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Abstract

An improved direct colorimetric method for determining the concentration of urinary hippuric acid as an index of toluene exposure was described. One tenth ml of urine was diluted with 0.4 ml 0.01 M phosphate buffer H 6.9 and mixed with 0.5 ml pyridine. The mixture was layered on 0.2 ml benzenesulfonyl chloride. The reaction was started by mixing for one min with a mechanical shaker. The colored solution was allowed to stand for 30 min, diluted with 5 ml ethanol, and absorbance measured at 410 nm within 30 min after the dilution. The coefficient of variation of this method was 6% and the recovery 103% when urine contains about 0.2-0.5 mg hippuric acid per ml of urine. The concentration was linear up to 2.0 mg per ml hippuric acid in a specimen.

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AN IMPROVED DIRECT COLORIMETRIC METHOD FOR THE QUANTITATIVE ANALYSIS OF URINARY HIPPURIC ACID AS AN INDEX OF TOLUENE EXPOSURE

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Abstract. An improved direct colorimetric method for determining the concentration of urinary hippuric acid as an index of toluene exposure was described. One tenth ml of urine was diluted with 0.4 ml 0.01 M phosphate buffer pH 6.9 and mixed with 0.5 ml pyridine. The mixture was layered on 0.2 ml benzenesulfonyl chloride. The reaction was started by mixing for one min with a mechanical shaker. The colored solution was allowed to stand for 30 min, diluted with 5 ml ethanol, and absorbance measured at 410 nm within 30 min after the dilution. The coefficient of variation of this method was 6% and the recovery 103% when urine contains about 0.2–0.5 mg hippuric acid per ml of urine. The concentration was linear up to 2.0 mg per ml hippuric acid in a specimen.

Toluene is widely used as an industrial solvent. About 68% of toluene inhaled by man is excreted as urinary hippuric acid (HA). The quantitative analysis of urinary HA should give, therefore, an indication of toluene exposure (1).

For quantitative determination of HA in urine, several methods have been developed. Ogata *et al.* (2) improved the method introduced by Umberger and Fiorese (3) in which HA was extracted with ethyl acetate. Recently Tomokuni and Ogata (4) introduced a direct colorimetric method which eliminated the extraction process. This method was simpler than other procedures hitherto reported, but some variation was found in the value from sample to sample, and the recovery was relatively low. In the present paper, an improved method of direct colorimetric determination of HA is reported which minimizes error and increases recovery.

MATERIALS AND METHODS

Materials and Equipments

Urine was collected on the day of analysis from healthy men employed in this laboratory. All reagents used were of reagent grade. Benzenesulfonyl chloride (BSC) was from Merck Chemical, Rhyway, N. J., U. S. A. HA and

sodium hippurate were from Wako Pure Chemical Ind. Ltd., Osaka. A Hitachi Perkin-Elmer 139 spectrophotometer (Perkin-Elmer Corp., Norwalk, Conn., U. S. A.) was used to measure the absorbance of colored solution. A high performance liquid chromatograph (Hitachi type 633, HLC) equipped with 4.0 mm $\phi \times$ 150 mm stainless steel column packed with LiChrosorb RP 18, 10 μm (Merck) and UV detector at 254 nm wave length was used to determine urinary HA.

Methods

Original direct method (4). Fresh urine (0.5 ml) was pipetted into a test tube, and 0.5 ml of pyridine was added and mixed. Then, 0.2 ml of BSC was added to this solution which was then mixed manually. The colored solution was allowed to stand for 30 min at room temperature, then diluted to 5 ml with ethanol and mixed well. Absorbance was determined at 410 nm against ethanol as reference. The standard HA and blank (water) were treated by the same procedures.

Improved direct method. BSC (0.2 ml) was pipetted into a test tube. In another test tube, urine (0.1 ml) was diluted with 0.4 ml of 0.01 M phosphate buffer (pH 6.9) and mixed with 0.5 ml of pyridine. All of the urine-pyridine mixture was layered on the BSC in such a way as to prevent immediate mixing of the two solutions, shaken vigorously for one min with a mechanical shaker, allowed to stand for exactly 30 min at room temperature and diluted with 4 ml ethanol. The absorbance was measured at 410 nm within 30 min after the dilution. The standard and the blank were treated by the same procedure.

