METABOLISM OF PHTHALIDYL THEOPHYLLINE IN RAT LIVER

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Phthalidyl theophylline (PH-TH) in rat liver was metabolized by a hydrolase to theophylline (TH) and 2-carboxybenzaldehyde, and the latter was further metabolized to 2-hydroxymethylbenzoic acid by an nicotinamide adenine dinucleotide-dependent reductase. The hydrolase could be strongly inhibited by acetazolamide, therefore, urinary excretion of PH-TH metabolites was significantly retarded when PH-TH and acetazolamide were coadministered to rats. The results from in vivo experiments suggest that PH-TH was efficiently absorbed from the gastrointestinal and metabolized extensively by the liver.

Keywords — phthalidyl theophylline (PH-TH); rat liver metabolism; urinary excretion; acetazolamide inhibition.

INTRODUCTION

Theophylline (TH) is an orally effective bronchodilator widely used for the treatment of asthma.1) Since TH is a fairly water-soluble polar molecule and its duration of action is short, several attempts have been made to increase its lipophilicity and modify its bioavailability.2) In the present study, when the phthalidyl moiety was introduced to TH as a leaving group, the water solubility of TH decreased 100-fold. Phthalidyl theophylline (PH-TH) was found to be stable in aqueous media in the physiological pH range but was readily hydrolyzed in animal tissues, liberating TH. According to Clayton et al.3) the 3-acyloxy function of the phthalidyl moiety of talampicilline (phthalidyl D-α-amino-3-benzylpenicilline) was hydrolyzed by a plasma esterase and D-α-amino-3-benzylpenicilline was produced in the blood. However, the enzyme that liberates the parent compound from PH-TH differs from that of talampicilline. This report describes the degradation profile of PH-TH in aqueous media, the metabolism of PH-TH in rat tissues and in isolated hepatocytes, and the characteristics of enzymes involved in PH-TH metabolism in the liver.

MATERIALS AND METHODS

Chemicals — TH and 2-hydroxymethylbenzoic acid were purchased from Nakarai Chem. Co., phthalide, 2-carboxybenzaldehyde and TPCK (tosylamino-2-phenyl chloromethyl ketone) from Aldrich Chem. Co., DIFP (diisopropyl fluorophosphate), PMSF (phenylmethyl sulfonyl fluoride), NEM (N-ethylmaleimide), eserine, parathione, acetazolamide and α-chymotrypsin (bovine pancreas) from Sigma. To synthesize PH-TH, 1.8 g TH, 2.1 g bromophthalalide and 1.3 ml triethylamine were dissolved in 20 ml dimethylformamide and the mixture was maintained at 25 °C for 18 h. The crystals that formed were removed by filtration and the filtrate was evaporated to dryness in vacuo. The residue was dissolved in warm CHCl₃ and crystallized from the solvent. mp 235 °C, UV λmax nm (ε): 276 (9067). Anal. Caled for C₁₉H₁₂N₄·O₄: C, 57.69; H, 3.87; N, 17.94. Found: C, 57.09; H, 3.84; N, 17.92.

Animals — Ten-week-old male Wistar rats, weighing 300-320 g, were used. The rats were maintained at 25 °C on a cycle of 12 h light and 12 h dark with free access to laboratory chow and water.

