Sensitive and valid assay for reliable evaluation of drug interactions mediated by human organic anion transporter 1 and 3 using 5-carboxyfluorescein

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Abstract
Drug interactions can induce significant clinical impacts, either by increasing adverse effects or by decreasing the therapeutic effect of drugs, and thus, need to be explored thoroughly. Clinically significant drug interactions can be induced by organic anion transporter 1 (OAT1) and OAT3 when concomitant medications competitively interact with the transporters. The purposes of this study were to develop and validate a sensitive and selective analytical method for 5-carboxyfluorescein (5-CF) and optimize the experimental conditions for interaction studies. An analytical method using high-performance liquid chromatography (HPLC) equipped with a fluorescence detector was validated for accuracy, precision, matrix effect, recovery, stability, dilutional integrity, and carry-over effect. In addition, the 5-CF concentration, incubation period, and washing conditions for interaction study were optimized. Using a valid analytical method and optimized conditions, we performed an interaction study for OAT1 and OAT3 using 26 test articles. Some of the test articles showed strong inhibitory potency for the transporters, with IC50 values close to or less than 10 μM. The valid analysis method and optimized systems developed in this study can be utilized to improve the predictability of drug interactions in humans and consequently aid in successful disease treatment by maintaining appropriate systemic exposures.

Keywords
Organic anion transporters (OATs) · 5-carboxyfluorescein (5-CF) · Drug interactions · Method validation · High-performance liquid chromatography (HPLC)

Introduction
Organic anion transporters (OATs) belong to solute carrier 22 (SLC22) family and include OAT1, OAT2, OAT3, OAT4, OAT7, OAT10, and urate transporter 1 (URAT1) [1]. These transporters have 12 transmembrane domains with carboxy- and amino-termini on the intracellular side [2, 3]. The first identified transporter was OAT1 from rat kidneys, which showed classical para-amino hippuric acid (PAH) transport profiles in a sodium-independent manner with saturable profiles by increasing intracellular concentrations of PAH [4, 5]. Thus, initial studies have focused on the kidneys; however, it was confirmed in subsequent studies that the expression of OATs is also observed in several barriers, including the liver, brain, and placenta [1, 6, 7]. OATs transport numerous endogenous organic anions, such as neurotransmitter metabolites, steroid hormones, and cyclic nucleotides to regulate homeostasis or deliver biological signals across barriers [8–10]. In addition, many exogenous compounds such as β-lactam antibiotics, diuretics, and nonsteroidal anti-inflammatory drugs (NSAIDs) are known to be substrates of OATs [11–13]. Some compounds are not transported by OATs but have the capability to inhibit transporters by binding to certain transporter surfaces [14].

As OATs regulate the transmembrane transport of several drugs, interactions with concomitant medications that
are inhibitors of identical OAT isozymes can occur, which may lead to alterations in pharmacokinetics as well as the efficacy and toxicology of drugs. For example, it has been reported that concomitant administration of methotrexate with NSAID or β-lactam antibiotics increases systemic exposure to methotrexate, thereby increasing toxicity. This may result in suppression of bone marrow or damage to the intestinal epithelium [15, 16]. It is presumed that inhibition of OAT activity through concomitant drugs reduces renal clearance of methotrexate and drugs subsequently remain in the body at high concentrations for longer periods compared to when administered alone. Thus, drug interactions through OATs need to be thoroughly evaluated for safe drug administration and effective disease treatment. In this context, the Food and Drug Administration (FDA) of the United States and European Medicines Agency (EMA) recommend evaluating the interaction potentials of drug candidates for OATs in drug development programs [17, 18]. Clinically significant drug interactions are usually mediated by OAT1 and OAT3, which are located in the basolateral membrane of the renal proximal tubule [19, 20].

The evaluation of drug interactions mediated by transporters conventionally begins with in vitro experiments. Because OAT1 and OAT3 mediate transport of substrates into cells, an uptake study that measures the amount of substrate transported into cells in the presence or absence of potential inhibitors is generally performed to predict drug interactions through transporters. The use of sensitive and selective substrates for the transporters is a pivotal element of the uptake study, and the employment of a valid analysis method and experimental conditions are critical to obtain accurate data for the subsequent predictions of clinical drug interactions. Some radiolabeled substrates have been used for drug interaction studies; however, they require special protection when used and disposed of and are usually expensive. Substrates that are safer for experimenters and less burdensome may aid in drug interaction studies to obtain more information to facilitate effective and safer drug regimens.

