To a dried extract containing hippuric acid (HA), 1.0 mL of acetic anhydride and 2.0 mL of 0.5% p-dimethylaminobenzaldehyde (DAB) solution in pyridine were added, and the solution was kept at 40 °C for 1 h after thorough mixing. The absorbance was then determined at 458 nm against a blank containing acetic anhydride, DAB, and pyridine. This method was in good agreement with Beer's law within 1 to 100 μg and the mean ± SD absorbance for 20 μg HA was 0.939 ± 0.013 (n = 5). The apparent molar absorptivity of 2.5 × 10^4 mol/cm, and the relative standard deviation of 1.4% was calculated.

The determination of hippuric acid (HA) in urine is of great significance, mainly for testing of liver function (1), diagnosis (2-4), and estimation of the detoxication of alkylbenzenes and drugs (5-7).

In this paper a convenient and reproducible method based on colorimetry is presented. About 40 papers dealing with methods of determination of HA have been published. These methods depend on column chromatography (8), extraction (9), colorimetry (10-12), gas chromatography (14-17), thin-layer chromatography (18), paper chromatography (19), fluorimetry (20), titration (21), and determination of radioactivity (22). The method most widely used at present is based on gas chromatography. Applying this method to the determination of HA in liver homogenates of rat and eel (instead of urine), we had difficulties with overlapping peaks. The method to be described here is applicable to urine as well as liver homogenates.

Two colorimetric methods have been presented for the determination of HA. With the method of Umbreger (10) which was later modified by Ogata, the color is produced in a mixture of the HA-containing sample with benzene-sulfonyl chloride and pyridine. With the method reported by Gaffney et al. (19) and Ogata et al. (23), DAB is used. Gaffney et al. employed paper chromatography. They detected the HA spot by spraying a 4% solution of DAB in acetic anhydride which contained a few crystals of sodium acetate with subsequent heating of the chromatogram at 130-150 °C for 1-2 min. The absorbance of the color was determined after elution of the spot. Ogata et al. improved this method. Their reaction mixture additionally contained silica gel.

The colorimetric procedure reported in this paper is based on the DAB method.

**EXPERIMENTAL**

**Chemicals and Instruments.** Analytical grade chemicals from Wako Pure Chemical Industries Ltd (Osaka, Japan) were used. A Hitachi-139 Spectrophotometer was used for measuring the absorbance at 458 nm and a Hitachi-124 Spectrophotometer for recording the absorption spectra.

**Determination of HA. Extraction of HA from Urine.** Urine (0.1 mL), 10 μL 6 N HCl, ca. 20 mg NaCl, and 1 mL ethyl acetate in a 10-mL test tube were mixed for 30 s with a Vortex mixer (Thermomix Inc., Japan). After 5 min, a 0.1-mL aliquot of the ethyl acetate layer was transferred to a 20-mL test tube and evaporated to dryness under reduced pressure.

**Extraction of HA from Liver Homogenates.** Method A: for homogenates containing more than 20 μg HA per 0.5 mL. Rat liver (10 g) was homogenized in 90 mL of 1.15% KCl in a Potter-Elvejem Teflon pestle homogenizer and then centrifuged at 1000 X g for 10 min.

To a 10-mL glass stoppered centrifuge tube, 0.5 mL of the supernatant and 20 μL 6 N HCl, 20 μL glacial acetic acid, ca. 150 mg NaCl, and 2.5 mL ethyl acetate were added. The mixture was titrated with a Vortex mixer for 30 s and centrifuged at 1500 X g for 5 min. The ethyl acetate layer was dried over anhydrous sodium sulfate. An aliquot (0.5 mL) was then evaporated under reduced pressure.

Method B: for homogenates containing less than 20 μg HA per 0.5 mL. To a 10-mL glass stoppered centrifuge tube 0.5 mL of the supernatant of the homogenate and 50 μL 6 N NaOH, ca. 150 mg NaCl, and 5 mL ethyl acetate were added. The mixture was agitated with a Vortex mixer. It was then centrifuged at 1500 X g for 10 min and the ethyl acetate layer was removed as thoroughly as possible. The aqueous layer was acidified with 0.1 mL 6 N HCl and extracted with 5 mL n-hexane by vortexing and centrifuging. After the hexane layer was removed as thoroughly as possible, the aqueous layer was extracted with 5 mL ethyl acetate by vortexing and centrifuging. The extract was dried with anhydrous sodium sulfate and an aliquot (3.5 mL) was evaporated under reduced pressure.

**Development of the Color and Its Measurement.** To the dried HA-containing residue, 1.0 mL of acetic anhydride and 2.0 mL of 0.5% DAB solution in pyridine were added in turn. After thorough mixing, the solution was kept at 40 °C for 1 h. The absorbance was then determined at 458 nm against a blank containing acetic anhydride, DAB, and pyridine.
RESULTS

Time Course of Color Development. As is shown in Figure 1, it takes about 1 h at 40 °C or up to 2 h at 30 °C to develop the maximum absorbance. The color is stable for at least one day.

Absorption Spectra under Varying Ratios of Acetic Anhydride and Pyridine. In a total volume of 3 mL with constant amounts of DAB and HA, the color was developed in the presence of different ratios of acetic anhydride and pyridine. As shown in Figure 2, there was a minute red shift with increasing pyridine concentration. The maximum absorbance at 458 nm was obtained with a pyridine/acetic anhydride ratio of 2.

