Factors Affecting Glucuronidation Activity in Caco-2 Cells

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Summary: Presystemic intestinal metabolism reduces the intestinal absorption and bioavailability of orally administered drugs. The factors affecting glucuronidation activity in Caco-2 cells seeded in Transwell (4.7 cm²) require clarification to establish an in-vitro system to assess intestinal glucuronidation metabolism for novel drug development.

α-Naphthol (α-NA), a substrate for UGT1A6 in Caco-2 cells, has often been used as a model substrate for glucuronidation. α-Naphthol glucuronidation activity increased from 7 to 21 culture days after seeding in Transwell and stabilized after 21 days. The higher the passage number of Caco-2 cells, the larger the variance of glucuronidation activity, but apical pH did not significantly influence glucuronidation in the pH range of 5.5 to 7.4. When the passage number ranged from 83 to 159, Km,app was highest at passage number 130. In contrast, Vmax,app increased with the passage number. This indicates that the kinetic parameters for glucuronidation in Caco-2 cells are dependent on the passage number of the cells. These results should be useful for establishing the experimental conditions for Caco-2 cells that predict intestinal glucuronidation activity in vivo.

Key words: glucuronidation; glucuronide transport; Caco-2 cells; passage number; kinetic parameters

Introduction

The liver is a primary organ for drug metabolism. After oral dosing, there is a contribution to bioavailability and the first-pass effect. However, intestinal metabolism also has an impact on bioavailability and the first-pass effect for orally administered drugs19 because they have to pass through intestinal tissue to enter the systemic circulation, and drugs are attacked by enzymes during the absorption process.20 Thus, the impact of intestinal drug metabolism on absorption cannot be ignored, but no experimental method to assess intestinal metabolism has been established. In the 1980s, Noordhoek et al. reported kinetic studies on the intestinal oxidation metabolism by P450,3,4 and recently, studies of intestinal drug metabolism by P450s (CYPs) have increased.15,16 However, there have been few studies on intestinal glucuronidation, a type of conjugation metabolism.

Caco-2 cells have been used as a tool to study intestinal transport (absorption),19 but glucuronidation metabolism in Caco-2 cells has not been studied kinetically. Once the experimental conditions to assess drug glucuronidation metabolism in Caco-2 cells have been established, they should be applicable to high-throughput screening for the development of orally active drugs. At present, however, the factors affecting glucuronidation activity in Caco-2 cells are unclear. For example, it is well known that pH on the mucosal side of the human intestinal tract ranges from approximately 5.5 to 7.0, but the pH effect on glucuronidation activity is unclear. The dependency of glucuronidation activity on the passage number is also unknown, although it was reported that α-naphthol glucuronidation in a microsomal fraction prepared from Caco-2 cells was higher in Caco-2 cells at passage number 121 compared to passage number 78. Furthermore, the influence of culture days on glucuronidation activity remains to be clarified. Therefore, in this report, we have studied the following factors: culture days after seeding, apical side pH and passage number. α-Naphthol was used as the model substrate, because an inverse correlation was observed between intestinal metabolism and the absorption of α-naphthol in rats,10 and Caco-2 cells had UGT1A6 for α-naphthol glucuronidation.5,9

Materials and Methods

α-Naphthol and α-naphthyl β-D-glucuronide were
Factors Affecting Glucuronidation in Caco-2 Cells