5-carboxyfluorescein (5-CF; 3',6'-dihydroxy-3-oxospiro[2-benzofuran-1,9'-xanthene]-5-carboxylic acid) is a green fluorescent compound that contains monocarboxylic acid, presenting the substrate profiles for OAT1 and OAT3 [21]. Some drug interaction studies for OAT1 and OAT3 have been performed using 5-CF as a substrate, with the detection method using a fluorometer without separation from coexisting compounds [22, 23]. However, some treatment compounds used with 5-CF for the determination of inhibitory effects may interfere with fluorescence measurements, thereby masking the inhibitory effect of the test articles. In addition, the uptake profiles of 5-CF for OAT1 and OAT3 in cell-based systems have not yet been fully investigated, and non-optimized experimental conditions with 5-CF are being used to evaluate OAT1- or OAT3-mediated drug interactions.

In this study, we aimed to develop and validate a sensitive and selective analytical method for 5-CF detection using chromatographic techniques combined with a fluorescent detection system. In addition, experimental conditions to determine the drug interactions through OAT1 and OAT3 using 5-CF were optimized. Finally, the combination of the validated analytical method and optimized experimental conditions was successfully applied to evaluate OAT1- and OAT3-mediated drug interactions.

### Methods

#### Materials

5-CF, rhodamine 123 (internal standard; IS), ammonium formate, phosphate buffered saline (PBS), triton X-100, poly-D-lysine, and dimethyl sulfoxide (DMSO) were purchased from Sigma–Aldrich (St. Louis, MO, USA). 26 test articles (amodiaquine, buspirone, clarithromycin, cyclosporin A, desipramine, diclofenac, diltiazem, domperidone, doxoru- bicin, edoxaban, erythromycin, fluorescein isothiocyanate [FITC], glimepiride, lisinopril, loperamide, metoprolol, novobiocin, oseltamivir, piroxicam, prazosin, procainamide, propranolol, quercetin, quinidine, terfenadine, and verapamil) used for the interaction study were obtained from Sigma–Aldrich (St. Louis, MO, USA) or Selleckchem (Houston, TX, USA). High-performance liquid chromatography (HPLC) grade methanol and water were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

#### Chromatographic conditions and detection method

To determine 5-CF concentration in the cell lysate, a Shimadzu LC-20 HPLC system (Shimadzu Co., Kyoto, Japan) connected to a fluorescence detector (RF-20A, Shimadzu Co.) was used. The chromatographic resolution of 5-CF and IS was performed using a reverse-phase HPLC column (Synergi™ 4 µm polar-RP 80 Å, 150 × 2.0 mm; Phenomenex, Torrance, CA, USA) with an isocratic composition of 50 mM ammonium formate in water and methanol (20:80% v/v) at a flow rate of 0.8 mL/min. The total run time per sample was 4 min and the autosampler temperature was set at 4 °C. The wavelengths for excitation and emission were set to 495 and 517 nm, respectively, which produced the maximum intensity in fluorescence spectra. Instrument control and data analysis were performed using LC Solution Software (Version 1.25; Shimadzu Co.).
**Calibration standards and quality control (QC) samples**

Stock solutions of 5-CF or IS were prepared at a concentration of 30 mM in DMSO. Standard working solutions of 5-CF were obtained by serially diluting the stock solutions with methanol. An IS working solution was prepared using an IS stock solution at a final concentration of 30 nM in methanol. Blank cell lysates were spiked with each standard working solution to generate final concentrations of 1, 2, 5, 20, 50, 200, 500, and 1000 nM. QC samples were prepared at final concentrations of 3 nM (low QC [LQC] level), 40 nM (medium QC [MQC] level), and 800 nM (high QC [HQC] level) in the cell lysate.

**Sample preparation**

An aliquot of 30 μL of the cell lysate was added to 200 μL of methanol containing IS (30 nM). The mixture was vortexed for 4 min and then centrifuged at 12,000 rpm for 4 min. The supernatant was transferred into an LC vial and 10 μL was injected into the HPLC system.

**Method validation**

The analysis method of 5-CF was validated in terms of selectivity, sensitivity, linearity, and intra- and inter-day accuracy and precision. The selectivity was evaluated by comparing chromatograms of the blank cell lysate, cell lysate spiked with only 5-CF, cell lysate spiked only with IS (zero blank), and cell lysate spiked with both 5-CF and IS. The lower limit of quantification (LLOQ) was determined as the concentration with acceptable precision and accuracy and ≥ 5 times the analyte response of the zero blank. The linearity of the assay was determined using calibration standards containing 5-CF at concentration range of 1–1000 nM. Linear regression analysis was performed using a constructed calibration curve consisting of the y-axis of the peak area ratio of 5-CF to IS, and the x-axis of the nominal concentration ratio of 5-CF to IS. The accuracy and precision of the analytical method were determined for four different concentrations (LLOQ, LQC, MQC, and HQC) in a single day (n = 5) and for 3 days and calculated as described previously [24].

