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Technical note

Evaluation of intestinal metabolism and absorption using the Ussing chamber system equipped with intestinal tissue from rats and dogs

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ABSTRACT

The purpose of this study was to evaluate the intestinal metabolism and absorption in a mini-Ussing chamber equipped with animal intestinal tissues, based on the transport index (TI). TI value was defined as the sum of drug amounts transported to the basal-side component (X^{corr}) and drug amounts accumulated in the tissue (T^{corr}), which are normalized by AUC of a drug in the apical compartment, as an index for drug absorption. Midazolam was used as a test compound for the evaluation of intestinal metabolism and absorption. The metabolite formulation of midazolam was observed in both rats and dogs. Ketoconazole inhibited the intestinal metabolism of midazolam in rats and improved its intestinal absorption to a statistically significant extent. Therefore, the mini-Ussing chamber, equipped with animal intestinal tissues, showed potential to use the evaluation of the intestinal metabolism and absorption, including the assessment of species differences.

1. Introduction

Prediction of oral bioavailability is one of the key factors in the drug development stage. Rats and dogs are often used in preclinical studies in order to characterize pharmacokinetic (PK) properties of drug candidates prior to clinical studies. However, the PK properties of drugs in rats and dogs are often different from those in humans, due to species differences. For example, the expression of drug metabolic enzymes in the small intestine such as CYP3A was quite different among animals. Regarding species difference in intestinal CYP3A metabolism, midazolam exhibited the highest intestinal activity in the monkey, followed by humans > rats [1]. It has been reported that intestinal intrinsic clearance values for human CYP3A substrates in rats and dogs appeared to be lower for most of the compounds, and showed moderate correlation with those in humans [2]. The species differences should be considered to fill a gap in PK properties among species. Bioavailability is estimated to be the multiple of: the fraction of a dose absorbed (F_a); the fraction of a drug passing though the gut wall without metabolism (F_g); and the fraction of a drug passing through the liver (F_h). In order to predict each fraction in humans, there are several *in vitro* and *ex vivo* experimental systems. Regarding the prediction of F_a in humans, we previously developed an evaluation method using a mini-Ussing chamber, equipped with intestinal tissues from animals and humans [3,4]. The transport index (TI) values of three drugs with different levels of membrane permeability (i.e. FD-4, metoprolol, and atenolol) in small intestinal tissues in rats and dogs showed a good correlation with those in humans; however, the correlation of TI values in monkeys was lower compared to rats and dogs [4]. In this system, the TI values are used to predict the fraction of a dose absorbed (F_a). An advantage of this system is to take into account not only the change in drug solubility during incubations, but also the tissue accumulation of a drug. However, the usefulness of the intestinal metabolism in the mini-Ussing chamber has not yet been investigated.

Midazolam is a typical substrate of CYP3A in humans [5] and animals [6]. The oral bioavailability of midazolam ranged from 24% to 46% in humans [7–9]. The $F_a * F_g$ and F_h were estimated to be 57% and 56%, respectively, in clinical studies [8]. This indicates that bioavailability of midazolam is affected by both intestinal and hepatic first-pass effects. Regarding absorption, midazolam was classified in the BCS Class I, namely a highly soluble and permeable drug class, based on the

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Loc-I-Gut approach [10–12]. To the best of our knowledge, there is no report of midazolam indicating its specificity of intestinal drug transporters. Midazolam exhibited no effects on the P-gp-mediated transports [13]. Therefore, it is expected that midazolam is absorbed *via* passive diffusion.

In this study, intestinal metabolism of midazolam was examined using a mini-Ussing chamber system, equipped with intestinal tissues in rats and dogs. Intestinal metabolism and absorption of midazolam was assessed based on the TI values.

2. Materials and methods

2.1. Materials

Midazolam, spiperone, 1′- and 4-hydroxymidazolam (1-OH and 4-OH, respectively), and ketoconazole were purchased from Sigma-Aldrich (Boston, MA, USA). All other reagents used were of the highest purity or HPLC-grade.

2.2. Animal intestinal tissues

Animal jejunum tissues were obtained from male Sprague-Dawley (SD) rats and male beagle dogs. Our investigations were performed after approval by our local ethical committee at Otsuka Pharmaceutical Co., Ltd. and were in accordance with *"Principles of Laboratory Animal Care (NIH publication # 85 – 23)"*. The isolated tissues were placed in an ice-cold transport buffer immediately after removal from each animal. The transport buffer (pH = 7.4) was composed of: 128 mM NaCl; 5.1 mM KCl; 1.4 mM $CaCl_2$; 1.3 mM $MgSO_4$; 21 mM $NaHCO_3$; 1.3 mM KH_2PO_4; and 10 mM NaH_2PO_4. The buffer including the isolated tissues was used to the laboratory within 10 min under the ice cold.