Liver Extracts — The livers of rats under ether anesthesia were perfused with saline and then excised. Livers were homogenized with 10 volumes of 50 mM phosphate buffer, pH 7.4, centrifuged first for 10 min at 20000 × g, then for 60 min at 105000 × g with a Hitachi SC20BA centrifuge at 4 °C. The supernatant was concentrated 12-fold with Centriflo CF 25 (Amicon Corp). A 6-ml portion was loaded on a Sephadex G-150 superfine column (2.6 × 32 cm), which had been equilibrated with 50 mM phosphate buffer, pH 6.8, containing 100 mM NaCl, and eluted with the same buffer at 4 °C. Of the 5-ml fractions collected, fraction No. 22 to 26 which displayed PH-TH hydrolase activity were combined and used to study PH-TH hydrolase kinetics.
Isolated Hepatocytes — The liver cells were isolated according to Moldéus et al. as described previously. The viability of the hepatocytes, according to LDH latency test, was 96–98%. PH-TH in 0.5 mM was incubated with 1.5 × 10⁶ cells/ml isolated hepatocytes suspended in Krebs-Henseleit buffer, pH 7.4, containing 1% bovine serum albumin and other additives at 37 °C under an atmosphere of O₂:CO₂ (95:5). After 10 to 40 min of incubation, 2-ml aliquots of the reaction mixture were transferred to a test tube with 0.5 ml of 0.6 N HClO₄ containing 100 μg acetonilide as an internal standard for high performance liquid chromatography (HPLC), and PH-TH and its metabolites were extracted with 4 ml ethyl acetate with vigorous shaking for 10 min. A portion of the organic layer was dried with Na₂SO₄ and evaporated in vacuo. The residue was then dissolved in the mobile phase of HPLC and subjected to HPLC analysis.

PH-TH Hydrolysis Assay — PH-TH (0.5 mM) in 50 mM phosphate buffer, pH 8.0, and the enzyme preparation were incubated at 37 °C with shaking. At time intervals, a 2-ml portion was taken into a tube with 0.5 ml 0.6 N HClO₄ containing 100 μg acetonilide (internal standard) and extracted with 4 ml ethyl acetate as described above. A portion of the solvent was evaporated, then the residue was dissolved in the mobile phase for HPLC and chromatographed on a Nucleosil 10 C₁₈ column.

2-Carboxybenzaldehyde Reductase Assay — Liver extracts and 0.5 mM 2-carboxybenzaldehyde were incubated at 37 °C with shaking in phosphate buffer, pH 7.0, in the presence of 2 mM nicotinamide adenine dinucleotide (NADH). After 10 min of incubation, a 2-ml portion was transferred to a test tube with 0.5 ml 0.4 N HClO₄ containing 250 μg benzoic acid (internal standard) and 4 ml ethyl acetate. The tube was vigorously shaken for 10 min, centrifuged for 5 min at 2000 rpm, and then the organic layer was subjected to HPLC analysis.

HPLC — HPLC was performed on a Nucleosil 10 C₁₈ column (0.4 × 30 cm) with 50 mM KH₂PO₄ (pH 2.4)–CH₃CN (3:1) as the mobile phase. PH-TH and its metabolites were detected at a wave length of 262 nm using a Jasco TWINCLE liquid chromatograph equipped with a variable-wavelength ultraviolet (UV) detector (Japan Spectroscopic Co.). The retention time for PH-TH, TH, 2-carboxybenzaldehyde, 2-

hydroxymethylbenzoic acid, acetonilide and benzoic acid were 10.8, 3.6, 5.5, 5.1, 7.6 and 9.3 min, respectively, at a flow rate of 1.0 ml/min at 25 °C.

Urinary Excretion — PH-TH suspended in 0.5% carboxymethyl (CM)-cellulose (10 mg/ml) was administered p.o. to rats at a dosage of 15 mg/kg. The rats (average body wt.: 310 g) were placed in a Ballman cage Model KN-326 and urine-collecting adaptors were attached to their penises. The rats were forced to void urine using ethyl ether at 2-h intervals and 2-ml portions of urine were analyzed by extracting the metabolites with 4 ml ethyl acetate.

RESULTS AND DISCUSSION

Degradation of PH-TH in Aqueous Media

PH-TH is stable in buffers at acidic or neutral pH. When 1 mM PH-TH was maintained at 37 °C at various pH values, the rate of degradation followed apparent first-order kinetics with quantitative formation of TH and 2-carboxybenzaldehyde. The rate constants at various pH are shown in Table I. Our results suggest that TH could be generated from PH-TH very slowly at physiological pH in various tissues.

