6	-
3,4-Dihydroxyphenylacetic acid	34DHPhA	14.1	15.6	24.0
Tryptophan	Try	14.7	13.4	3.00
5-Hydroxyindole-3-acetic acid	5HIAA	16.7	18.0	26.5
4-Hydroxyphenylacetic acid	4HPhA	17.2	18.5	23.1
4-Hydroxy-3-methoxybenzoic acid	4H3MeOBA	17.3	18.8	24.1
3-Methoxymandelic acid	3MeOMA	18.3	19.4	25.3
4-Hydroxy-3-methoxyphenylacetic acid	4H3MeOPhA	18.9	19.6	23.1
2-Hydroxyphenylacetic acid	2HPhA	19.3	20.7	25.3
Indolelactic acid	ILA	22.0	22.6	28.3
Indole-3-acetic acid	IAA	24.2	24.4	25.9
4-Methoxyphenylacetic acid	4MeOPhA	24.1	24.5	-
3-Methoxyphenylacetic acid	3MeOPhA	24.2	25.2	23.3
Indole-3-propionic acid	IPA	26.5	26.9	26.2

Experimental conditions: 1) The column was 15 cm long, 4.1 mm i.d. packed with TSKLS 410 (ODS silica gel). The gradient went from 0.01 M phosphoric acid to 60% acetonitrile in 0.01 M phosphoric acid. The gradient mode was No. 7 of Waters model 660 solvent programmer and the interval was 30 min. The column temperature was 24 $\pm$ 2°C. The flowrate was 1 mL/min and the chart speed was 0.5 cm/min. 2) 0.05 M phosphoric acid was used instead of 0.01 M. The other conditions were the same as for 1). 3) The column was 15 cm long, 4.1 mm i.d. packed with TSK 540 DEAE ion-exchanger. The gradient elution went from 5% acetonitrile-water to 50% acetonitrile-water with 0.5 M ammonium acetate buffer at pH 4.5. The gradient was the same as for 1. The column temperature was 40°C. The flowrate and the chart speed were 1 mL/min and 0.5 cm/min.

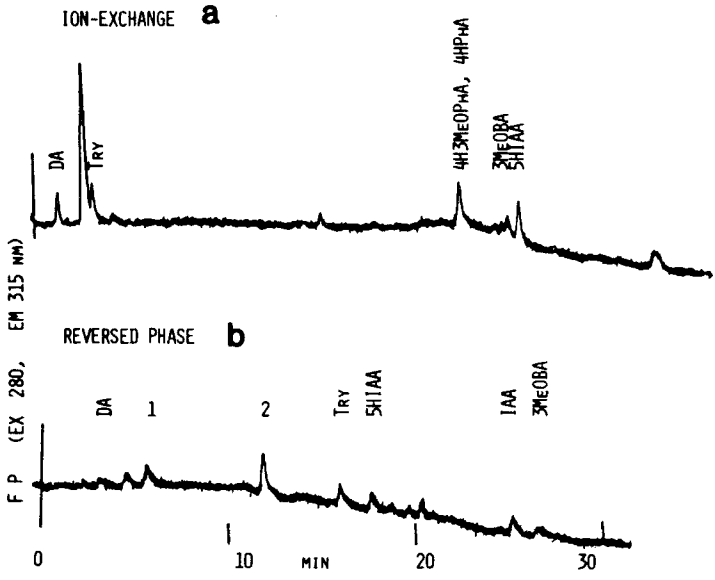

Fig. 1-a) An ion-exchange liquid chromatogram of a new-born baby's urine sample. The details of the experimental conditions are given in Table I. The sample volume was 20 µL.

b) A reversed-phase liquid chromatogram of a new-born baby's urine sample. The details of the experimental conditions are given in Table I. The sample was the same as in Fig. 1-a and the injected volume was 20 µL. The concentration of phosphoric acid in the eluent was 0.01 M.

groups: The first group includes new-born babies, whereas the second group includes the patients with cancer and the last group the healthy persons. The results of the analyses of urine of cancer patients and normal subjects were collected in Fig. 6-a, and those of new-born babies and normal subjects were summarized in Fig. 6-b. The analyses were carried out under different conditions, however the chromatograms identified by an asterisk were those of the same urine and were analyzed in two systems.

