COMPARATIVE STUDY OF COLORIMETRIC METHOD USING DIAZOTIZATION REACTION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD IN DETERMINATION OF PARA-AMINOHIPPURIC ACID

Masao KIGUCHI and Jun-ichi SUDO

Department of Toxicology and Clinical Pharmacology, Faculty of Pharmaceutical Sciences, Higashi-Nippon-Gakuen University, Ishikari-Tobetsu, Hokkaido 061-02, Japan

Accepted March 22, 1987

Abstract—In this study, colorimetric method and high-performance liquid chromatographic (HPLC) method were improved and established, respectively, in order to minimize analytical errors in determination of para-aminohippuric acid (PAH) in rat urine and plasma. In terms of the colorimetric method, an operative step following addition of Tsuda reagent was modified as follows: after the addition of Tsuda reagent, reaction mixture was kept at 40°C for 70 min before spectrophotometry. Linearities were observed both in the higher range of 0 and 2.5 to 12.5 μg and in the lower range of 0 and 100 to 1,000 ng per test tube, and its practical detection limit was 100 ng per test tube. In terms of HPLC method, using a reversed-phase column (Nucleosil 5 C18), PAH was separated by a mobile phase of acetonitrile/50 mM KH₂PO₄ (pH 2.8)=9/95. Linearities were observed in the higher range of 0 and 10 ng to 2 μg and in the lower range of 0 and 1 to 10 ng per injection, and its practical detection limit was 1 ng per injection. These results denote that the above two methods are applicable to routine PAH determination. In addition, our HPLC method is considered to be applicable to microassay of PAH, because its sensitivity is more sensitive and minimization of volume system is more easily achieved as compared with the colorimetric method.

Key words: Para-aminohippuric acid, diazotization, high-performance liquid chromatography, urine, plasma, rat.
Masao KIGUCHI and Jun-ichi SUDO

INTRODUCTION

In screening of nephropathy in experimental animals, clearance of para-aminohippuric acid (PAH) is routinely determined as one of the major diagnostic tools. Also, renal capacities in secretion and accumulation of PAH have been investigated in relation to renal proximal tubular damage (Child and Dodds, 1967; Yamazaki et al., 1981; Kuo et al., 1982; Sudo et al., 1984), because renal PAH is known to be handled mainly in the proximal tubules (Cortney et al., 1965; Tune et al., 1969).

In the above cases, PAH is generally determined spectrophotometrically through diazotization of the para-amino group (Smith et al., 1945; Brun, 1950; Kanai and Kanai, 1982). However, in determinations of urinary and plasma PAH concentrations, we found that constant absorbances were not obtained according to the method of Kanai and Kanai (1982) modifying the methods of Smith et al. (1945) and Brun (1950). Namely, minute errors both in time and temperature of reaction following addition of Tsuda reagent, brought large deviations to absorbance measurement.

In order to resolve this problem, we tried to improve and establish the two methods, a colorimetry and a high-performance liquid chromatography (HPLC) for PAH determinations. This study was designed to compare the validities and accuracies of these two methods in determination of PAH.

MATERIALS AND METHODS

Chemicals and Materials. All reagents were of the highest grade available and purchased from Wako Pure Industries, Ltd. (Osaka, Japan). A reversed-phase HPLC column (Nucleosil 5 C18; particle size, 5 μ; 4 mm X 250 mm; Macherey-Nagel Co.; Dueren, F.R.G.) was obtained from Wako Pure Chemical Industries, Ltd. Wister rats were obtained from Sankyo Labo Service Co. (Tokyo, Japan).

Apparatus. Absorbance was measured using a Hitachi-320 spectrophotometer with a constant temperature cuvette holder (Hitachi Ltd.; Tokyo, Japan). The HPLC system consisted of a Model 510 solvent delivery pump fitted with a Model 710B WISP autosampler and a Model 481 Lamda-Max LC spectrophotometer, all from Waters Assoc. (Milford, MA, U.S.A.). A Model ERC-5310 column oven (Erma Inc.; Tokyo, Japan) was used to maintain column temperature. For recording chromatograms, a Model FBR-252A flatbed recorder (TOA Electronics Ltd.; Tokyo, Japan) was used. A Model 3390A Hewlett-Packard recording integrator (Hewlett-Packard Co.; Avondale, PA, U.S.A.) was used to determine peak area.