Modified direct method (5). Urine specimen (0.05 ml) was pipetted into a test tube, and 0.5 ml of pyridine was added and mixed. Then, 0.2 ml of BSC was added to this solution and mixed. After being allowed to stand for 30 min the colored solution was diluted with 3 ml of ethanol. Absorbance was determined at 410 nm against ethanol.

Modified Umberger's method (2, 3). One milliliter of fresh urine was placed in a tube with stopper, the pH adjusted to about 2.0 with HCl, and the specimen saturated with NaCl to facilitate extraction of HA. HA was completely extracted with 4 ml of ethyl acetate, and the extract transferred to a test tube and dried at about 70°C in a water bath. The specimen was taken up in 0.5 ml of pyridine and 0.2 ml of BSC added. The mixture was allowed to stand for 30 min at room temperature, diluted with 4.3 ml chloroform, and the absorbance was read at 380 nm against a pyridine-BSC mixture.

High performance liquid chromatography. Standard HA solution or urine (0.9 ml) and 0.1 ml of the internal o-methyl HA solution (10 mg ml) were placed in a tube with stopper, the pH adjusted to 2.0 with HCl, and the solution nearly saturated with NaCl (0.3 g). HA and o-methyl HA were extracted with 4 ml of ethyl ether-methanol (9 : 1 v/v). One ml of extract was transferred to another test tube. After drying, 0.5 ml of methanol was added and the solution was injected into HLC. A favorable mobile phase for separation of urinary glycine conjugates was methanol-water-acetic acid (20 : 80 : 0.2, v/v) mixture. Flow rate was 1.2 ml per min, producing pressure was 60 kg per cm^2.

RESULTS

Influence of water on color development. A possible influence of water on color development was tested because azalactone from HA has been formed under non-aqueous conditions (3). Four 0.5 ml specimens of pyridine dissolved HA were mixed with 0.05, 0.20, 0.50 and 1.00 ml water respectively, and the color developed by both original and improved direct methods. As shown in Table 1, color

TABLE 1. COEFFICIENT OF VARIATION OF HA STANDARD ABSORBANCE IN PYRIDINE SOLUTION (0.1 MG/ML), AND AT THE VOLUMES OF WATER ADDED; ORIGINAL AND IMPROVED METHODS (N=8)

Method		Water volume added (ml)			
		0.05	0.20	0.50	1.00
Original method	Mean	0.157	0.076	0.190	0.272
	SD	0.015	0.006	0.022	0.033
	SD/Mean	0.100	0.080	0.118	0.122
Improved method	Mean	0.121	0.069	0.146	0.239
	SD	0.003	0.003	0.006	0.018
	SD/Mean	0.024	0.041	0.058	0.077

The values of Mean and SD are expressed as absorbance at 420 nm. and SD/Mean is coefficient of variation.

intensity was lower when 0.05 or 0.20 ml water was added, and higher when 0.50 or 1.00 ml was used. The difference in the absorbances might be caused by the formation of colored substances with different absorption curves due to the different water content in the respective reaction systems. The color was most stable and reproducible when 0.5 ml water was present. Therefore, a system in which 0.5 ml water is present was employed in further experiments.

Effect of hydrogen ion concentration on color development. The pH values of normal urine so far encountered ranged from 5.0 to 7.8 and the effect of pH value on color development was tested in this pH range. The standard HA solution (0.2 mg/ml) was diluted with 0.01 M phosphate buffer of pH 4.9, 6.9 and 7.9, the color developed and the intensity measured at 410 nm. The readings were 0.271, 0.294 and 0.371 respectively. In pH 7.9 solution the color intensity was higher, but color fading was slightly faster than that in solution of pH 6.9; in addition, adjusting pH of urine to 7.9 was rather difficult with the phosphate buffer. Therefore, the urine was diluted with 0.01 M phosphate buffer of pH 6.9 in further experiments. The coefficient of variation of urinary HA level was 5% by this procedure.