Recovery and matrix effects were evaluated using QC samples in three replicates. The recovery was determined by dividing the mean peak area of an analyte added before sample preparation (set 3) by the mean peak area ratio of an analyte spiked in the post-preparation matrix (set 2). The mean peak area of an analyte spiked in the post-preparation matrix (set 2) was compared with that of an analyte spiked in the mobile phase (set 1) to calculate the matrix effect. The recovery and matrix effects were calculated for IS (30 nM) in the same manner.

The stability of 5-CF in cell lysates was evaluated at two QC levels (LQC and HQC) in triplicate under various analytical handling and storage conditions: benchtop, long-term storage, freeze–thaw, and autosampler storage. The stability of the samples was assessed by comparing the peak area of the analyte to that of the IS in the samples stored under the conditions stated previously with those obtained from freshly prepared QC samples. The stability of the stock solutions of 5-CF and IS was also evaluated after storage at −20 °C for 60 days.

For the quantification of samples with concentrations higher than the upper limit of quantification (ULOQ), dilutional linearity was evaluated using samples with a five-fold concentration of HQC. To evaluate the carry-over effect, blank samples were analyzed immediately after the samples at the ULOQ, and the peak area was compared with that of LLOQ samples.

**Cell culture and determination of OAT1 or OAT3 expressions**

Madin–Darby canine kidney II (MDCK II)/FRT cells expressing human OAT1 or OAT3 were provided by Dr. Sukjae Chung of Seoul National University (Seoul, Korea). The cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with high glucose containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 U/mL penicillin/0.1 mg/mL streptomycin, and 1% non-essential amino acids (NEAA; Sigma–Aldrich, St. Louis, MO, USA) in a humidified atmosphere containing 5% CO2 at 37 °C. The expression of OAT1 or OAT3 was confirmed using quantitative polymerase chain reaction (qPCR) using specific primers for the transporters (forward 5′-CCACCT CTTCTCTGCGCTTCAT-3′ and reverse 5′-GTCTGT TTCCTTTTTCGCTTC-3′ for OAT1, forward 5′-CGT CTTCTCTCTATCATTCTGTT-3′ and reverse 5′-CTG GCTCTGCTTTGGCTTCTTG-3′ for OAT3).

**Uptake study**

The uptake profiles of 5-CF by human OAT1 or OAT3 were determined using an uptake study that was slightly modified from a previously reported method [25, 26]. Briefly, cells were seeded in 96-well plates (SPL Life Science, Korea) coated with poly-D-lysine at a density of 2 × 10^4 cells/well and incubated for 1 day. The transport medium was prepared using a commercially available Hanks’ balanced salt solution (HBSS; Gibco, Grand Island, NY, USA) with pH adjusted to 7.4. On the day of the uptake study, cells were washed twice with pre-warmed transport medium and pre-incubated with the buffer at 37 °C for 20 min to equilibrate the cell conditions. After pre-incubation, 5-CF in the transport buffer was loaded into the cells and incubated for the designated
period. The cells were then washed three times with washing buffer (iced cold transport buffer) and lysed with 0.1% Triton X-100 in PBS by shaking at 100 rpm for 1 h.

To evaluate the effect of bovine serum albumin (BSA; Sigma–Aldrich, St. Louis, MO, USA) on the washing procedure, various concentrations of BSA (0.1–10%) were prepared in washing buffer. To determine the inhibitory properties of the test articles, a transport buffer containing both 5-CF and the test article was added to the cells, followed by the procedures described previously.

All measurements of 5-CF concentration in the uptake study were performed using the analytical method validated in this study, and the method sufficiently covered concentrations of 5-CF measured in the cell lysates in this study. The measured concentrations were normalized through protein amount quantified using the BCA protein assay kit (Thermo Fisher, Waltham, MA, USA) and incubation time, and the amount of 5-CF uptake was presented in units of pmol/mg protein/min.

Data analysis

For the determination of $K_m$ values for the uptake of 5-CF by OAT1 or OAT3, the Michaelis–Menten kinetic equation was used as shown in the following equation:

$$V = \frac{V_{\text{max}} \times C}{K_m + C} \tag{1}$$

where $V$ is the uptake velocity of 5-CF into the cells, $C$ is the concentration of 5-CF treated in the cells, $V_{\text{max}}$ is the maximum uptake velocity, and $K_m$ is the Michaelis Menten constant, which indicates the 5-CF concentration needed to achieve half of $V_{\text{max}}$.

Statistical analysis

Data are presented as mean ± standard deviation (SD). Statistical significance was determined using the $p$ value from the unpaired Student’s $t$ test. In this study, a $p$ value less than 0.05 was considered statistically significant.