As seen in Fig. 6, the urine samples from the first two groups contain a relatively large amount of the compound under peak No. 2. This compound

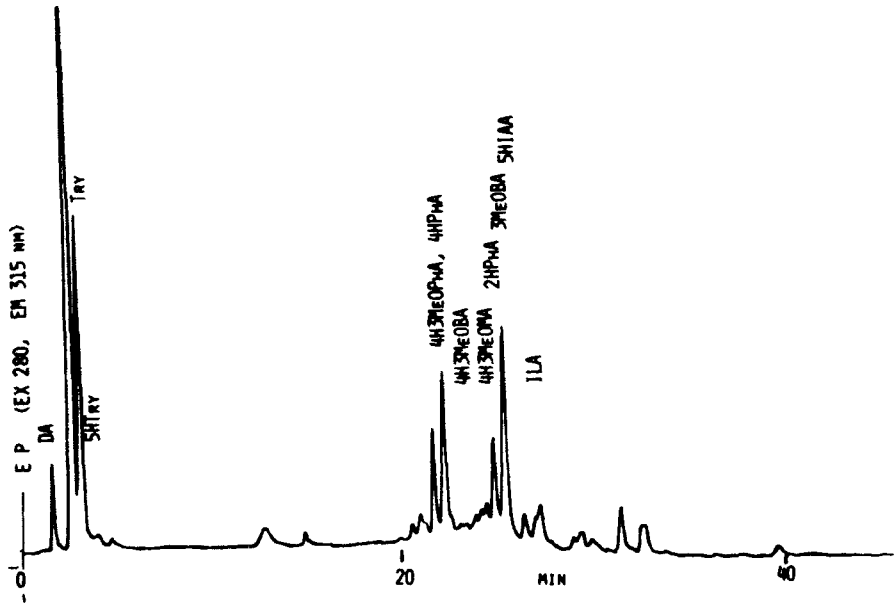

Fig. 2 An ion-exchange liquid chromatogram of a healthy boy's urine sample. The details of the experimental conditions are given in Table I. The sample volume was 20 µL.

was very polar, and unfortunately the compound could not be identified from the retention time and seems not to be an oxidation product of tryptophan. The retention time of peak No. 1 obtained with 0.05 M phosphoric acid in the eluent was close to that of 4-hydroxy-3-methoxymandelic acid, but such a large peak was not found on the chromatograms in 0.01 M phosphoric acid as the eluent.

The analysis of the fingerprint of the chromatograms, hence, the comparison of peak height ratio may help to find irregular metabolism related to health condition of people and may minimize the effect of food intake. This type of approach was applied to monitor patients [7] and used in cancer research [8]. It seems that for one person's urines whether it was collected on different days or in the morning or evening the fingerprints

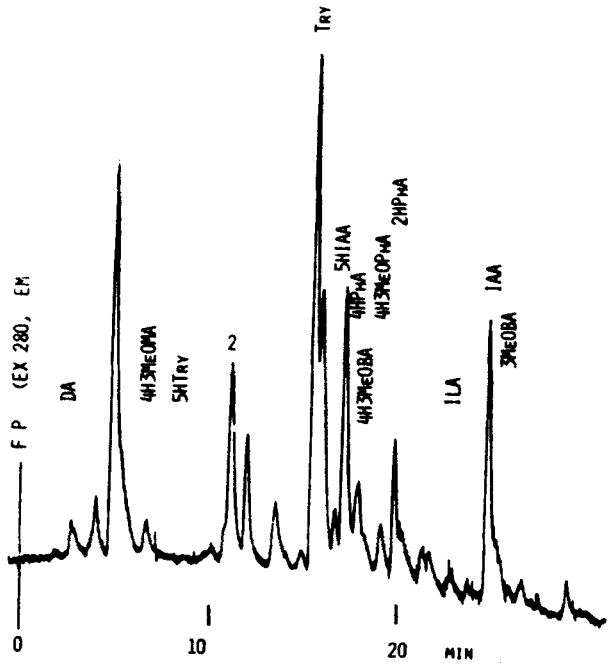

Fig. 3 A reversed-phase liquid chromatogram of a healthy boy's urine sample. The details of the experimental conditions are given in Table I. The sample was the same as in Fig. 2 and the volume was 20 µL. The concentration of phosphoric acid in the eluent was 0.01 M.

are similar. They are different compared with other persons. This means that introducing liquid chromatography in regular physical tests could help to detect irregular health conditions. The problem encountered for such applications is the long time of analysis and therefore, it may be used only in special cases.