Glucuronidation metabolism in Caco-2 cells: Caco-2 cells (American Type Culture Collection, MD, USA) were seeded onto Transwell inserts with 1 × 10⁶ cells in DMEM supplemented with 10% FBS, 1% MEM non-essential amino acids, 100 U/mL penicillin and 100 μg/mL streptomycin, pH 7.4. Cells were given fresh culture medium every day and were maintained in an atmosphere of 5% CO₂/95% air at 37°C for 21 to 28 days. The medium in the Transwell was removed and the remaining medium was washed out twice with buffer. α-Naphthol solution (1.5 mL)³⁰ at pH 7.4 and pre-warmed to 37°C was added to the apical side, and buffer (2.7 mL)³⁰ at pH 7.4 and pre-warmed to 37°C was added to the basal side. To study the apical pH effects, α-naphthol solution (1.5 mL) at pH 5.5 or 6.5, pre-warmed to 37°C, was added to the apical side. Two hundred μL was periodically sampled from the apical and basal sides over 60 min, and mixed with 200 μL solution of 0.1 mM 2,4-dihydroxy benzoic acid dissolved in 10% perchloric acid in a microtube. For each sample removed, 200 μL of α-naphthol solution and buffer was added to the apical and basal sides, respectively. The mixture in the microtube was centrifuged at 11,000 x g for 5 min using a KM-15200 benchtop centrifuge (Kubota, Tokyo, Japan). The resultant supernatant was applied in the HPLC assay. After 60 min, Caco-2 cells in the Transwell were washed twice with cold buffer, and 1.0 mL and 1.5 mL solution of 0.1 mM 2, 4-dihydroxy benzoic acid dissolved in 10% perchloric acid and 0.1% Triton X-100 were added to the apical and basal sides, respectively. After 30 min, the mixture was centrifuged at 11,000 x g for 5 min using a KM-14500 benchtop centrifuge (Kubota, Tokyo, Japan). The resultant supernatant was applied to the HPLC to determine the α-naphthol glucuronide in Caco-2 cells. HPLC assay: α-Naphthol and α-naphthol glucuronide were determined according to a previously reported method.³¹ The HPLC system consisted of a pump (880-PU, Jasco Co, Tokyo, Japan) with a flow rate of 1.5 mL/min, a UV detector (875-UV, Jasco) set at 286 nm, an integrator (D-2500, Hitachi Ltd, Tokyo, Japan) and an ODS column (80T, Tosoh, Tokyo, Japan). The mobile phase was composed of 47% methanol, 0.5% acetic acid and 10 mL/L tetrahydroammonium bromide.

Data analysis: Metabolic clearance was calculated as:

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\text{Metabolic clearance} = \frac{X_{\text{met}}}{\text{AUC}_{\text{muc}}} \]

where \(X_{\text{met}}\) is the total amount of α-naphthol glucuronide in the apical and basal sides and in the cells. \(\text{AUC}_{\text{muc}}\) is the area under the concentration curve of α-naphthol on the apical side.

Statistical analysis of the data was performed using the Tukey multiple comparison post test following analysis of variance.

Apparent kinetic parameters (\(K_{m, \text{app}}\) and \(V_{\text{max,app}}\)) were obtained by fitting data to the Michaelis-Menten equation using a non-linear fitting program, MULTI.¹²

Results

Influence of culture days on α-naphthol glucuronidation: Figure 1 shows the influence of culture days on α-naphthol glucuronidation in Caco-2 cells with various passage numbers. In Caco-2 cells with a passage number of 78, glucuronidation activity was not dependent on the culture days. Meanwhile, in Caco-2 cells with a passage number of 131, the glucuronidation activity was remarkably dependent on the culture days.

Influence of passage number on glucuronidation activity: The influence of the passage number of Caco-2 cells on glucuronidation activity is summarized in Table 1. The passage number had no significant influence on metabolic clearance in Caco-2 cells cultured for 7 days. In contrast, metabolic clearance in Caco-2 cells cultured for 21 days significantly increased as the passage number increased. There was a tendency for metabolic clearance in Caco-2 cells cultured for 28 days to increase with the passage number, and a significant difference was observed between passage numbers 78 and 128.

p130 p.2 [100%]
Table 1. Influence of passage number on metabolic clearance ratio (clearance at passage (P) number/clearance at passage number 78).

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<td>P128</td>
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Data represent the mean ± S.E. (n = 3).

