SIMPLIFIED ASSAY METHOD FOR 3-HYDROXYANTHRANILIC ACID IN HUMAN URINE BY FLUOROMETRY AND ITS NORMAL VALUE

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Estimations of human exposure levels to aromatic nitro- and amino-compounds (ANA) are often made by Bratton-Marshall's diazo reaction. In Japan, the environmental conditions of the ANA industries have recently improved remarkably. The workers in ANA plants excrete somewhat higher levels of diazotizable substances in the urine than healthy controls and the contributory ratio of the exposed ANA substances to the total diazotizable ones in the urine appears to be of the order of 10 to 20%, most of the time.

Under such circumstances, it is necessary to measure the contributory ratio of physiological ANA substances such as hydroxyanthranilic acid to the total diazotizable substances. A simplified assay method for hydroxyanthranilic acid in the urine was devised employing cation exchanging resins for the separation of the hydroxyanthranilic acid from other contaminating substances in the urine, and using fluorometry in alkaline solution for intensifying the fluorescence of hydroxyanthranilic acid.

The reproducibility of the fluorimetric method was tested by an enzymatic assay method for hydroxyanthranilic acid determination in human urine, and the correlation coefficient was satisfactorily high ($r=0.890$, $p<0.01$). The normal excretory value of hydroxyanthranilic acid in the human urine ranges from 1.71 to 11.2 ($\mu\text{g}/\text{min}$), which is equivalent to 10 to 70 $\mu\text{moles}/\text{day}$.

The diazo colour development in body fluids such as the urine, blood, etc. by the Bratton-Marshall reaction,¹⁾ is enhanced after intake of aromatic nitro- and amino-compounds (ANA) of medical or industrial use. Examples of the former are aspyrine and pyrimidone, and of the latter are aniline and nitrobenzene. Urine samples from normal subjects develop a faint red-purple tint by the Bratton-Marshall diazo reaction¹⁾. Such colour development of normal urine after the diazo-reaction is due to physiological diazotizable ANA compounds such as 3-hydroxyanthranilic acid (3-OHAA) which is one

of tryptophan metabolites, kynurenine, xanthurenic acid, indole derivatives, etc.²⁾³⁾ The Bratton-Marshall reaction is a relevant method for monitoring the degree of ANA exposure⁴⁾⁵⁾ of workers in the ANA industries.

The current exposure conditions in the Japanese ANA industries have been found to give 0.3 to 2.0 (mg/mg of creatinine) of diazotizable substances (paminophenol (PAP) being used as the calibration standard) in the workers' urine⁵⁾. The already present physiological diazo value (the normal value) ranges from 0.17 to 0.45 (mg/mg of creatinine)⁴⁾⁵⁾. Current evidence regarding urinary total diazotizable substances, thus, mean that estimations of the ANA workers' exposure levels by the diazo-reaction may lead to some obscurity. One method for obtaining accurate estimates of ANA exposure levels using a simple detecting reaction like the Bratton-Marshall reaction, would be to evaluate the contribution of physiological diazotizable substances (such as 3-OHAA) with employing another biochemical assay method which gives the estimate of the physiological diazo value in addition to the Bratton-Marshall reactions and then to subtract the former physiological value from the total value. There is an evidence that hydroxyanthranilic acid reduces methemoglobin,⁵⁾ which is increased by heavy exposure to ANA substances. A simplified and accurate assay method for 3-hydroxyanthranilic acid is thus desired.

METHODS

1. *Reagents:*

Cation exchanging resin, Dowex 50W $\times$ 2, was purchased from Sigma Chemicals Ltd. D,L-Tryptophan, kynurenic acid, D,L-3-hydroxytryptophan, indole-3-carboxyaldehyde, and D,L-kynurenin sulfate were purchased from Wako Pure Chemicals Company. 3-Hydroxyanthranilic acid (3-OHAA) was obtained from Nakarai Chemicals Company. All reagents used were of analytical grade, unless indicated otherwise.

2. *Preparation of the column:*

Dowex 50W $\times$ 2 was subsuspended in glass-distilled and deionized water (water) overnight, washed several times with water, 2 N HCl, water, 2 N NaOH and water. This serial washing was repeated twice. The resin was then resuspended in water until use. The washed Dowex resin was poured into a glass column (Fig. 1) of 0.7 cm in diameter, until the resin reached a height of 4 cm. The column had a reservoir and was plugged with glass wool.

3. *Application of the urine sample to the column:*

The column was washed twice with 2 volumes to 0.1 N HCl before applying the sample to it. Ten to 20 ml of urine sample was diluted to 40 ml by adding water and HCl solution, the final concentration of which was adjust to 0.1 molar in the diluted sample. The diluted urine sample containing 0.1 molar HCl was then applied to the column.