Collection of Urine and Plasma. Urine and plasma were collected as reported previously (Sudo et al. 1983). A Wistar male rat weighing 220 g was anesthetized intraperitoneally with 50 mg/kg body weight of sodium pentobarbital, and was intubated for free respiration, after which a left jugular vein was catheterized with a
Determination of Para-Aminohippuric Acid

polyethylene tube (PE-50). Two ml of saline containing 0.2 % PAH was injected as a prime, followed by infusion of saline containing 0.225 % PAH at a rate of 0.2 ml/kg body weight/min; an equilibration period of 60 min was allowed. The left ureter was then catheterized with a polyethylene tube (PE-10) through the retroperitoneal approach (Sudo et al., 1983). Urine collection was begun at 120 min after the start of operation. Urine was collected for 10 min, after which its abdominal cavity was opened by ventral incision in order to obtain the blood through the abdominal aorta. The blood was put into a heparinized tube and centrifuged (1,700 X g, 10 min, 4°C) for separation of plasma.

Colorimetric Assay of PAH. PAH concentrations in plasma and urine samples were determined by modifying the method of Kanai and Kanai (1982). In terms of plasma, 300 μl of plasma was mixed with 2.1 ml of water. In terms of urine, 10 μl of urine was mixed with 10 ml of water, and 2.4 ml of this mixture was used for the below steps. To these mixtures in volume of 2.4 ml, 600 μl of 1 N trichloroacetic acid was further added for deproteinization. They were kept in ice for 20 min, and centrifuged (1,700 X g, 10 min, 4°C) to obtain the supernatants. PAH in each sample was diazotized as below. To 2.5 ml of standard solutions and the supernatants obtained from the plasma and urine, 500 μl of 2 N HCl was added and mixed. Following addition of 50 μl of 0.2 g/dl sodium nitrite, the mixtures were kept for 5 min. Next, 50 μl of 25 g/dl of urea solution was added and kept for 10 min. After 30 μl of 0.2 g/dl Tsuda reagent (1-(β-diethylaminoethyl)-α-naphthylamine oxalate) was added, the mixtures were incubated at 40°C for 70 min. Absorbance was measured at 570 nm, and PAH concentration was calculated from the regression line based on the standard solutions.

HPLC Assay of PAH. For deproteinization, 10 μl of plasma and urine samples were added to 90 μl and 10 ml of 5 % perchloric acid solutions, respectively. After centrifugation (10,062 X g, 10 min, 4°C), the supernatants were used for the HPLC assay. Conditions of HPLC were as follows: column, Nucleosil 5 C18 (5 μ, 4 mm X 250 mm); mobile phase, acetonitrile/50 mM KH2PO4 (pH 2.8)=5/95; injection volume, 20 μl; flow rate, 1 ml/min; column temperature, 40°C; detection wave, 254 nm; detection sensitivity, 0.02 A.U.F.S. The amount of PAH in each sample was calculated by measuring the peak areas and heights from the regression line based on the standard solutions.

Calculation. Values were represented in mean ± standard deviation (S.D.). Error rates (%) were calculated in S.D. X 100/mean. Relation coefficients(r) were calculated by the ordinary method.

RESULTS AND DISCUSSION

First, in order to test the validity and accuracy in the colorimetric method of PAH, changes in color development at various temperatures were investigated with time elapsed following addition of Tsuda reagent. Fig. 1 shows the results in the

303
color development of standard solution containing 12.5 μg PAH per test tube, under 20, 30, 40 and 50°C. After the addition of Tsuda reagent, the absorbances revealed rapid increases, with the color development showing a dependency on the temperatures. The color development in 50°C reached a maximal level at 40 min, after which it was slightly and gradually discolored. The color development in 40°C reached a stationary level later than 70 min. The color development in other temperatures increased up to 90 min, and reached neither the maxima nor the stationary levels.

![Graph showing color development of PAH at various temperatures.]

**Fig. 1** Changes in color development of PAH at various temperatures. PAH standard solution (12.5 μg PAH per test tube) was treated as represented in Materials and Methods. After adding Tsuda reagent, its absorbance at 20, 30, 40 and 50°C was continuously monitored in a constant temperature cuvette.

