COLORIMETRIC DETERMINATION OF URINARY GLYCINE CONJUGATES

Masana OGATA, Reiko SUGIHARA, Hiroko ASAHARA
and Shohei KIRA

*Department of Public Health, Okayama University Medical School,
Shikatacho, Okayama 700 Japan*

(Received January 26, 1977)

A quantitative procedure is described for determining urinary glycine conjugates. The color development was recognized after addition of pyridine and benzenesulfonyl chloride to glycine conjugates. The dried residue of ethyl acetate extract from urine was dissolved in pyridine and benzenesulfonyl chloride was added. After addition of chloroform to the mixture, the absorbance was determined at 420 nm (extracting method). In the improved procedure of direct colorimetric method, pyridine was added to urine and mixed; then benzenesulfonyl chloride was diluted with mixture of dimethylsulfoxide and ethanol, and absorbance was determined at 430 nm (improved direct method).

To separate each glycine conjugate, paper chromatographic procedure was carried out. Urine specimen each was directly developed on filter paper with butanol—acetic acid—water or isopropanol—ammonia—water. On the other hand, ethyl acetate extract from urine was developed with toluene—acetic acid—water. Comparing the spots with authentic sample spots, each location was determined by illuminating with ultraviolet light. Then, each spot was extracted with pyridine. Benzenesulfonyl chloride was added to the extract; the absorbance was determined at 420 nm. Complete separation of urinary glycine conjugates could be done by two-way chromatography.

The normal values of urinary glycine conjugates were also compared by extracting method and improved direct method.

Determination of urinary hippuric acid and *m*- or *p*-methylhippuric acid by benzenesulfonyl chloride has been used in testing the liver function following benzoic acid administration and tests of exposure to vapors of toluene and *m*- or *p*-xylene.¹⁾ A quantitative method for urinary hippuric acid was described by Ogata, Tomokuni and Takatsuka²⁾ with an ethyl ether extract and by Tomokuni and Ogata³⁾ employing the colorimetric method of Umberger and Fiorese⁴⁾

In this paper, improved direct determination of glycine conjugates as a simple method and colorimetric determination of glycine conjugates after paper chromatography as a complete separation method are described.

MATERIALS AND METHODS

Materials

Urine specimens for determining normal hippuric acid values were collected from healthy volunteers in Okayama City.

Benzenesulfonyl chloride (BSC) and hippuric acid (HA) were purchased from Merck Chemical, Rhyway, N. J.; *m*-methylhippuric acid (*m*-MHA) was obtained from Tokyo Kasei Ind. LTD.; salicyluric acid (SA), nicotinuric acid (NA) and isonicotinuric acid (INA) were purchased from Nippon Kankoshikiso Kenkyusho.

Model DK-2A ratio-recording spectrophotometer (Beckman Instruments Corp., Calif.) and Hitachi Perkin-Elmer 139 spectrophotometer (Perkin-Elmer Corp. Norwalk, Conn.) were used for colorimetric determinations.

Methods

Simple colorimetric determination (Method A)

Method A-1 (Extracting method). One milliliter of fresh urine and 1 ml of standard glycine conjugate solution were pipetted into a stoppered tube. The pH was adjusted to about 2.0 with HCl, and the solution was nearly saturated with NaCl (0.3 g) to increase the extracting rate. Glycine conjugates in urine were extracted twice with 2 ml of ethyl acetate. Thereafter, 0.2 ml of the extract was transferred to a test tube and dried at 70°C in a water bath. Glycine conjugates were determined by the colorimetric method of Umberger and Fiorese⁴⁾ for hippuric acid determination. Each glycine conjugate standard solution and a blank (water) were treated in the same way.

Method A-2 (Improved direct method). Fresh urine (0.2 ml) or glycine conjugate standard solution was pipetted into a test tube, and 0.5 ml of pyridine was added and mixed. Then 0.2 ml of BSC was added and mixed. The colored solution was allowed to stand for 30 min at room temperature, then diluted to 5 ml with a mixture of dimethylsulfoxide (DMSO) and ethanol (19 : 1, by vol.); the absorbance of colored products was measured at their absorption maximum, respectively.