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Fig. 1. Column for separation of 3-hydroxyanthranilic acid from human urine samples.

4. Apparatus:

An Hitachi MPF4 spectrofluorometer, Cary 14 and Hitachi 101 spectrophotometers were used for the study. Water at 37°C was made to circulate around the cell chamber of the spectrophotometer, from a water bath equipped with a thermostat, when the enzymatic assay was performed.

5. Enzymatic assay:

An enzymatic assay method for 3-OHAA was established by referring to the papers of Ishiguro et al.⁷⁾ and Savage and Prinz⁸⁾. The detailed procedure of the enzymatic method will be published elsewhere. Only a summary of the method, for comparison with the fluorimetric method, is given here.

The reaction mixture consisted of 0.1 ml of 40 mM MnCl_2 , 0.2 ml of 1 mg/ml (H_2O) catalase, 0.5 ml of 2 mM hypoxanthine, 0.2 ml of sample and 0.1 M sodium phosphate buffer (pH 7.40) added to make the volume of the medium up to 4.0 ml. The enzyme reaction was initiated by adding 50 μl of xanthine oxidase solution (in the ammonium sulfate solution used directly from the purchased bottle).

6. *Normal value measurement:*

Three healthy office clerks were requested to void urine 3 to 5 times during the working period for 3 days, and 5 university students were also requested to do the same. Each urine sample was transferred immediately to a polyethylene bottle containing 0.5 ml of concentrated HCl and the sample was frozen in a refrigerator (-40°C) until assay.

RESULTS AND DISCUSSION

For the analysis of 3-OHAA in the biological fluids, the following steps of analysis were followed: (1) separation and purification, and (2) identification. The separation was performed by column chromatography with Dowex 50 W $\times$ 2 and 3-OHAA was identified by fluorometry in alkaline solution.

(1) Elution pattern of 3-OHAA from the column:

Ten to 20 ml of urine was acidified with HCl and diluted to 40 ml with water as described under method. The sample was then applied to the column. When the sample had passed through the column, consecutive addition to the column of 50 ml of 0.1 M HCl, 100 ml of 0.2 M HCl and 100 ml of 1.0 M HCl was made, and a final addition of a 1 M HCl aliquot was used for the determination of 3-OHAA in the urine. The elution pattern of the human urine sample with authentic 3-OHAA added was the same as that of the authentic 3-OHAA solution (Fig. 2). The fraction eluted from the column with 1 M HCl had the same excitation (315 nm) and emission (390 nm) fluorescence peaks as the authentic 3-OHAA after the addition of 2 M NaOH to the aliquot. The elution pattern was the same as that in Tompsett's reports^{9a,b}.

The elution speed was 2.0 ml/min. Fig. 3 shows the relation between the fluorescence intensity and pH of the solvent containing the 3-OHAA fraction from the Dowex column

Fig. 2. Elution pattern of 3-hydroxyanthranilic acid from the Dowex 50W $\times$ 2 column (H^+ type).

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Fig. 3. The pH-fluorescence relation.

Numbers on the abscissa indicate the pH of the solution which was added to the aliquot of the eluate from the column.

(pH-fluorescence relation). It is well-known that the fluorescence intensity of a substance depends closely upon the solvent's pH¹⁰. Detailed examination of the 3-OHAA fluorescence in solvents of different pH's revealed that the 3-OHAA fluorescence was intensified in a solvent of alkaline pH. The fluorescence intensity was measured at 315 nm excitation and 390 nm emission wavelengths. The measurements of the pH-fluorescence relation was made by the following procedure. Two volumes of the buffer were added to one volume of the eluate, or 3-OHAA in 1 M HCl. The buffers used in the study are listed in Table 1. All of the solute concentrations in the buffer were made 0.1 molar. Maximum fluorescence of 3-OHAA in 1 M HCl after the addition of 2 volumes of the buffer was obtained, when 0.1 M borate buffer (pH 10.0) was added to the sample. Alkaline buffer caused the fluorescence of 3-OHAA to be intensified. For convenience

Table 1. Relative fluorescence intensity of 3-OHAA at different pH's of the medium.

pH	Buffer used	Relative fluorescence intensity (%)
1.00>	HCl	0.00
1.88	pyrophosphate	0.00
2.48	glycine	0.00
3.00	barbiturate	0.05
3.89	acetate	0.29
6.98	phosphate	4.90
8.00	triethanol amine	31.0
9.00	tris-hydroxymethylaminomethane	28.7
10.0	borate	38.0
14.0<	NaOH	21.5

of the measurement, 2.0 M NaOH was adopted for routined 3-OHAA assay.

