COMPARATIVE EVALUATION OF COLORIMETRY, GLC AND HPLC FOR THE DETERMINATION OF URINARY HIPPURIC ACID

Kenji HASEGAWA, Kazunori SEIJI, Shoji SHIOJIMA, Akio KOIZUMI and Masayuki IKEDA

Center of Occupational Medicine, Tohoku Rosai Hospital, Dainohara 4-chome, Sendai 980, Japan

†Department of Environmental Health, Tohoku University School of Medicine, Seiryo-cho, Sendai 980, Japan

(Received November 12, 1979)

Human urine samples, 81 from the non-exposed, 36 from those with intensive exposures to toluene, and 23 from the less intensively exposed, were analyzed for hippuric acid by colorimetry, gas liquid chromatography (GLC), and high performance liquid chromatography (HPLC). The colorimetric method always gave higher values than the GLC method. In the case of the non-exposed, the value by the former method was more than double the value by the latter. When the colorimetric values were plotted on the abscissa against the GLC values on the ordinate, the slope of the calculated regression line was 0.444 for the non-exposed while it was 0.882 for the exposed. The value was higher as the exposure was more intensive. A conversion formula between colorimetric and GLC values was calculated from the values observed and it was

\[ y = x (1 - 1.78e^{-0.43x}) + 0.50 \]

where \( x \) and \( y \) are the colorimetric and GLC values (mg/ml), respectively, with a correlation coefficient of 0.82. The equation is however not applicable when \( x \) is smaller than 0.6. The HPLC values essentially agreed with the GLC values, the regression line being

\[ y = 0.993z + 0.009 \]

where \( y \) and \( z \) are the GLC and HPLC values (mg/ml), respectively. The correlation coefficient, \( r \), was 0.994.

The significance of urinary hippuric acid is well established as a measure of exposure of factory workers to toluene\(^1,2\), one of the most popular constituents in various industrial thinner preparations and solvents. As the method of determination, colorimetry after the reaction of hippuric acid with benzenesulfonyl chloride\(^3,4\) was once widely accepted, but has been gradually replaced by gas liquid chromatographic (GLC)\(^5,6\) and high performance liquid chromatographic (HPLC)\(^7-10\) methods, because of possible higher
specificity of the latter two methods. All of the three methods are, however, still in use currently especially because the colorimetric method can cope with much larger numbers of samples than the other two methods. Reports are yet rather scanty on the comparative evaluation of the three methods. It is the purpose of the present communication to make quantitative comparison of the urinary hippuric acid levels as determined by the colorimetric, GLC, and HPLC methods, and to establish a conversion formula so that the results obtained by any one of the three leading methods can be compared with those obtained by other methods.

**MATERIALS AND METHODS**

Urine samples were collected from factory workers engaged in the application of toluene-containing adhesives to produce rubber bags. GLC analyses of workroom air and adhesive constituents revealed no solvent other than toluene. Correspondingly, no methylhippuric acids, xylene metabolites, were detected in urine when analyzed by the GLC system which can separate methylhippuric acids from hippuric acid. Two groups of samples were studied; the samples in Group 1, 36 in total, were obtained from 7 female workers by repeated collection of urine when they were exposed to toluene at around 40-100 ppm as measured with carbon-felt personal dosimeters (details of the determinations are to be given elsewhere by Koizumi et al.), while those in Group 2 were collected from 23 workers (4 males and 19 females) in the morning when the exposure was less intensive. In addition, control urine specimens were sampled from 81 subjects (50 males and 31 females) with no known occupational exposure to toluene-containing chemicals. All the samples were kept frozen at −20°C until analyzed.

Colorimetric method for hippuric acid determination was based on the chromophore-forming reaction of hippuric acid with benzenesulfonyl chloride, and carried out after Ogata et al. as follows; hippuric acid in 1.0 ml of urine after the addition of 2 drops of 36% HCl and ca. 0.3 g of NaCl was extracted into 4.0 ml of ethyl acetate with vigorous shaking for 3 min. An aliquot, 0.2 ml, of the ethyl acetate was taken to dryness. The residue was mixed step-wise with 0.5 ml of pyridine and then 0.2 ml of benzenesulfonyl chloride. The mixture was kept standing at room temperature for 30 min, and subjected to the measurement of absorbance at 380 nm after the clarification with 4.3 ml of chloroform. For GLC analysis, to 1.0 ml of urine were added 0.3 g of NaCl, 2 drops of 36% HCl, and 0.4 mg of tridecylic acid in 0.2 ml of methanol as an internal standard. The mixture was shaken vigorously for 3 min with 4.0 ml of ethyl acetate. The organic layer was dessicated with 0.5 g of Na2SO4 and evaporated to dryness. The residue was treated with diazomethane in ether for methyl esterification of hippuric acid. After removal of the ether by evaporation, the residue was taken up in 0.1-0.2 ml of methanol, of which 1-2 μl was injected into a GLC. The GLC analysis with 4% Apiezon M on Chromosorb G (AW-DMCS, 80-100 mesh) columns was carried out after Caperos and Fernandez, with two modifications that the apparatus used was a Hitachi
When urine samples collected from the people with no known exposure to toluene were analyzed by the three methods (Table 1), it was apparent that the colorimetric method gave higher values than the GLC and HPLC methods, regardless of the sexes of the subjects studied; the difference between the former geometric means and the latter two was about 0.5 mg/ml. The hippuric acid level in the urine from female subjects tended to be higher than that from the males (p<0.01) as analyzed by GLC or HPLC, while no significant (p>0.05) difference could be detected when the colorimetric values were compared.