Colorimetric determination after paper chromatography (Method B)

One-way chromatography

Method B-1. Urine (0.2 ml) and standard solution of glycine conjugates were developed on filter paper (40×40 cm, Tokyo Filter Paper Company, NO. 51) with butanol—acetic acid—water (4 : 1 : 1) (Method B-1-1), or isopropanol—ammonia—water (20 : 1 : 2) (Method B-1-2). The locations of spots were found by illuminating the filter paper with ultraviolet light with maximum intensity of 253 nm. The spots were marked, cut out and extracted twice with 3 ml of pyridine, and 0.4 ml of BSC was added to the pyridine extract (1.0 ml). Thereafter, 3.6 ml of chloroform were added to the solution above and mixed well. The

DETERMINATION OF URINARY GLYCINE CONJUGATES

absorbance of the stable colored solution was determined at 420 nm against chloroform; the blank (water) was treated in the same way.

Method B-2. Ethyl acetate extract (0.2 ml) from urine (1.0 ml) by method A-1 was spotted on filter paper and developed with toluene—acetic acid—water (100 : 50 : 2.3). The extraction and coloration procedures were the same as those of Method B-1.

Two-way chromatography

Ethyl acetate extract (0.2 ml) from urine (1.0 ml) by Method A-1 was spotted on filter paper developed with a solvent pair of isopropanol—ammonia—water (1st) and butanol—acetic acid—water (2nd). After developing and drying by a fan, 4% of dimethylaminobenzaldehyde (DAB) solution mixed in acetic anhydride saturated sodium acetate was sprayed thoroughly on the paper.

RESULTS

Simple colorimetric determination

Absorption spectra of colors are shown in Fig. 1 and Fig. 2 obtained with HA, m-MHA, SA, NA and INA standards by Method A-1 and A-2. The absorption maxima of colors for HA and m-MHA were 420 nm, 445 nm and 475 nm and 475 nm, NA and SA were 430 nm, 450 nm and 480 nm, INA was 430 nm, 455 nm and 485 nm, by Method A-1.

Fig. 1. Absorption spectra obtained with glycine conjugate standards by Method A-1.

Fig. 2. Absorption spectra obtained with glycine conjugate standards by Method A-2.

By method A-2, the absorption maxima of colors for HA, m-MHA and NA were 430 nm, SA was 440 nm, and INA was 455 nm and 510 nm, respectively. In this paper, for colorimetric determination of glycine conjugates, optical density was measured at 420 nm by Method A-1 and 430 nm by Method A-2, for convenience.

The effect of reaction time was examined on the intensity of color produced with HA standard by Method A-1 and A-2. The color development by both methods was most intense at 5 min after the addition of BSC, and thereafter gradually decreased, except for the interval of 30 to 60 min when no change was observed. The color intensity gradually decreased after the dilution. The ratio of optical density at 30 min to that at 5 min after the reaction was 87% by Method A-1 and 98% by Method A-2.

Calibration curves for each glycine conjugate measured by Method A-1 and A-2 are shown in Fig. 3 and Fig. 4, and follow the Beer-Lambert's law.

Urinary glycine conjugate concentration measured by Method A-1 and A-2 were compared. The relationship between the values obtained by these two methods can be described by a regression line. The regression equation is as follow: $Y = 0.81X - 0.22$, where X represents the values obtained by Method A-1 and Y the values obtained by Method A-2. The correlation coefficient is 0.89.

Recoveries of HA and sodium hippurate by Method A-1 and A-2 ranged from 75% to 103% (Table 1).

DETERMINATION OF URINARY GLYCINE CONJUGATES

Fig. 3. Calibration curves for glycine conjugate standards measured by Method A-1.

Fig. 4. Calibration curves for glycine conjugate standards measured by Method A-2.

Colorimetric determination after paper chromatography

The R_f values of HA, *m*-MHA, SA, NA and INA were: by Method B-1-1, 81, 81, 81, 61 and 61, respectively; by Method B-1-2, 34, 42, 10, 22 and 22, respectively; and by Method B-2, 45, 63, 47, 21 and 21, respectively. These data indicate that SA was

separated by Method B-1-2; and HA and m-MHA by Method B-2. INA was hardly separated from NA by paper chromatography but it was measured at 510 nm as having a different absorption maximum from other glycine conjugates by Method A-2. The recovery of HA added to urine ranged from 96% to 106% by Method B-1-1 and the recovery was from 93% to 99% by Method B-2 (Table 1).

Using two-way paper chromatography by Method B-1-2 followed by Method B-2, complete separation of those acids recognized (Fig. 5). Spots detection with ultraviolet light, extraction with pyridine and coloration with BSC could be done as the same way in the one-way chromatogram.