The relative fluorescence intensity ratio of a certain substance to the 3-OHAA fluorescence intensity after addition of 2 M NaOH to the sample in 1.0 M HCl was measured. The results are shown in Table 2. Separation of anthranilic acid from 3-

Table 2. Relative fluorescence intensity of different substances related to tryptophan metabolism, as compared to that of 3-hydroxyanthranilic acid.

Substance	Fluorescence intensity relative to 3-OHAA in 2M NaOH
3-hydroxyanthranilic acid	1.00
3-hydroxypyridine	0.37
anthranilic acid	0.88
kynurenine	0.048
xanthrenic acid	0.072
kynurenic acid	0.059
D,L-tryptophan	0.0098
p-aminohippuric acid	0.12
pyridoxal phosphate	1.20

The excitation wavelength was 315 nm and the emission wavelength was 390 nm (Hitachi MPF-4 spectrofluorometer).

Fig. 4. Test of the reproducibility of the fluorimetric assay. An enzymatic assay method was adopted for comparison with the fluorimetric assay (see text).

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OHAA was not possible by this method. In the eluted fraction in 1.0 M HCl, therefore, after the addition of 2 M NaOH, both 3-OHAA and anthranilic acid (AA) emit the same intensity of fluorescence per molar concentration at the selected emission and excitation wavelengths. With human urine, however, the effect of contaminant AA was negligible, since only trace of AA, at most is excreted quantitatively as its glycine or glucuronic acid conjugates¹¹⁾¹²⁾, which were eluted from the Dowex column in the previous fractions.

The recovery rate of the authentic 3-OHAA added to the column ranged 75.0 to 81.0%, and the coefficient of variance (C.V.) was 1.23% (the sample numbers were 50 and the added 3-OHAA ranged from 200 to 500 $\mu\text{g}/100\text{ ml}$). The C.V. was sufficiently small.

The reproducibility of the assay method for 3-OHAA required testing by using another assay method for 3-OHAA. The catalase manganese coupling cinnabaric acid condensation reaction⁷⁾⁸⁾ was selected as the test, since the two assay systems are basically different from each other. The results compiled were in good agreement with each other between 2.0 and 5.5 μg of 3-OHAA in 1.0 ml of the medium. The correlation coefficient was statistically significant ($r=0.890$, $P<0.01$; Fig. 4). The enzymatic method tends to give somewhat lower estimates for concentration of 3-OHAA. That

Table 3. Urinary excretion of 3-hydroxyanthranilic acid in healthy male subjects ($\mu\text{g}/\text{min}$).

Time	Day when sample was taken										
	23 April, 1979			24 April, 1979			25 April, 1979				
	Name (age)										
	I.N. (65 yrs)			T.D. (53 yrs)			M.Z. (28 yrs)				
8 : 00 to 9 : 00 a.m.	4.66	—	4.53	5.31	5.01	7.77	2.90	5.31	5.77		
11 : 00 a.m.	5.33	—	5.63	6.06	7.87	4.47	2.17	3.50	3.60		
13 : 00 p.m.	8.45	—	7.17	9.60	8.50	5.77	3.82	2.93	3.46		
15 : 00 p.m.	2.39	2.51	8.26	10.80	11.20	4.96	3.61	2.95	2.45		
16 : 30 p.m.	3.18	3.88	7.92	11.10	6.42	3.50	3.40	4.51	3.00		
Time	Name (age)										
	H.D. (20 yrs)			K.W. (20 yrs)		O.N. (20 yrs)		H.Y. (21 yrs)		Y.N. (21 yrs)	
	9 : 00 to 9 : 45 a.m.		7.50		11.10		1.71		2.19		4.36
	11 : 35 a.m.		4.40		8.48		2.41		9.07		5.60
13 : 15 p.m.		7.20		2.56		3.00		2.61		9.83	
15 : 25 p.m.		7.70		6.57		5.29		4.27		7.70	

Data from office clerks are shown in the upper part of the table; those in the lower part are from students. The estimation shown in the upper part of the table was performed for three consecutive day per a person.

may be due partly to absorbance change of the flavin enzyme, xanthine oxidase. Confirmatory experiments on this point are now in progress.

The excretory rate of 3-OHAA into the urine of healthy male subjects ranged from 1.71 to 11.2 $\mu\text{g}/\text{min}$, as shown in Table 3. These urinary excretory values were equivalent to 10 to 70 $\mu\text{moles}/\text{day}$, which was within the reported normal range of urinary values for 3-OHAA^{11) 12)}.

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