Degradation of PH-TH in Tissue Homogenates

When 0.5 mM PH-TH was incubated with homogenates of various rat tissues, TH and 2-carboxybenzaldehyde were produced as the major metabolites. PH-TH was resistant to plasma esterase but the 1-lacton function of phthalidyl moiety was readily hydrolyzed by tissue enzymes. The specific activity of the hydrolytic enzyme was highest in the liver, which

<table>
<thead>
<tr>
<th>pH</th>
<th>K × 10⁻³ (h⁻¹)</th>
</tr>
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<tbody>
<tr>
<td>4</td>
<td>0.3</td>
</tr>
<tr>
<td>5</td>
<td>0.5</td>
</tr>
<tr>
<td>6</td>
<td>1.8</td>
</tr>
<tr>
<td>7</td>
<td>10.8</td>
</tr>
<tr>
<td>8</td>
<td>215.9</td>
</tr>
<tr>
<td>9</td>
<td>1661</td>
</tr>
</tbody>
</table>

PH-TH (50 μg/ml) was dissolved in 0.1 M buffers (μ = 0.5) of various pH at 37 °C and the PH-TH remaining at 37 °C was periodically determined by HPLC. pH 4 and 5: acetate buffer; pH 5, 6 and 7: phosphate buffer; pH 8 and 9: borate buffer.
TABLE II. *PH-TH* Hydrolase Activities in Rat Tissue Homogenates

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Hydrolase activity (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>2.25 ± 0.18</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.64 ± 0.02</td>
</tr>
<tr>
<td>Small intestine</td>
<td>0.97 ± 0.23</td>
</tr>
<tr>
<td>Heart</td>
<td>0.58 ± 0.03</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.36 ± 0.03</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.25 ± 0.02</td>
</tr>
<tr>
<td>Lung</td>
<td>0.21 ± 0.03</td>
</tr>
<tr>
<td>Brain</td>
<td>0.13 ± 0.03</td>
</tr>
</tbody>
</table>

*Mean ± S.E. (n = 3).* Tissues obtained from 10-week-old male Wistar rats were homogenized with 50 mM phosphate buffer, pH 7.0, and then incubated with 0.5 mM *PH-TH* as substrate.

was followed by kidney and small intestines (Table II). The results indicated that a large portion of *PH-TH* absorbed from the gastrointestinal tract could be metabolized in the liver.

*Metabolism of *PH-TH* in Isolated Hepatocytes*

As shown in Fig. 1, the disappearance of *PH-TH* from the incubation medium was accompanied by quantitative formation of TH

![Graph showing metabolic pathways](image)

**FIG. 1. Metabolism of *PH-TH* in Isolated Rat Hepatocytes**

*PH-TH* (0.5 mM) was incubated with 1.5 × 10⁶ cells/ml isolated hepatocyte suspensions at 37 °C. At the specified intervals, *PH-TH* and metabolites were extracted with ethyl acetate and determined by HPLC as described in the text.

TABLE III. Effect of Enzyme Inhibitors on *PH-TH* Hydrolase

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibition (% control)</th>
<th>Inhibitor</th>
<th>Inhibition (% control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIFP</td>
<td>2</td>
<td>Acetazolamide</td>
<td>100</td>
</tr>
<tr>
<td>PMSF</td>
<td>0</td>
<td>Sulfanilamide</td>
<td>83</td>
</tr>
<tr>
<td>TPCKa)</td>
<td>0</td>
<td>Eserine</td>
<td>1</td>
</tr>
<tr>
<td>NEM</td>
<td>45</td>
<td>Parathionine</td>
<td>28</td>
</tr>
</tbody>
</table>

*PH-TH* hydrolase activity in 105000 × g supernatant fraction of rat liver extracts was determined with 0.5 mM *PH-TH* as substrate in the presence or absence of 1 mM inhibitors. *a)* Because of solubility, 0.065 mM TPCK was used.
and, 2-carboxybenzaldehyde and 2-hydroxymethylbenzoic acid; the proportion of 2-carboxybenzaldehyde/2-hydroxymethylbenzoic acid decreased when the incubation time was prolonged. Subsequent studies with 105,000 × g supernatant fraction of liver extract revealed that 2-carboxybenzaldehyde was enzymically reduced to 2-hydroxymethylbenzoic acid in the presence of NADH. Nicotinamide adenine dinucleotide phosphate (NADPH) was about 50% as effective as NADH. A small amount of phthalide was formed from α-hydroxymethyl benzoic acid during extraction.