The results obtained with the total of 140 urine samples from both the exposed and the non-exposed are shown in a scatter diagram (Fig. 1) taking colorimetric values on the abscissa and GLC values on the ordinate. Because a larger number of samples

Table 1. Hippuric acid concentration in the urine samples from the non-exposed subjects as determined by colorimetry, GLC and HPLC.

<table>
<thead>
<tr>
<th>Methods</th>
<th>Geometric means* (mg/ml)</th>
<th>95% Confidence ranges* (mg/ml)</th>
<th>Ranges of distribution** (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males (n=50)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colorimetry</td>
<td>0.681</td>
<td>0.286—1.622</td>
<td>0.194—1.385</td>
</tr>
<tr>
<td>GLC</td>
<td>0.126</td>
<td>0.024—0.662</td>
<td>0.035—0.766</td>
</tr>
<tr>
<td>HPLC</td>
<td>0.124</td>
<td>0.027—0.562</td>
<td>0.025—0.675</td>
</tr>
<tr>
<td>Females (n=31)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colorimetry</td>
<td>0.734</td>
<td>0.292—1.844</td>
<td>0.228—1.653</td>
</tr>
<tr>
<td>GLC</td>
<td>0.236</td>
<td>0.036—1.532</td>
<td>0.030—0.938</td>
</tr>
<tr>
<td>HPLC</td>
<td>0.241</td>
<td>0.044—1.305</td>
<td>0.050—1.050</td>
</tr>
<tr>
<td>Total (n=81)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colorimetry</td>
<td>0.701</td>
<td>0.287—1.711</td>
<td>0.194—1.653</td>
</tr>
<tr>
<td>GLC</td>
<td>0.161</td>
<td>0.025—1.013</td>
<td>0.030—0.938</td>
</tr>
<tr>
<td>HPLC</td>
<td>0.159</td>
<td>0.029—0.883</td>
<td>0.025—1.050</td>
</tr>
</tbody>
</table>

* Calculated with an assumption of log-normal distribution.\(^{13}\)
** Minimum and maximum values observed.
were analyzed, the values from the non-exposed are spotted separately from the values from the exposed and shown in the upper part of the figure on expanded scales.

The non-exposed group gave a regression line (shown as a broken line in Fig. 1) with an equation of

$$y = 0.444x - 0.090 \quad (n=81, \ r=0.599)$$  \hspace{1cm} (1)

where $y$ is hippuric acid (mg/ml) measured by the GLC method, while $x$ is hippuric acid (mg/ml) measured by the colorimetric method. The correlation coefficient, $r$, was 0.599. When the same was figured out with the Exposed Group 1, the equation for the regression line was

$$y = 0.893x - 0.512 \quad (n=36, \ r=0.973)$$  \hspace{1cm} (2)
URINARY HIPPURIC ACID BY COLORIMETRY, GLC AND HPLC

and with the Exposed Group 2, it was

\[ y = 0.847x - 0.200 \quad (n=23, \ r=0.927) \]  (3)

When the data of the two exposed group were combined, the equation (the solid line)

\[ y = 0.882x - 0.408 \quad (n=59, \ r=0.963) \]  (4)

was obtained.

All the lines shown by the Eqs. (1) to (4) have small but positive intercepts on the abscissa. It is also apparent that the slope in Eq. (4) for the exposed, 0.882, is almost twice as large as that in Eq. (1) for the non-exposed, 0.444. The value in Eq. (2) for the Exposed Group 1, 0.893, with intensive exposure appears to be somewhat larger than its counterpart in Eq. (3) for the Exposed Group 2, 0.847, those in which were exposed less intensively.

