A METHOD FOR THE PREPARATION OF CALIBRATION CURVES FOR ACETAMINOPHEN GLUCURONIDE AND ACETAMINOPHEN SULFATE IN RABBIT URINE WITHOUT USE OF AUTHENTIC COMPOUNDS IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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A method for the preparation of calibration curves for acetaminophen glucuronide (NAPAG) and acetaminophen sulfate (NAPAS) in rabbit urine without use of authentic compounds in high-performance liquid chromatography was examined. Rabbits were dosed intravenously with acetaminophen (NAPA, 30 mg/kg). Urine was collected and diluted. A plot of the peak area ratio of NAPAG to internal standard against NAPA concentration after the hydrolysis of diluted urine with β-glucuronidase was linear and passed through the origin. A linear tendency was also observed in the plot of the peak area ratio of NAPAS to internal standard against NAPA concentration calculated by the difference between the peak area ratio of NAPA after the hydrolysis with β-glucuronidase and that with β-glucuronidase/arylsulfatase. Thus, once the calibration curve has been prepared following the enzyme hydrolysis of NAPAG and NAPAS, then the concentration of NAPAG and NAPAS in the sample solution can be calculated from the peak of NAPAG and NAPAS, respectively. The method is simple, and has the advantage that pure standards of the individual NAPA metabolites are not required.

Keywords — acetaminophen; N-acetyl-p-aminophenol; acetaminophen glucuronide; acetaminophen sulfate; HPLC; calibration curve; β-glucuronidase; β-glucuronidase/arylsulfatase

Acetaminophen (N-acetyl-p-aminophenol, NAPA) is a widely used mild analgesic and antipyretic agent. Studies on the metabolism of NAPA have shown that major routes of elimination involve sulfation (acetaminophen sulfate, NAPAS) and glucuronidation (acetaminophen glucuronide, NAPAG). Methods for NAPA analysis have been reviewed elsewhere.\(^1\) They include ultraviolet spectrometry after extraction and colorimetric methods involving chemical hydrolysis to p-aminophenol, reaction with dyes and phenolic ring nitration. In the previous report,\(^2\) we developed a fluorometric procedure for the determination of NAPA and its conjugate in blood.

Measurement of conjugate formation has been limited to indirect approaches, namely selective enzyme hydrolysis and measurement of the liberated NAPA. Frequently two enzymes are employed, a non-specific preparation containing both β-glucuronidase and arylsulfatase and a purified form of either β-glucuronidase or arylsulfatase. Thus one conjugate is assayed directly while the other conjugate is assayed by difference. Routine confirmation of complete and specific hydrolysis is essential.

A number of high-performance liquid chromatographic (HPLC) methods for simultaneous assay of NAPA and its conjugates in biological fluids have appeared in the literature.\(^3\)–\(^8\) An authentic compound of NAPA conjugate is needed for the preparation of the calibration curve. The synthesis of drug conjugates is, however, usually difficult and time-consuming.

The present study was undertaken to prepare the calibration curves for NAPAG and NAPAS in rabbit urine without using authentic compounds in an HPLC method.

MATERIALS AND METHODS

Materials — NAPA was of pharmaceutical grade and was purchased from Yamanouchi Pharmaceutical Co., Ltd. (Tokyo, Japan). Theophylline was obtained from Nakarai Chemicals, Ltd. (Kyoto, Japan). NAPAS was synthesized as reported by Cummings et al.\(^9\) NAPAG was generously donated by Dr. Josiah N. Tam (McNeil Consumer Products, Fort
Washington, PA, U.S.A.) and by Dr. R. S. Andrews (Sterling Winthrop, Newcastle-upon-Tyne, Great Britain). β-Glucuronidase (13000 UF/ml) was obtained from Tokyo Zohki Kagaku Co., Ltd. (Tokyo, Japan). β-Glucuronidase/arylsulfatase (101250 UF/ml, 800000 UR/ml) was purchased from Boehringer Mannheim GmbH (Mannheim, Federal Republic of Germany). All other chemicals were of reagent grade.

**Preparation of Buffer Solution** — The composition of acetate buffer solution used was 0.1 M acetic acid-0.1 M sodium acetate (1:2, v/v), pH 5.0.