Color stability. The color stability of the reaction mixtures is shown in Figure 1. The fading of the color 60 min after the start of reaction was 7.3% in the

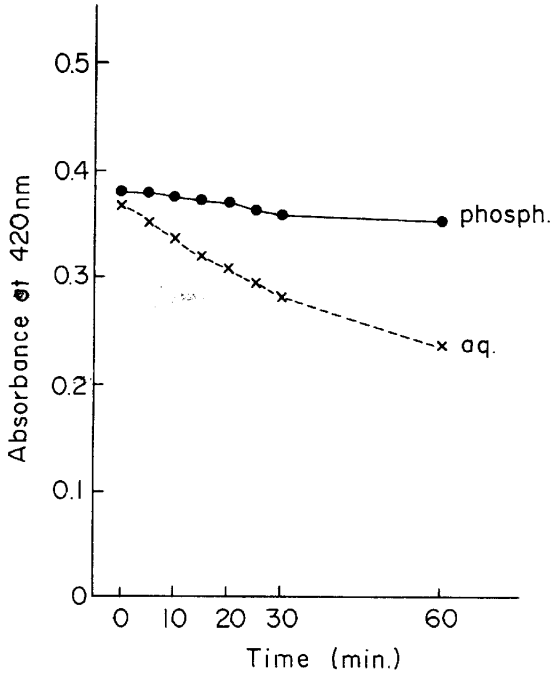

Fig. 1. The color stability of the reaction mixture after the addition of ethanol. One tenth ml of HA solution (0.2 mg/ml) was diluted with 0.4 ml water or 0.01 M phosphate solution of pH 6.9, mixed with 0.5 ml pyridine, and layered on 0.2 ml BSC. Zero time is the time immediately after shaking the layered solution. phosph.; HA, pyridine, BSC and 0.01 M phosphate buffer (pH 6.9), and aq.; HA, pyridine, BSC and aqueous solution. Concentration of HA is 0.2 mg/ml.

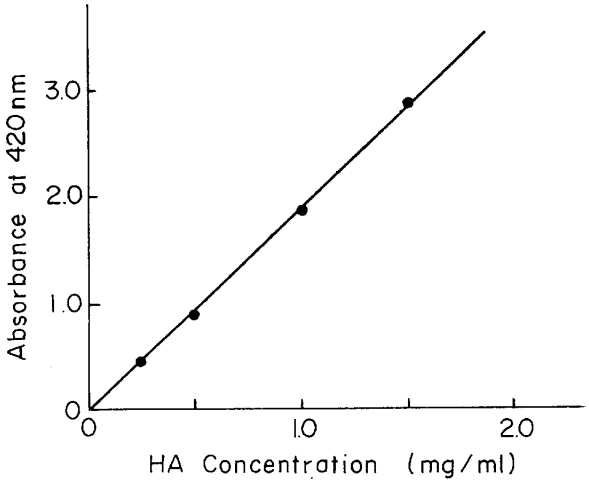

Fig. 2. Calibration curve for HA measured by the improved direct method.

buffered solution and 35.5% in water respectively when 0.2 mg per ml HA solution was used.

Calibration curve. A calibration curve for HA measured by the improved direct method is shown in Figure 2. The concentration-color intensity relationship follows the Lambert-Beer law at least up to 2.0 mg per ml HA in the sample.

Recovery. The recoveries expressed by per cent when 0.5, 1.0 and 2.0 mg per ml of sodium hippurate was added to urine were about 103 ± 5 (mean ± SD), 102 ± 7 and 105 ± 6 by the improved direct method, and 74 ± 14, 75 ± 20 and 87 ± 6 by the original direct method.

