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Quantification of intracellular and extracellular digoxin and ouabain by liquid chromatography/electrospray ionization tandem mass spectrometry

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ABSTRACT

A liquid chromatography/tandem mass spectrometry method for the determination of intracellular accumulation in addition to transcellular transport of digoxin and ouabain in renal epithelial HK-2 cells was developed. The solid-phase extraction Bond Elut® C18 (100 mg/1 mL) cartridge was used for the extraction of digoxin and ouabain from extracellular (medium) and intracellular (cell lysate) matrices. Chromatographic separation was performed on a CAPCELL PAK C18 MGII column (2.0 mm × 150 mm, 5 μm). This method covered a linear range of 0.5–1000 ng/mL of concentrations in medium and 0.5–1000 ng of concentrations in cell lysate for digoxin and ouabain. The intra-day precision and inter-day precision of analysis were less than 11.9%, and the accuracy was within ±11.6%. The total run time was 16 min. Our method was successfully applied to the transport experiments of digoxin and ouabain by HK-2 cell monolayers.

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1. Introduction

Digoxin is a cardiac glycoside and is clinically used for the treatment of heart failure and cardiac arrhythmias and reduces the rate of hospitalizations [1]. For the safe and effective treatment, it is important to perform therapeutic drug monitoring of digoxin because digoxin has a narrow therapeutic window. Digoxin is mainly excreted into urine. Renal dysfunction is one of risk factors for increasing the plasma concentration of digoxin, resulting high incidence of adverse events [2].

It is reported the contribution of membrane transporters to the renal handling of digoxin [3,4]. Solute carrier (SLC) O4C1 (organic anion transporting polypeptide (OATP) 4C1) and ABCB1 (P-glycoprotein) are involved in the renal tubular secretion of digoxin. OATP4C1 is a member of SLCO (OATP) family and is localized in the basolateral membrane of the proximal tubule, and transports cardiac glycosides (digoxin and ouabain), thyroid hormones (triiodothyronine and thyroxine), cAMP, bile acids (chenodeoxycholic acid and glycocholic acid), estrone 3-sulfate, methotrexate, and sitagliptin [5–7]. P-glycoprotein is a best characterized efflux transporter and digoxin is a typical substrate of this transporter. Digoxin is therefore used as a probe drug for the evaluation of potential drug–drug interaction mediated by P-glycoprotein [8].

It has been demonstrated the transcellular transport of digoxin using intestinal epithelial Caco-2 cells or ABCB1-overexpressing MDCK cells [9,10]. However, little information is available about the transcellular transport of digoxin by renal epithelial cells, which are expressed both OATP4C1 and P-glycoprotein. HK-2 cell line is an *in vitro* model of human proximal tubules [11]. Jenkinson et al. [12] reported that this cell line retains both OATP4C1 and P-glycoprotein expression. To reveal the contribution of these transporters to the renal secretion of digoxin, it is useful to determine the digoxin levels not only extracellular component but also

Abbreviations: LC/MS/MS, liquid chromatography/tandem mass spectrometry; LLOQ, lower limit of quantification; OATP, organic anion transporting polypeptide; SLC, solute carrier; SRM, selected reaction monitoring.

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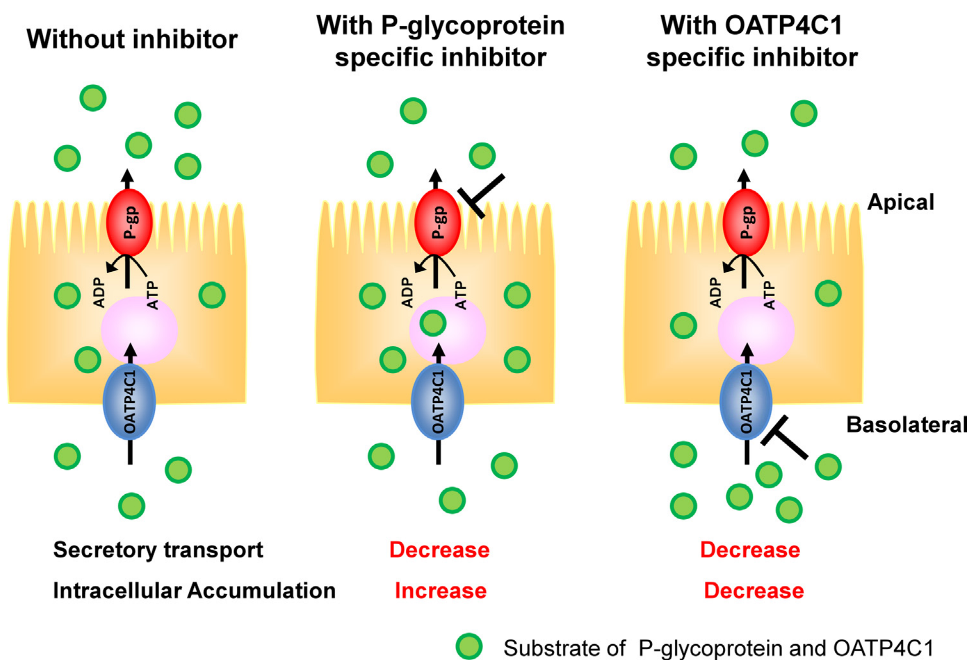