**PH-TH Metabolizing Enzymes**

**PH-TH Hydrolase** — PH-TH hydrolase activity found in liver homogenate was quantitatively recovered in the 105,000 × g supernatant fraction. The molecular weight of the enzyme was estimated to be 25,000 by Sephadex G-150 column chromatography in comparison with the gel filtration profile of enzyme markers (Dalton standard MS-II). The specific activity of PH-TH hydrolase fractionated by the Sephadex column was 70 nmol/min/mg protein and the $K_m$ value for PH-TH was about $6 \times 10^{-4}$ M. The enzyme was strongly inhibited by sulfonamides and to a lesser extent by SH inhibitors (Table III): 50% inhibition was attained by as little as $10^{-6}$ M acetazolamide in a separate experiment. Although chymotrypsin is known to hydrolyze several cyclic esters, its activity is not inhibited by acetazolamide. Furthermore, DIFP, PMSF and TPCK, which inhibited bovine pancreatic chymotrypsin, did not affect PH-TH hydrolase activity at 2 mM concentration (data not shown). Therefore, it appears that PH-TH hydrolase is a different enzyme from chymotrypsin.

**2-Carboxybenzaldehyde Reductase** — The enzyme which reduces 2-carboxybenzaldehyde to 2-hydroxymethylbenzoic acid emerged from a Sephadex G-150 column in a fraction corre-

![FIG. 2. Gel Chromatography of Liver Extracts](image)

The 105,000 × g supernatant of liver extracts was concentrated 12-fold and the 6-ml portion (protein: 186 mg) was chromatographed on a Sephadex G-150 column (2.6 × 32 cm) as described in the text. PH-TH hydrolizing activity ($\bigcirc \quad \bigcirc$), 2-carboxybenzaldehyde reducing activity ($\bullet \quad \bullet$) and protein concentration ($\Delta \quad \Delta$) in the fraction are shown.
sponding to a molecular weight of approximately 70000 (Fig. 2). The activity of the partially purified enzyme toward 3- or 4-carboxybenzaldehyde was negligibly small. It was also found that pyrazole and o-phenanthroline, which inhibit alcohol dehydrogenase,9 or barbital, an inhibitor of aldehyde reductase,9 did not inhibit 2-carboxybenzaldehyde reductase. The same enzyme activity was found in rat liver slices by Shiobara and Ogiso,10 but has not been classified as any of the known reductases or dehydrogenases.

**Urinary Excretion of PH-TH Metabolites**

Since acetazolamide strongly inhibited PH-TH hydrolase, simultaneous administration of acetazolamide was expected to alter the excretion profile of PH-TH metabolites. As shown in Fig. 3, excretion of TH and 2-hydroxymethylbenzoic acid was retarded significantly though their cumulative amounts were similar whether or not acetazolamide was coadministered. Approximately 2.1 mg of 2-hydroxymethylbenzoic acid was excreted into urine within 24 h per rat in the control experiment. Since 4.65 mg of PH-TH was orally administered to a rat, more than 90% of 2-hydroxymethylbenzoic acid formed from PH-TH was recovered in urine. This means that PH-TH was efficiently absorbed from gastrointestinal tracts and liberated 2-carboxybenzaldehyde in the liver which was further metabolized to 2-hydroxymethylbenzoic acid. Unlike the in vitro experiments, 2-carboxybenzaldehyde was not detected in the urine, probably because 2-carboxybenzaldehyde is very rapidly metabolized to 2-hydroxymethylbenzoic acid in vivo.

Several sustained-release TH preparations have been produced to improve its short duration of action,11,12 and the use of PH-TH in combination with acetazolamide may meet this need. It should also be noted that PH-TH could display different tissue distribution characteristics from TH. Different disposition of TH via PH-TH may lead to a finding of still undiscovered pharmacological action of TH.13

**REFERENCES**


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