Instead of analyzing all the components in urine, some target compounds can be analyzed in a short time with isocratic elution, for example indole-3-acetic acid and 5-hydroxyindole-3-acetic acid. 5-Hydroxyindole-3-acetic acid is the end product of the metabolism of serotonin and is recognized as an important compound related to carcinoid tumors [9-16]. The selective

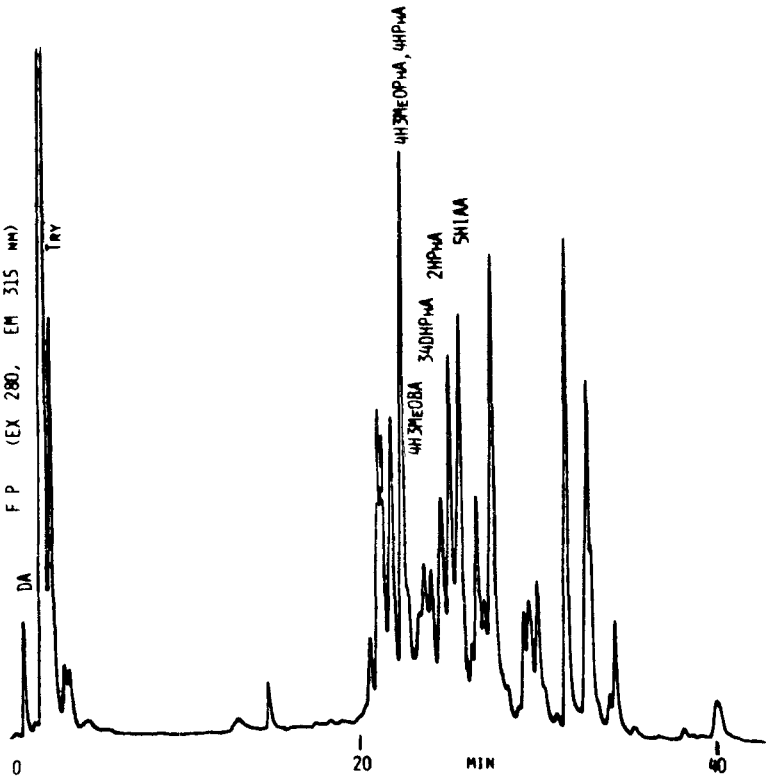

Fig. 4 An ion-exchange liquid chromatogram of a urine sample from a patient with cancer. The details of the experimental conditions are given in Table I. The sample volume was 10 µL.

separation of these compounds from urine was done on an macro-porous ion-exchange resin [17]. The column efficiency was comparable with a bonded ion-exchange silica gel, however, the stability of the packing was superior to bonded silica gels. In addition, when a urine sample was passed through alumina or Dowex 1X8 columns like for purification of 4-hydroxy-3-methoxy-mandelic acid, the fingerprint of the fluorescent compounds was not significantly changed but over 30% of the aromatic acids were lost and such a pre-treatment is not acceptable for the analysis of aromatic acids. No pressure

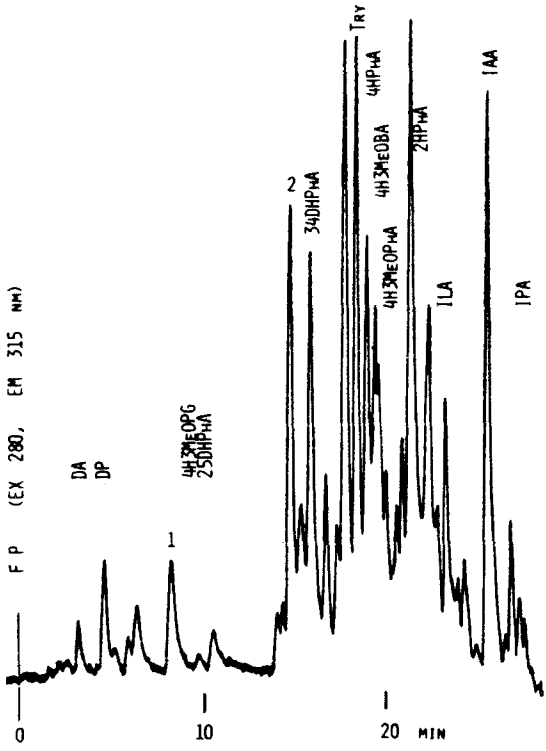

Fig. 5 A reversed-phase liquid chromatogram of a urine sample from a patient with cancer. The details of the experimental conditions are given in Table I. The sample was the same as in Fig. 4 and the volume was 10 µL. The concentration of phosphoric acid in the eluent was 0.05 M.