2.3. Transport experiment in mini-Ussing chamber

The mini-Ussing chamber system, equipped with intestinal tissues from animals, was prepared according to the method reported previously [3,4]. Isolated intestinal tissues, in which the muscle layer was removed with tweezers, were mounted vertically in mini-Ussing chambers. The available area for the permeation study was 0.07 cm^2. The volume of each side was 1.35 mL, and the solution temperature was maintained at 37 ˚C in a water-jacketed reservoir. The solutions used for pre-incubation on apical and basal sides were as follows: for apical, the transport buffer containing 5 mM mannitol; for basal, the transport buffer containing 5 mM D-glucose and 1% (w/v) bovine serum albumin. The system was maintained by continuously infusing 95% O_2/5% CO_2 gas, before and during the transport experiments. The concentrations of midazolam in the apical side were set at 50 μg/mL in DMSO (final concentration; 0.5%). The transport study was performed for 2 h according to the report by Nejdfors et al. [14]. The apical-side buffer (10 μL) was collected at: 0.083 h; 0.25 h; 0.5 h; 1 h; 1.5 h; and 2 h. The basal-side buffer (600 μL) was collected at: 0.5 h; 1 h; 1.5 h; and 2 h. The collected buffer from each side was replaced with an equivalent volume of fresh buffer at each sampling point. After collecting the final sample, the tissues were homogenized with 1 mL basal transport buffer under ice-cold condition to determine the accumulated amount of drugs.

2.4. Calculation of transport index

In the mini-Ussing chamber system, the TI values were used to estimate the permeability based on the actual drug concentration in the apical compartment. TI value was defined to be the sum of the percent of the dose transported and tissue-accumulated corrected by the AUC value of the parent drug in the apical compartment based on the mass balance of tested compound (Eq. (1)).

$$TI = X^{corr} + T^{corr} \tag{1}$$

where X^{corr} and T^{corr} are the percent of the dose transported to the basal side compartment and that of the dose accumulated in the tissue, respectively, which were corrected by the AUC of midazolam in the apical compartment of drugs (Eqs. (2) and (3)).

$$X^{corr} = \left[\frac{2\cdot(C_{apical}^{t_0}\cdot t_3)\cdot(C_{basal}^{t_3}\cdot V_{basal})}{\sum_{i=1}^{3}((C_{apical}^{t_{i-1}} + C_{apical}^{t_i})\cdot(t_i - t_{i-1}))} + \sum_{i=4}^{6}\left(\frac{2\cdot(C_{apical}^{t_0}\cdot(t_6 - t_3))\cdot(C_{basal}^{t_i}\cdot V_{basal} - C_{basal}^{t_{i-1}}\cdot(V_{basal} - V_{sampled}))}{(C_{apical}^{t_{i-1}} + C_{apical}^{t_i})\cdot(t_i - t_{i-1})}\right)\right] \cdot \frac{1}{C_{apical}^{t_0}\cdot V_{apical}}\cdot 100(\%) \tag{2}$$

$$T^{corr} = \frac{2\cdot C_{apical}^{t_0}\cdot t_6\cdot T}{\sum_{i=1}^{6}(C_{apical}^{t_{i-1}} + C_{apical}^{t_i})\cdot(t_i - t_{i-1})}\cdot\frac{1}{C_{apical}^{t_0}\cdot V_{apical}}\cdot 100(\%) \tag{3}$$

where $C_{apical}^{t_i}$ and $C_{basal}^{t_i}$ mean the drug concentrations in the apical side and basal side at time t_i, respectively. V_{apical}, V_{basal} and $V_{sampled}$ represent the volumes of apical, basal compartments, both 1.35 mL, and sampled volume from basal compartment, 0.6 mL, respectively. T is the amount of the drug accumulated in the intestinal tissues in the end of transport study. Furthermore, t_0 to t_6 mean 0, 0.083, 0.25, 0.5, 1, 1.5 and 2 h, respectively.

2.5. Inhibitory study

Transport of midazolam using the Ussing chamber system was conducted according to the method described in Miyake et al. [3,4] in the presence and absence of ketoconazole. Ketoconazole was solved in DMSO (0.5%) and added to apical sides (final concentration, 50 μg/mL) at pre-incubation time. The basal-side buffer (600 μL) was collected at: 0.5 h; 1 h; 1.5 h; and 2 h. The collected buffer from each side was replaced with an equivalent volume of fresh buffer at each sampling point. After collecting the final sample, the tissues were homogenized with 1 mL basal transport buffer under ice-cold conditions to determine the accumulated amount of midazolam and its metabolites.

2.6. Analytical method

Midazolam, 1-OH, and 4-OH were quantified in the multiple reaction monitoring (MRM) mode with the LC-MS-MS system (Quattro-Micro, Waters, Milford, USA), where spiperone is used as an internal standard (IS). Collected buffer (0.5 mL) was mixed with acetonitrile (0.1 mL) including IS and centrifuged at 14,000 rpm for 5 min. The supernatant was filtrated through a microfilter (pore size 0.2 μm; Millipore, Tokyo) and then subjected to the LC-MS-MS system. Analytical column was Acquity UPLC BEH C18 (2.1 × 50 mm, 1.7 μm; Waters). Mobile phase A was water:acetonitrile:formic acid (95:5:0.1, v/v). Mobile phase B was acetonitrile: formic acid (100:0.1, v/v). Flow rate was 0.5 mL/min. The gradient condition for elution was as follows: 0–0% B (0.0–0.5 min); 100% B (0.5–4.7 min); and 0% B (4.7–5.5 min). The mode for electrospray ionization (ESI) and the transition of *m/z*, namely *m/z* of the protonated/deprotonated molecular ion and predominant production, are summarized in Table 1. The range of the standard curve was 5–5000 ng/mL. The coefficient of variation for the standard curve ranged from 0.1 to 20.0% and the accuracy was ranged from 0.1 to 20.0%.