Influence of Concentration of DAB on Color Development. HA (15 μg), 1.0 mL acetic anhydride and 2.0 mL of pyridine containing different concentrations of DAB were kept at 30 °C for 2 h. As shown in Figure 3, almost the same absorbance was found in the range of 0.375 to 1.0% DAB in pyridine. A final concentration of 0.33% DAB was, however, chosen as standard concentration to avoid HA-independent color development. Figure 3 also shows that the color is developed in the absence of DAB. It took, however, more than 3 h until the absorbance reached a constant value.

Influence of Water on Color Development. The color was developed under standard conditions except that varying amounts of water were added to the reaction mixture. As shown in Figure 4, up to 30 μL of water do not influence the color development significantly.

Calibration Curves. (a) Calibration Curve without Extraction. The calibration curves which were prepared using a standard ethyl acetate solution of HA showed fine linearity within 1 to 100 μg. Moreover as shown in Table I, this method is precise to 1.4% relative standard deviation for 20 μg HA. The apparent molar absorptivity was 2.5 × 10^4/mol/cm at 458 nm.

(b) Calibration Curves with Extraction. The calibration curves, which were prepared using an HA aqueous standard solution according to the extraction procedures for urine or

Table I. Reliability of Color Development under the Standard Condition

<table>
<thead>
<tr>
<th>HA, μg</th>
<th>N</th>
<th>Mean ± SD absorbance at 458 nm</th>
<th>Rel std dev, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>5</td>
<td>0.466 ± 0.015</td>
<td>3.2</td>
</tr>
<tr>
<td>20</td>
<td>5</td>
<td>0.939 ± 0.013</td>
<td>1.4</td>
</tr>
<tr>
<td>30</td>
<td>5</td>
<td>1.444 ± 0.017</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Using a standard ethyl acetate solution of HA, the color was developed according to the procedure in Experimental after ethyl acetate was evaporated.
away after elution with methanol. Work is in progress to present method with the gas chromatograph method by Rf

1496

dimethylaminobenzal)oxazol-5-one, Schueler et al. identify the colored substances. Substance with the

190

contributed to the absorbance at 458 nm (see Figure 5). Absorption spectra of the eluted spots with methanol indicated yellow spots & one which was synthesized by Attenburrow et al. (24).

5

3

2

1

0.70, 0.48, and 0.42) could be separated. The absorption spectra of the eluted spots with methanol indicated that the colored compound with the Rf value of 0.42 mainly contributed to the absorbance at 458 nm (see Figure 5). This substance may be 2-phenyl-4-(1-hydroxyethylidene)oxazol-5-one which was synthesized by Attenburrow et al. (24). The substance with the Rf value of 0.70 may be 2-phenyl-4-(p-dimethylaminobenzal)oxazol-5-one, which was synthesized by Schueler et al. (25) and Gaffney et al. (19). The spot with the Rf value of 0.48 was paler than the other two. The color faded away after elution with methanol. Work is in progress to identify the colored substances. Seventeen urine samples were used for comparing the present method with the gas chromatograph method by

DISCUSSION

The colorimetric method for determination of HA, presented in this paper, is simpler, more reproducible, and about 5 times more sensitive than the previous colorimetric methods. With thin-layer chromatography of the colored substance on silica gel developing with toluene/acetone acid 9:1, three yellow spots (Rf 0.70, 0.48, and 0.42) could be separated. The absorption spectra of the eluted spots with methanol indicated that the colored compound with the Rf value of 0.42 mainly contributed to the absorbance at 458 nm (see Figure 5). This substance may be 2-phenyl-4-(1-hydroxyethylidene)oxazol-5-one which was synthesized by Attenburrow et al. (24). The substance with the Rf value of 0.70 may be 2-phenyl-4-(p-dimethylaminobenzal)oxazol-5-one, which was synthesized by Schueler et al. (25) and Gaffney et al. (19). The spot with the Rf value of 0.48 was paler than the other two. The color faded away after elution with methanol. Work is in progress to identify the colored substances. Seventeen urine samples were used for comparing the present method with the gas chromatograph method by

Figure 5. Absorption spectra of two yellow spots separated on a thin-layer chromatogram. The solid lines represent the absorption spectra in methanol and the dotted lines in the mixed solution of acetic anhydride and pyridine (1:2)

Table II. Recovery Studies of Hippuric Acid Added to Urine and Liver Homogenate by the Present Method

<table>
<thead>
<tr>
<th>HA added, HA(μg)/mL of urine covered</th>
<th>Re-added, HA(μg)/mL of liver homogenate covered</th>
<th>Recovery, %</th>
<th>SD</th>
<th>Rf</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>100</td>
<td>3.4</td>
<td>0.70</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>100.0</td>
<td>2.6</td>
<td>0.48</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>100.1</td>
<td>2.3</td>
<td>0.42</td>
</tr>
</tbody>
</table>

a HA was extracted according to Method B. b HA was extracted according to Method A.

Buchet et al. (14), and from data a correlation coefficient of 0.977 and the regression line of Y = 0.896X + 0.090 was calculated (Y = the present method).

With the present method the absorbance can also be measured at 489 nm in good agreement with Lambert–Beer’s law. Compared with measurements at 458 nm, the sensitivity is about 70%.

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LITERATURE CITED


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