drop was observed in this system after analysis of over 100 injections and therefore this system was very useful to analyze urine directly after filtration. Examples of chromatograms are shown in Figs. 7-9 and the samples are the same as those in Figs. 1-5.

The peaks of 5-hydroxyindole-3-acetic acid and indole-3-acetic acid were not contaminated by indolepropionic, indoleglyoxylic, indolepyruvic and indolelactic acids. The detection limits of 5-hydroxyindole-3-acetic and

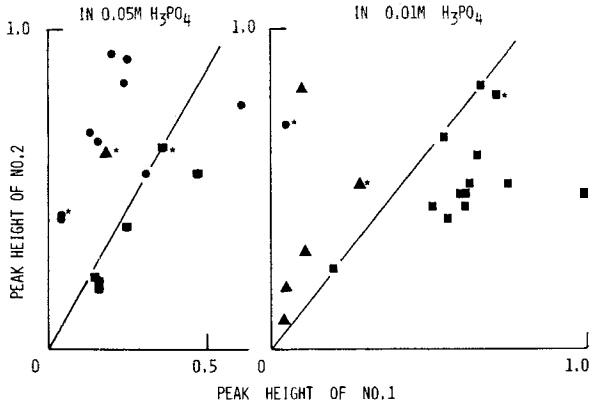

Fig. 6 Peak height ratio of Nos. 1 and 2 in reversed-phase liquid chromatography. The experimental conditions are given in Table I.
*: indicates the same samples in different chromatographic modes.
●: cancer patient's urine
▲: new-born baby's urine and
■: normal subject's urine.

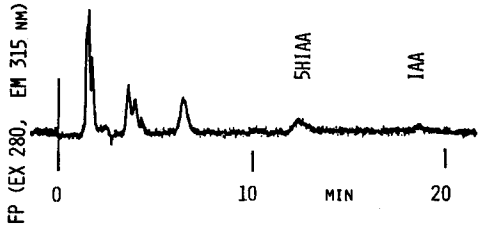

Fig. 7 Chromatogram of a new-born baby's urine sample. The sample volume was 20 μL. The column was 15 cm long, 4.1 mm i.d. and packed with Hitachi 3013N. The eluent was a 25% acetonitrile-water mixture containing 0.05 M ammonium acetate, 0.01 M octylsodiumsulfate. The flowrate was 1 mL/min. The chart speed was 0.5 cm/min and the column temperature was 40° C.

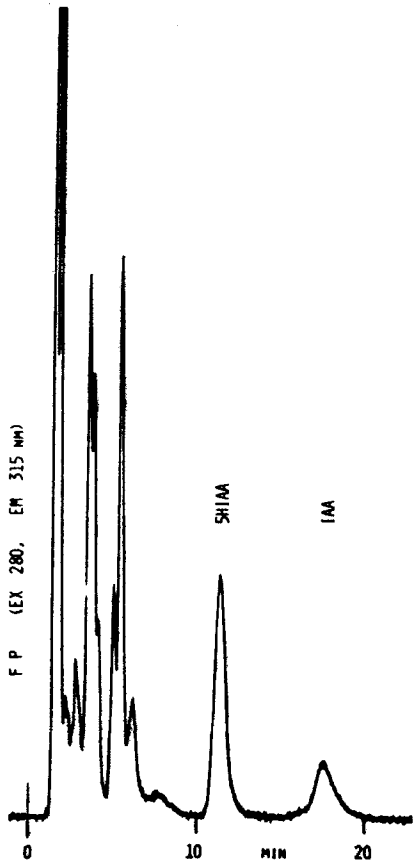

Fig. 8 Chromatogram of a one and half year old boy's urine sample. The sample volume was 20 µL. The chromatographic conditions are the same as in Fig. 7.

indole-3-acetic acids were respectively 3 and 4 ng. The distribution of these acids is summarized in Fig. 10.