**Analytical Methods** — HPLC was used in these studies. We used a LC-6A pump (Shimadzu Co., Ltd., Kyoto, Japan), a model 7125 sample injection valve fitted with a 200 µl sample loop (Rheodyne Inc., CA, U.S.A.), an UVIDEC-100-II variable wavelength ultraviolet (UV) absorbance detector (Japan Spectroscopic Co., Ltd., Tokyo, Japan) and a Chromatopac C-R3A recorder (Shimadzu Co., Ltd.). The column (Cosmosil packed column, Nakarai Chemicals, Ltd.) was 150 × 4.6 mm i.d. containing a bonded octadecylsilane-silica gel of 5 µm particle size. The column was used at room temperature. A mixture of acetonitrile and 0.05 M sodium sulfate (pH 2.7, adjusted with phosphoric acid)(1:9, v/v) was used as the mobile phase at a flow rate of 1.5 ml/min. The chromatographic mobile phase was filtered through a 0.45 µm pore size membrane filter (Toyo Roshi Co., Ltd., Tokyo, Japan). The sample was withdrawn using a Hamilton syringe and loaded onto the column through a Rheodyne valve.

**Preparation of Calibration Curves** — The standard solutions (200 µg/ml NAPA and NAPAS) were dissolved in distilled water. The internal standard solution was prepared to contain 200 µg of theophylline/ml in distilled water. β-Glucuronidase and β-glucuronidase/arylsulfatase were diluted to 6.5 and 40 times its original volume with acetate buffer solution, respectively. Blank urine was diluted to 10 times its original volume with acetate buffer solution. Diluted urine (2.0 ml) was added to 0.2, 0.4, 0.6, 0.8, 1.0, 1.5 and 2.0 ml of drug solution, and each was diluted to 4.0 ml with acetate buffer solution. These drug solutions (2.0 ml) were hydrolyzed with β-glucuronidase/arylsulfatase (0.5 ml) at 37 °C for 24 h. After the hydrolysis, this solution was mixed with 2.0 ml of the internal standard solution and an aliquot of the solution was chromatographed. In the case of the calibration curve of NAPAS, the hydrolysis with β-glucuronidase/arylsulfatase was omitted. Calibration curves were prepared by using 4 samples at each concentration and plotting peak area ratio versus drug concentration. Calibration curves were calculated by a least—squares linear regression analysis.

**Animal Experiments** — Male albino rabbits (2.2—2.6 kg) were used throughout the study. The animals were housed in an air-conditioned room and maintained on a standard laboratory diet. The rabbits were fasted overnight but had free access to water. After fasting, rabbits were dosed intravenously (via an ear vein) with NAPA (30 mg/kg) dissolved in 0.9% NaCl. Urine was collected at appropriate time intervals through a catheter inserted into the bladder. Urine was diluted to 10—40 times its original volume with acetate buffer solution. These solutions (2.0 ml) were hydrolyzed with β-glucuronidase/arylsulfatase (0.5 ml) or β-glucuronidase (0.5 ml) at 37 °C for 24 h. After the hydrolysis, this solution was mixed with 2.0 ml of the internal standard solution and an aliquot of the solution was chromatographed.

<table>
<thead>
<tr>
<th>Substance</th>
<th>k(^a)</th>
<th>(t)(^b) (min)</th>
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</thead>
<tbody>
<tr>
<td>Solvent front ((t_0))</td>
<td>0</td>
<td>1.1</td>
</tr>
<tr>
<td>NAPAG</td>
<td>0.896</td>
<td>2.2</td>
</tr>
<tr>
<td>NAPAS</td>
<td>2.298</td>
<td>3.7</td>
</tr>
<tr>
<td>NAPA</td>
<td>2.878</td>
<td>4.4</td>
</tr>
<tr>
<td>Theophylline</td>
<td>3.606</td>
<td>5.2</td>
</tr>
</tbody>
</table>

\(a\) \(k' = (t - t_0) / t_0\), capacity factor \hspace{1cm} \(b\) Retention time.
RESULTS AND DISCUSSION

NAPAG, NAPAS, and NAPA were analyzed after modifying the method described by Jung and Zafar. The conditions finally chosen utilized a microparticulated bonded reverse-phase packing with a mobile phase consisting of acetonitrile and 0.05 M sodium sulfate (1:9, volume ratio). A detector wavelength of 254 nm was selected. The retention times and capacity factors for NAPAG, NAPAS, NAPA, and theophylline are shown in Table I. All compounds tested have retention times different from theophylline and thus do not interfere. Calibration curves were prepared by using blank diluted urine to which known amounts of NAPA and NAPAS were added. Peak area ratios of NAPA and NAPAS to the internal standard, theophylline, were used to construct the calibration curves. Figure 1 shows the calibration curves for NAPA and NAPAS. The ratio of peak area of each drug to the internal standard were linearly related to drug concentration. Similar linear relationship was obtained in the case of calibration curve for liberated NAPA after the hydrolysis of NAPAS with β-glucuronidase/arylsulfatase. The statistical parameters of the calibration curves are summarized in Table II; the correlation coefficients were 0.999 and the intercepts were negligible. The precision of the assay for NAPAS and NAPA was determined by the analysis of 4 samples at each concentration. From these results, it was confirmed that NAPAS could be determined as NAPA after the hydrolysis of NAPAS with β-glucuronidase/arylsulfatase.