In the original methods of Smith et al. (1945) and of Brun (1950) and its modified method of Kanai and Kanai (1982), the absorbance in color development was proposed to be measured at 10 min after the addition of Tsuda reagent. In this time point of 10 min, the absorbance in 50°C was approximately 2.2 times of that in 20°C. In addition, the color reaction in this 10 min advanced very rapidly. Accordingly, we considered that the absorbance should not be measured in this time point, and chose the following conditions for the color development: After the addition of the Tsuda reagent, the mixture was kept at 40°C for 70 min, and then the absorbance was determined.

Based on this modification, PAH standard solutions at various concentrations were determined following the incubation at 40°C for 70 min for the color development. A linearity was observed in a range of 0 and 2.5 to 12.5 μg per test
Determination of Para-Aminohippuric Acid
tube: $r=0.999$ in 6 points. When 1,000 ng PAH per test tube was determined, its absorbance was 0.077. Providing that the minimal detection in this colorimetric method could be achieved in 0.001 as absorbance, the detection limit was 12.9 ng per test tube. In practice, when low contents of PAH were determined, 100 ng per test tube showed an absorbance near 0.010. When other analytical factors were considered, this 100 ng per test tube was speculated to be the practical detection limit in this colorimetric method. In addition, PAH was furthermore determined in the lower range of 0 and 100 to 1,000 ng per test tube. This determination showed a linearity ($r=0.999$) in 6 points of 0, 100, 300, 500, 700 and 1000 ng per test tube.

Next, we tried to establish a new HPLC method for separation of PAH from interference substances in the urine and plasma samples. Fig. 2 shows the chromato-

![Chromatograms](image)

**Fig. 2.** Chromatograms of urinary and plasma PAH in HPLC method. Upper chromatogram: urine sample. Lower chromatogram: plasma sample. Retention time of PAH was 5.22 min in this employed HPLC system.
grams. The retention time of PAH was 5.22 min in this HPLC system. The PAH peak was completely separated from the other substances in the urine and plasma samples.

The calibration curve of PAH which was made by measuring peak areas in this HPLC system showed a linearity in a range of 0 and 10 ng to 2 μg per injection : \( r = 0.999 \) in 14 points, while no linearity was observed in the lower range. In contrast, concerning peak heights, a linearity was observed even in a range of 0 and 1 to 10 ng per injection : \( r = 0.999 \), in 6 points of 0, 1, 3, 5, 7 and 10 ng per injection. The detection limit was 0.5 ng per injection in signal to noise ratio of 3 : 1. From this result, the practical detection limit was considered to be more than 1.0 ng per injection.

Finally, concerning plasma and urinary samples obtained from a rat loaded PAH in saline, PAH determinations were carried out 6 times in order to compare the accuracies between the colorimetric method and the HPLC method. Table 1 shows the results. Analytical error rates were less than 10% in both methods. This fact denoted that accuracies in the both methods were almost equal.

In conclusion, in case of the colorimetric method using diazotization of PAH, incubation for more than 70 min in mild temperature (e.g., 40°C) following the addition of Tsuda reagent is considered to be necessary to obtain accurate values. Also, it was found that the accuracies were almost equal in the colorimetric and HPLC methods, while the practical detection limit in the HPLC was approximately 100 times more sensitive than that in the colorimetric method. Accordingly, these two methods are considered to be applicable for ordinary PAH determination. In addition, when microassay of PAH is needed, it would be enabled by means of

<table>
<thead>
<tr>
<th>Table 1. Comparison of values obtained by colorimetric method and HPLC method in urinary and plasma PAH determinations.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td>Urinary PAH</td>
</tr>
<tr>
<td>concentration (mg/ml)</td>
</tr>
<tr>
<td>Error rate (%)</td>
</tr>
<tr>
<td>Plasma PAH</td>
</tr>
<tr>
<td>concentration (mg/ml)</td>
</tr>
<tr>
<td>Error rate (%)</td>
</tr>
</tbody>
</table>

Concerning identical urine and plasma samples that were obtained from a rat, PAH determinations were done 6 times by colorimetric method and HPLC method, respectively. Urinary and plasma PAH concentrations were represented in mean ± standard deviation (S. D.) \( (N = 6) \). Error rates (%) were calculated in S. D. X 100 / mean.
minimization of the volume systems in the two methods. In particular, the minimization in volume system is easily achieved in the HPLC method in comparison to the colorimetric method. Thus, our HPLC method is considered to be applicable to microassay of PAH.

ACKNOWLEDGEMENTS

This research was supported by the grants from the special research foundation of Higashi-Nippon-Gakuen University (Grant 85PA-3 and 86PA-4).

REFERENCES