These findings suggest that the apparent hippuric acid concentration as measured by the colorimetric method consists of three components, \( \alpha, \beta \) and \( \gamma \), where \( \alpha \) is hippuric acid derived from toluene absorbed, \( \beta \) is hippuric acid of food origin, and \( \gamma \) is un-identified urine component(s) other than hippuric acid yet positive to benzenesulfonyl chloride-pyridine reaction. Without any toluene exposure, \( \alpha \) is zero, while \( \gamma \) is more than \( \beta \) as shown in Table 1; the ratio of \( \beta/\gamma \) is roughly 1/3 to 1/4. In the presence of toluene exposure, \( \alpha \) is positive and it goes up as a function of the exposure intensity. The ratio of \( \gamma/(\alpha+\beta+\gamma) \), or the share of \( \gamma \) in total “hippuric acid” as measured by the colorimetry, goes down with more intensive exposure. The amount of hippuric acid is measured as \( \alpha+\beta \) by the GLC method while it corresponds to \( \alpha+\beta+\gamma \) by the colorimetric method. When \( \alpha \) is far larger than \( \gamma \), the ratio of \( (\alpha+\beta)/(\alpha+\beta+\gamma) \) will approach 1. Accordingly, the slope of the regression line in Fig. 1 would be closer to 1 under the condition of \( \alpha >> \gamma > \beta \). The postulations that the coefficient on \( x \) is less than 1 when \( x \) is small while it should quickly converge to 1 as \( x \) increases suggests the following equation to obtain the best conversion formula between the colorimetric value and the GLC value;

\[ y = x(1 + Ae^{Bx}) + C \]  (5)

where \( y \) and \( x \) are as in Eq. (1), both \( A \) and \( B \) are negative constants, and \( C \) is a positive constant. The calculation to figure out \( A, B \) and \( C \) with least square fitting of the data given in Fig. 1 resulted in

\[ y = x(1 - 1.78e^{-0.48x}) + 0.50 \]  (6)

with the correlation coefficient, \( r \), of 0.82. The high \( r \) value indicates that Eq. (6) well fits with the practical experiences as summarized in Fig. 1. The values of \( y \)'s calculated with several integral \( x \)'s are given in Table 2 to avert complex calculation. The equation, however, has a minimum point of inflection when \( x \) is around 0.6 where \( y \) is about 0.28. In the range of \( x \) below this point, the equation is apparently not applicable.

Contrary to the complex relationship between colorimetric and GLC values, the GLC and HPLC values revealed remarkable agreements to each other as shown in Fig. 2. The regression line calculated was

\[ y = 0.993z + 0.009 \quad (n=140) \]

where \( y \) is hippuric acid (mg/ml) measured by the GLC method, while \( z \) is hippuric
acid (mg/mL) measured by the colorimetric method.

Table 2. The values of y's at several x's calculated with Eq. (6).

<table>
<thead>
<tr>
<th>x</th>
<th>1.0</th>
<th>2.0</th>
<th>3.0</th>
<th>4.0</th>
<th>5.0</th>
<th>6.0</th>
<th>7.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>y</td>
<td>0.34</td>
<td>0.99</td>
<td>2.03</td>
<td>3.23</td>
<td>4.46</td>
<td>5.69</td>
<td>6.89</td>
</tr>
</tbody>
</table>

For Eq. (6), see text.

x: Hippuric acid (mg/mL) measured by the colorimetric method.

y: Hippuric acid (mg/mL) measured by the GLC method.

Fig. 2. Comparison between the GLC and HPLC results

Symbols are as in Fig. 1. The line in the figure is a regression line calculated for all examinees with the least square fitting method, and expressed as

\[ y = 0.993z + 0.009 \quad (n=140, \ r=0.994) \]

in which y is hippuric acid (mg/mL) measured by the GLC method while z is hippuric acid (mg/mL) measured by the HPLC method.

acid (mg/mL) measured by the HPLC method. The line has a slope very close to one with a quite small intercept. The correlation coefficient, r, was as high as 0.994. It should be stressed that this close agreement held true even with the samples from the non-exposed people (the upper half in Fig. 2), in which hippuric acid concentrations were low with relative abundance of possible interfering substances. From such good agreements, it is possible to infer that Eq. (6) is applicable also as a formula to convert the colorimetric value, x, to the best estimate of the HPLC value, z, when y in the equation
is replaced with \( z \).

To conclude from the practical viewpoint, the GLC method and the HPLC method give essentially the same value at least under the conditions studied, while the value obtained by the colorimetric method requires mathematical correction following Eq. (6) for the comparison with counterpart value obtained by the GLC or HPLC method.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. Akira HARADA, Sanyo Electric Company Health Administration Center, Osaka, Japan, for the GLC-mass spectrometric analyses of the samples.

REFERENCES