Comparison between the values of urinary HA measured by HLC and by the improved method. The urine of 21 persons employed in this laboratory with no occupational exposure to toluene and m-xylene was examined. The corrected values of mean HA concentrations for the specific gravity of normal urine (1.024)* were 0.255 mg and 0.558 mg per ml of urine by HLC and the improved methods respectively. The regression equation between HA values obtained by HLC (X) and in the improved method (Y) was $Y = 1.37X + 0.21$ (correlation coefficient: $r = 0.98$). Differences between the values by the colorimetric methods and HLC will be due to the fact that there are urinary glycine conjugates such as o-, m-, and p-hydroxyhippuric acids other than HA (6). The normal value by the improved direct method was lower than that (0.8 mg/ml) reported by Pagnotto and Lieberman (7) using an ultraviolet spectrophotometric method.

Comparison of the improved direct method, the modified direct method and HLC. Ten urine samples from a special person** were analyzed by the improved and the modified direct methods and by HLC. HA concentrations thus measured were 0.73 ± 0.21 mg per ml (mean ± SD), 1.14 ± 0.41 and 0.28 ± 0.16 mg per ml respectively, and coefficients variation of (SD/mean) were 0.28, 0.36 and 0.57 respectively. Also with this urine, the improved direct method was shown to be more reliable than the modified direct method, because lower HA concentrations could be measured indicating improved quantitative recovery. The regression equation between HA values obtained by HLC (X) and the modified direct method (Y_1) was $Y_1 = 1.10X + 0.83$ ($r = 0.44$, $p < 0.20$ by t test), and between HLC (X) and the improved method (Y_2) was $Y_2 = 1.02X + 0.46$ ($r = 0.76$, $p < 0.01$ by t test). Recovery of added HA (0.50 mg/ml) by the improved and the modified direct methods was 100.9 ± 2.2% and 99.8 ± 11.1% respectively.

Comparison of the improved and the other original direct methods, and modified Umberger's mothod. Urines of 12 normal persons were analyzed for HA by three

* correctcd value = measured concentration of HA × (specific gravity of the urine − 1.000) /0.024.

** This special urine has been examined by Dr. Ikeda previously. Urinary HA value was shown to be higher when the urine was diluted than that when not diluted by an automated colorimetric determination based on the BSC reaction (8).

colorimetric procedures. Mean values were 0.655 ± 0.392 (mean ± SD), 0.488 ± 0.304 and 0.469 ± 0.397 for the original, the improved and modified Umberger's methods respectively. The regression equations between the original (Y) and the improved (X) direct methods and that between modified Umberger's (Y) and the improved direct (X) methods were $Y = 1.09X + 0.12$ ($r = 0.91$), and $Y = 1.17X - 0.10$ ($r = 0.91$) respectively as shown in Figure 3.

Normal values of urinary excretion of HA. The mean concentration of urinary HA measured by the improved method was 447 μg per ml ($N = 42$). The mean plus standard deviation was 719 μg per ml, and the upper rejection limit (5% level) was 1183 μg per ml. These values were calculated with a log normal distribution as the frequency distribution indicated (Fig. 4).