Fig. 1. Scheme of renal secretory drug transport with inhibitor of P-glycoprotein or OATP4C1.

intracellular component. When the secretory transport of digoxin in polarized cells is decreased, it is difficult to identify which transport process (basolateral uptake? or apical efflux?) is impaired. If information of intracellular levels of digoxin exists, we are able to clarify this issue (Fig. 1). When we use a specific inhibitor of P-glycoprotein, secretory transport of digoxin would be decreased, however intracellular accumulation would be increased. On the other hand, when we use a specific inhibitor of OATP4C1, intracellular accumulation as well as secretory transport would be decreased. Several groups reported the analytical methods for extracellular samples (culture medium or buffer) of digoxin by liquid chromatography/tandem mass spectrometry (LC/MS/MS) [13,14], however there are no reports of determination the intracellular samples by LC/MS/MS. In the present study, we developed the quantification method for the evaluation of intracellular accumulation in addition to transcellular transport of digoxin as well as ouabain in HK-2 cells.

2. Materials and methods

2.1. Chemicals

Digoxin and ouabain (purity ≥95%) was purchased from Sigma (St. Louis, MO). Dexamethasone (purity 97%) as an internal standard was obtained from Nacalai Tesque (Kyoto, Japan). HPLC-grade methanol and ammonium acetate were purchased from Wako (Osaka, Japan). Fig. 2 shows the chemical structures of digoxin, ouabain, and internal standard.

2.2. Chromatographic and mass spectrometric condition

Chromatographic separation was carried out using a Shimadzu Prominance 20A System (Shimadzu, Kyoto, Japan) with a Shiseido CAPCELL PAK C18 MGII column (2.0 mm × 150 mm, 5 μm). Mobile phase flow rate was 0.2 mL/min. Mobile phase A consisted of 20 mM ammonium acetate/methanol (80:20, v/v), and mobile phase B consisted of 20 mM ammonium acetate/methanol (10:90, v/v). Mobile phase B was increased from 0% to 100% in a linear gradient over 5 min and kept until 10 min. Then mobile phase B was decreased to 0% from 10 min to 11 min and kept until 16 min. The column temperature was maintained at 50 °C. The injection volume was 25 μL. The overall run time was 16 min.

Mass spectrometry was carried out on an API 3200 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA). Positive ionization electrospray mass spectrometry was performed. The ionspray voltage was set at 5500 V. The turbospray gas (N_2) probe was heated at 300 °C. Nitrogen was used as curtain gas, gas 1 and gas 2, and their flows were set to 25, 50, and 30 units, respectively. Unit mass resolution was set in both mass-resolving quadrupoles Q1 and Q3. Selected reaction monitoring (SRM) transitions monitored were m/z 798 to m/z 651 for digoxin, m/z 585 to m/z 403 for ouabain and m/z 393 to m/z 106 for internal standard, respectively. The declustering potential was set at 31, 46, and 106 V and the values of the collision energy were 25, 23, and 17 V for digoxin, ouabain, and internal standard, respectively. The dwell time was 250 ms. Data were collected and processed using Analyst 1.4.2 data collection and integration software (Applied Biosystems).

2.3. Cell culture

Human proximal tubule HK-2 cells obtained from American Type Culture Collection (Rockville, MD) were cultured in Dulbecco's Modified Eagle Medium/F-12 (1:1) (Gibco/Invitrogen, Grand Island, NY) with 10% fetal bovine serum (ICN Biomedicals, Inc., Aurora, OH) and 1% penicillin–streptomycin (Sigma). For the transport studies, HK-2 cells were seeded on polycarbonate membrane filters (3-mm pores, 4.71-cm^2 growth area) inside Transwell cell culture chambers (Costar, Cambridge, MA) at a density of 2.1×10^5 cells/cm^2. Transwell chambers were placed in 35-mm wells of tissue culture plates with 2.6 mL of outside (basolateral side) and 1.5 mL of inside (apical side) medium. Cells were grown at 37 °C under 5% CO_2, given fresh medium every 3 days and used day 7.

2.4. Sample preparation

Stock solutions of digoxin and ouabain were prepared in dimethylsulfoxide at a concentration of 2 mg/mL. A stock solution of internal standard was prepared in water/methanol

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