Fig. 2. Effect of apical side pH on glucuronidation activity in Caco-2 cells. Symbols: glucuronide detected in the basal side (hatched column), the apical side (closed column) and cells (open column). Data represent the means ± S.E. (n = 17).

Effect of apical side pH on glucuronidation activity: Figure 2 shows Caco-2 cell glucuronidation at apical side pHs of 5.5, 6.5 and 7.4. There was a slight tendency for glucuronidation activity to increase with the pH value, but it was not statistically significant. The fractions of glucuronide localized in cells, apical or basal sides to the total (sum of those) glucuronide were almost identical, and α-naphthol glucuronide was primarily transported to the basal side.

Effect of passage number on kinetic parameters: The glucuronidation rates at various concentrations of α-naphthol were studied in Caco-2 cells of various passage numbers. The Km,app and Vmax,app values versus passage number are shown in Figs. 3a and b, respectively. The profile of Km,app vs. passage number was bell shaped, and the highest value of Km,app (539.7 μM) was observed at passage number 130. On the other hand, the Vmax,app value was almost constant in passage numbers ranging from 83 (Vmax,app = 11.6 pmol/min/cm²) to 111 (Vmax,app = 13.7 pmol/min/cm²), and thereafter increased with the passage number.

Discussion
Dependency of glucuronidation activity and kinetic parameters on passage number: In passage numbers ranging from 83 to 159, Km,app at passage number 130 was the highest (Fig. 3a). On the other hand, Vmax,app
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increased with the passage number (Fig 3b). Abid et al. reported that α-naphthol glucuronidation in a microsomal fraction prepared from Caco-2 cells (UDP-glucuronotransferase 1α) was higher in cells at passage number 121 than 78. Our study supports this report, and expands the understanding of the kinetic characterization of glucuronidation activity in Caco-2 cells. In addition, this result indicates that caution is necessary in discussing kinetic parameter values obtained with Caco-2 cells, although Caco-2 cells are a useful tool to kinetically characterize drug glucuronidation metabolism. A study of the factors influencing kinetic parameters is the next target.

No significant effect of apical pH on glucuronidation: α-Naphthol glucuronidation was almost identical at any pH ranging from 5.5 to 7.4 (Fig. 2), indicating that glucuronidation activity is not significantly affected by apical side pH. α-Naphthol is almost unionized at pH ranging from 5.5 to 7.4, therefore, the membrane transport of α-Naphthol is not significantly affected by pH, and glucuronidation activity is not remarkably affected by apical pH ranging from 5.5 to 7.4. As it is well known that intraluminal pH ranges from around 5.5 to 7.0, the experiment using Caco-2 cells was performed at apical pH ranging from 5.5 to 7.4. The result indicates that apical pH around these values, which is the standard condition for Caco-2 cell experiments, does not remarkably affect glucuronidation activity.

More α-naphthol glucuronide was transported to the basal side than to the apical side. Apical pH ranging from 5.5 to 7.4 did not affect the transport of α-naphthol glucuronide, suggesting that its membrane transport was also not affected. Saxena and Henderson reported that α-naphthol glucuronide was transported by a multi-specific organic-ion transport system (MOAT4) in mouse L1210 cells. Therefore, the transport of more α-naphthol glucuronide to the basal side than to the apical side appeared to be due to the carrier-mediated transport of α-naphthol glucuronide across the basolateral membrane. Further experiments are required to clarify α-naphthol glucuronide transport.

In conclusion, α-naphthol glucuronidation activity in Caco-2 cells increased from 7 to 21 culture days after seeding in Transwell, but became constant after 21 days. Kinetic parameters (Km,app and Vmax,app) for α-naphthol glucuronidation were not constant in Caco-2 cells in passage numbers ranging from 83 to 159. Apical side pH did not significantly affect glucuronidation activity in the range of pH 5.5 to 7.4. This study highlighted factors affecting glucuronidation activity in Caco-2 cells. We will develop experiment conditions in vitro that reflect in vivo kinetic characteristics.

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