Table 1. Recovery of added HA from normal urine by the Method A and B.

Method	Concentration (mg/ml)							
	0.1	0.2	0.3	0.5	0.75	1.0	2.0	3.0
Method A-1	95±22	98±11	101±16	103± 6	98±12	89± 8	90±11	90± 6
Method A-2†	—	77± 2	77±18	75±14	79±24	75±20	87± 6	80± 2
Method B-1-1	—	—	—	100± 5	—	106± 3	—	96± 5
Method B-2	—	—	—	93± 1	—	96± 9	—	99±18

† Sodium hippurate

% (mean±SD)

Fig. 5. Two way separation of glycine conjugates.

The solvent pair is isopropanol—ammonia—water (1st)/toluene—acetic acid—water (2nd) and DAB is the location reagent.

DETERMINATION OF URINARY GLYCINE CONJUGATES

Normal value of urinary HA by Method A

HA levels of morning urine samples obtained by these two methods in Okayama City are shown for 179 and 225 individuals, aged 11 to 65 years, in Table 2 and Fig. 6.

The normal urinary glycine conjugate concentration, that was regarded as HA, was adjusted in some cases to a specific gravity of urine at 1.024. The frequency distribution

Fig. 6. Frequency distribution of the concentration of HA in normal urine.

Table 2. Normal values of urinary HA.

	Men			Women			Total		
	Mean	Mean+SD	Rej. limit.	Mean	Mean+SD	Rej. limit.	Mean	Mean+SD	Rej. limit.
Method A-1	0.55	0.83	1.24	0.62	0.91	1.31	0.59	0.88	1.12
Method A-2	0.34	0.66	1.24	0.36	0.65	1.14	0.35	0.65	1.19

Rej. limit.: Rejection limit (5% level)

unit: mg/ml

indicated Log-normal distribution. The mean concentration of urinary HA was 0.59 mg/ml by Method A-1 (179 persons) and 0.39 mg/ml by method A-2 (225 persons). The value was calculated as Log-normal distribution. The normal value of urinary HA by Method B-1-1 was 0.30 ± 0.11 mg/ml (mean $\pm$ SD, 10 persons).

DISCUSSION

The purpose for the development of this technique was to minimize errors in the determination of glycine conjugates. By Method A-2, water decomposes BSC and the colored products. Therefore, smaller volume of sample took advantage of more accurate reproducibility in Method A-2. A sample of 0.2 ml of urine was used instead of 0.5 ml. When the colored product was diluted with ethanol, about the half of samples became turbid. Therefore, centrifugation is necessarily to remove the turbidity before colorimetric determination. The use of a mixture of DMSO and ethanol avoids this step.

The data indicate that this method is sensitive not only to HA but to other glycine conjugates (MHA, SA, NA and INA) in urine. MHA, SA, NA and INA are derived from *m*- or *p*-xylene, salicylic acid, nicotinic acid and isonicotinic acid, respectively.

Urinary HA concentrations of normal persons by Method A-1 and A-2 indicated Log-normal distribution as described by Heath⁵⁾. Coffee beans and certain vegetables and fruits, particularly plums and cranberries are known to contain benzoic acid or precursors of benzoic acid, such as quinic acid. Therefore, HA excretion in urine increases after taking such foods. Glycine conjugate in urine of normal person is thought to be HA, except that the person took the medicament (i.e. salicylic acid) which was metabolised into glycine conjugate. In our case, as the urine of person took antipyretics and anti-inflammatory agents was omitted previously, the glycine conjugate measured by these methods can be thought to be HA.

As the colored solution from glycine conjugates showed similar absorption maxima by Method A-1 and A-2, except for INA, the separation of each conjugate by paper chromatography is necessary for the colorimetric determination of each conjugate.

DETERMINATION OF URINARY GLYCINE CONJUGATES

REFERENCES

- 1) Ogata, M., Tomokuni, K. and Takatsuka, Y. (1970). *Brit. J. Ind. Med.* **27**, 43.
- 2) Ogata, M., Tomokuni, K. and Takatsuka, Y. (1969). *Brit. J. Ind. Med.* **26**, 330.
- 3) Tomokuni, K. and Ogata, M. (1972). *Clin. Chem.*, **18**, 349.
- 4) Umberger, C.J. and Fiorese, F.F. (1963). *Clin. Chem.*, **9**, 91.
- 5) Heath, D.F. (1967). *Nature*, **213**, 1159.