Rabbits were dosed intravenously with NAPA (30 mg/kg) and urine was collected. Urine was diluted to 10—40 times its original volume with acetate buffer solution. Chromatograms obtained following incubation of diluted urine with each of the enzymes are shown in Fig. 2. NAPAG, NAPAS, NAPA, and internal standard peaks were well resolved with retention times of 2.2, 3.7, 4.4, and 5.2 min, respectively, and no interfering peaks appeared in the blank diluted urine. The NAPA peak was not found in Fig. 2(B). β-Glucuronidase used was selective in the action as shown in Fig. 2(C). Addition of β-glucuronidase had little effect on the NAPAS peak. In Fig. 2(D), incubation with β-glucuronidase/arylsulfatase essentially removed the NAPAG and NAPAS components.

From these results, it appears that calibration curves for NAPAG and NAPAS in rabbit urine can be prepared without use of authentic compounds in the HPLC method. As shown in Fig. 3, the plot of the peak area ratio of NAPAG to the internal standard (Fig. 2(B)) against NAPA concentration after hydrolysis with β-glucuronidase (Fig. 2(C)) was linear and passed through the origin. In Fig. 4, a linear tendency was observed.

![Figure 1. Calibration Curves for NAPA and NAPAS](image)

**Table II. Statistical Parameters of the Calibration Curves for the Assay of NAPAS and NAPA in Rabbit Urine**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Correlation coefficient ( r )</th>
<th>Regression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Intercept</td>
</tr>
<tr>
<td>NAPAS</td>
<td>0.999</td>
<td>0.008</td>
</tr>
<tr>
<td>NAPA</td>
<td>0.999</td>
<td>0.008</td>
</tr>
<tr>
<td>NAPA(^a)</td>
<td>0.999</td>
<td>0.002</td>
</tr>
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\(^a\) NAPAS was analysed as NAPA after the hydrolysis of the sample with β-glucuronidase/arylsulfatase.
FIG. 2. Chromatograms of NAPAG, NAPAS and NAPA
Conditions for HPLC were given in Methods. (A) Blank diluted urine spiked with theophylline (internal standard). (B) Diluted urine sample obtained after intravenous administration of NAPA (30 mg/kg), with theophylline added as an internal standard. (C) Diluted urine sample obtained after intravenous administration of NAPA (30 mg/kg) was hydrolyzed with β-glucuronidase. (D) Diluted urine sample obtained after intravenous administration of NAPA (30 mg/kg) was hydrolyzed with β-glucuronidase/aryl-sulfatase. a) NAPAG. b) NAPAS. c) NAPA. d) Theophylline.

in the plot of the peak area ratio of NAPAS to the internal standard (Fig. 2(C)) against NAPA concentration calculated by the difference between the peak area ratio of NAPA in Fig. 2(C)

FIG. 3. Calibration Curve for NAPAG Prepared after the Hydrolysis of Urine Sample with β-Glucuronidase
Correlation coefficient (r): 0.999.

FIG. 4. Calibration Curve for NAPAS Prepared after the Hydrolysis of Urine Sample with β-Glucuronidase and β-Glucuronidase/Arylsulfatase
Correlation coefficient (r): 0.927.
and that in Fig. 2(D). It appears that a small peak area of NAPAS causes the variance of the calibration curve. Probably the increase of peak area ratio of NAPAS will improve the linearity of the calibration curve.

Thus, once the calibration curve has been prepared following the enzyme hydrolysis of NAPAG and NAPAS, then the concentration of NAPAG and NAPAS in the sample solution can be calculated from the peak of NAPAG and NAPAS, respectively. This approach has the obvious advantage of constructing a calibration curve for the individual conjugate without preparing authentic compounds. For the spectrophotometric and fluorophotometric methods, the following assumptions were made: the extent of unchanged NAPA can be obtained from direct extraction with no incubation, both unchanged NAPA and hydrolyzed glucuronide can be obtained from $\beta$-glucuronidase incubation and unchanged NAPA, hydrolysed glucuronide and hydrolysed sulfate can be obtained from $\beta$-glucuronidase/aryl sulfatase incubation. Thus NAPAG and NAPAS can be calculated by difference. On the other hand, the HPLC procedure is available for simultaneous assay of NAPA, NAPAG and NAPAS.

In conclusion, the method presented here is simple and has the advantage that pure standards of the individual NAPA conjugates are not required. Furthermore, this method may be helpful in the HPLC determination of glucuronide and sulfate of drug. It is possible to identify each drug conjugate peak in chromatograms without authentic compounds by the use of data reported previously.

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REFERENCES