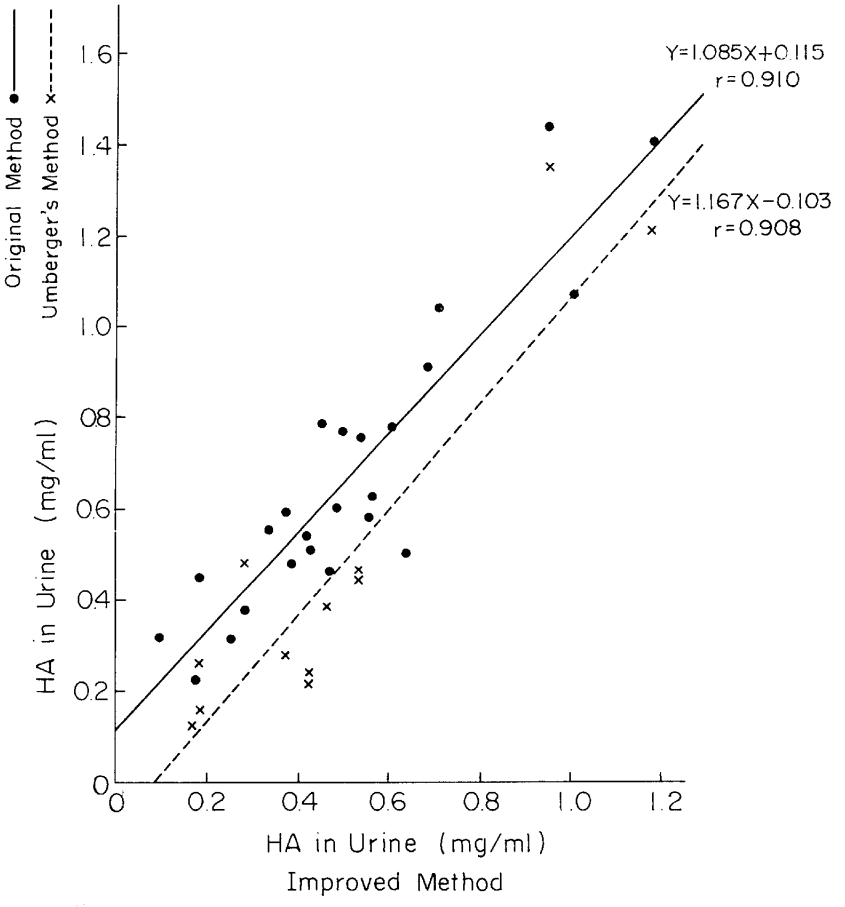

Fig. 3. Correlation between the improved method and the original direct method and between the improved method and modified Umberger's method for determination of urinary HA.

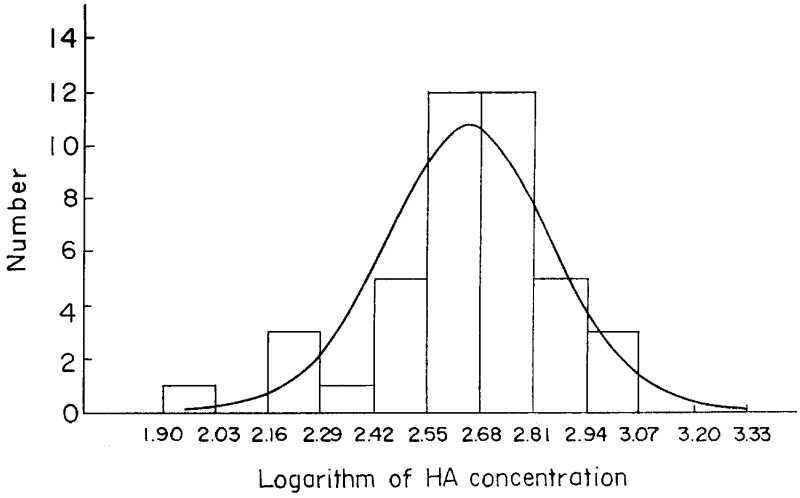

Fig. 4. Distribution curve of urinary HA concentration of a normal person.

DISCUSSION

The direct colorimetric methods for urinary HA determination were compared with each other and with modified Umberger's method and with that by HLC. The results and a review of the literature indicate that color development was influenced by order of addition and mixing conditions of the sample and reagents, water content and pH of the reaction system, reaction time, and fading. The improved direct colorimetric method described in this paper, that of layering the bufferized urine-pyridine mixture on BSC and mechanical shaking, controlled the factors which influence the color developement, and gave a satisfactory coefficient of variation of 6% and recovery of 103% when urine contained about 0.5–2.0 mg HA per ml. Because BSC has greater density than the urine-pyridine mixture, partial mixing and consequent onset of the color formation reaction of the two solutions was inevitable when BSC was poured on the urine-pyridine mixture. It was, therefore, practically impossible to run the color reaction for an accurately fixed time unless starting the reaction uniformly after layering as described. The method described is based on color development with BSC of glycine conjugates such as HA, hydroxyhippuric acids (normal urinal excrements) and methylhippuric acids derived from xylenes. It was, however, observed that increases of urinary HA accounted for increased excretion of glycine conjugates even after exposure to toluene using paper chromatography (2, 9), gas chromatography (10) and HLC (11). Therefore, the improved direct method in determing urinary HA would be useful to obtain an indication for the worker to be exposed to toluene.

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