The average amounts of 5-hydroxyindole-3-acetic and indole-3-acetic acids in normal subjects were 2.3 and 1.4 ppm respectively and those found for pathological subjects were 3.7 and 2.8 ppm respectively. The ratio between 5-hydroxyindole-3-acetic and indole-3-acetic acid concentrations in normal and

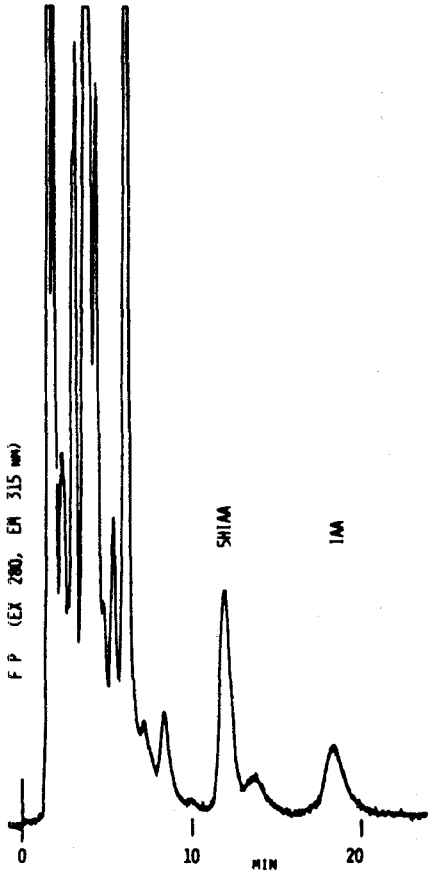

Fig. 9 Chromatogram of a urine sample from a patient with cancer. The sample volume was 10 μL. The chromatographic conditions are the same as in Fig. 7.

pathological subjects were respectively 1.7 and 1.3. There was no significant difference between these two subjects.

The reference values of indole-3-acetic acid concentration was 6.6 ppm [18], and 5-hydroxyindole-3-acetic acid concentrations were 0.6 ppm [18], 1.2 ppm [13], 0.5 - 4.0 ppm [10] and 3.7 ± 0.7 ppm [12] in normal subjects. In urine samples from patients with carcinoid tumors, stable cirrhosis and

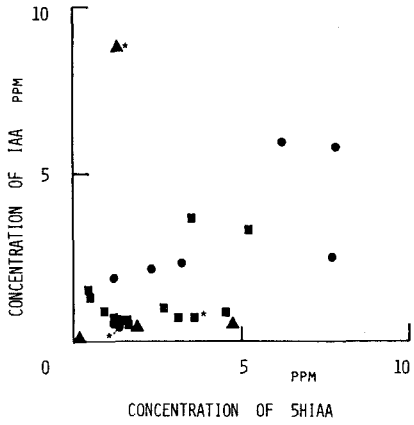

Fig. 10 Relationship between the amount of 5-hydroxyindole-3-acetic acid and indole-3-acetic acid in human urine.

*: indicates the same sample as in Fig. 6.

●: cancer patient's urine

▲: new-born baby's urine

■: normal subject's urine.

hepatic encephalopathy the concentrations were respectively 5.1 - 473 ppm [13], 4.4 ± 0.8 ppm and 4.6 ± 1.1 ppm [12].

Such small differences between normal and abnormal subjects could not be easily related to disease and it might be safer to look for metabolites instead of measuring the absolute value for one compound.

All the samples analyzed with the above systems were freshly collected and stored at -15° C until the analysis, therefore, a discussion about the concentration of each acid is not appropriate, but for pathological urine relatively large amount of proteins and the previously described acids were found.

CONCLUSION

Metabolism profiling is a very complex field of research, for a simple disease like acidurias, there are many possibilities that should be analyzed

[19]. The gas chromatography-mass spectrometer is presently the best analytical thechnique, but its power is not strong enough to solve all the chromatographyic problems related to certain diseases [18]. Liquid chromatography is a promising technique, but even for the chromatograms monitored with a fluorescent detector, the resolution and the time of analysis are presently not sufficient. One example is the analysis of indole-3-acetic and 5-hydroxyindole-3-acetic acids by a simplified system. One problem in the interpretation of urine analysis is the diet on the urine content.

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