Results

Method development for 5-CF analysis

Various analytical columns and mobile phases were evaluated to obtain symmetric peaks for 5-CF, without interference. The Synergi™ 4 µm polar-RP column exhibited the best peak shape with satisfactory separation from interferences compared to the Kinetex® C8 column (250×4.6 mm, 5 µm, 100 Å; Phenomenex, Torrance, CA, USA) or CAPCELL PAK C18 MGIII column (250×4.6 mm, 5 µm, 100 Å; Shiseido, Tokyo, Japan). An isocratic mobile phase composition of 50 mM ammonium formate:methanol = 2:8 was selected because it provided sufficient separation of the peaks for 5-CF and IS and adequate run times (4 min per sample). The retention time with the chosen LC condition was 1.9 and 2.8 min for 5-CF and IS, respectively.

Method validation for 5-CF analysis

No significant interference for the detection of 5-CF and IS was observed (Fig. 1). The LLOQ, which satisfies the acceptable criterion of more than 5 times the response at the retention time of the analyte in zero blank samples, was determined to be 1 nM with accuracy and precision within 80–120% from each of the five replicates in three separate runs. The calibration curves for 5-CF were linear in the concentration range of 1–1000 nM with coefficient of determination ($r^2$) > 0.99. The typical regression equation for the curve was $y = 0.2497x + 0.0075$, where $x$ is the concentration of 5-CF/IS and $y$ is the peak area ratio of 5-CF/IS. The calculated concentrations of > 75% non-zero calibration standards were within ±15% of the theoretical concentrations, except at the LLOQ, where the calculated concentrations were ±20% of the theoretical concentrations in each validation run, in accordance with the bioanalytical guidance. Intra- and inter-assay precision and accuracy were determined using four different QC levels (LLOQ, LQC, MQC, and HQC). The relative standard deviation (RSD), an indicator of precision, was less than 11.7% for both intra- and inter-day assays, and the accuracy ranged from 96.8–105%, as shown in Table 1, indicating that this analytical method is precise and accurate for the quantification of 5-CF in the samples obtained from the cell uptake study.

The recovery and matrix effects of this method were determined at three QC levels of 5-CF (LQC, MQC, and HQC) and one IS concentration (30 nM). The mean recoveries of 5-CF and IS were 93.2–106% and 99.5%, respectively (Table 2). The mean matrix effects of 5-CF and IS were 91.1–102% and 95.7%, respectively. The stability of 5-CF was determined using LQC and HQC, and the measured concentrations after storage at bench top conditions (room temperature) for 2 h, autosampler (4 °C) for 24 h or long term at ~20 °C for 60 days, or repeated freezing and thawing process (3 cycles) were more than 90% of the theoretical concentrations (Table 3). In addition, the stock solutions of 5-CF and IS were stable after storage at ~20 °C for 60 days. Collectively, it was determined that 5-CF was stable under all the storage and handling conditions tested in this study. For the study of $K_m$ value calculation, a high concentration of 5-CF must be quantified; thus, dilutional integrity was evaluated with a five-fold dilution. The accuracy and RSD of the diluted samples were 103 and 4.14%, respectively.
Table 1 Intra- and inter-day accuracy and precision of 5-CF in cell lysates

| Nominal concentration (nM) | LLOQ | LQC | MQC | HQC |
|----------------------------|------|-----|-----|-----|
| 1                          | 1.05 ± 0.06 | 3.09 ± 0.36 | 40.7 ± 3.8 | 815 ± 54 |
| 3                          | 5.42  | 2.92 | 1.84 | 1.91 |
| 40                         | 5.98  | 11.7 | 9.34 | 6.68 |
| 800                        |       |     |     |     |

Intra-assay (n = 5)
- Measured concentration (mean ± SD)
- Accuracy (RE, %)
- Precision (RSD, %)

Inter-day (n = 15)
- Measured concentration (mean ± SD)
- Accuracy (RE, %)
- Precision (RSD, %)

RE relative error expressed by the absolute value of [(calculated concentration-theoretical concentration)/theoretical concentration × 100%]. RSD standard deviation of concentration/mean concentration × 100%, SD standard deviation.
which can be applied to the analysis of samples with a concentration higher than the ULOQ (1000 nM). The carry-over effect was not observed in the analysis of the blank samples following the samples at the ULOQ.

**Optimization of experimental conditions with 5-CF for OAT1 and OAT3**

Expression of OAT1 or OAT3 in mock, OAT1, or OAT3-expressing cells was confirmed using qPCR. As mRNA expression was not detected in mock cells (Ct values were not available in mock cells), the relative expression levels of OAT1 or OAT3 between the cells could not be calculated. Instead, C_i values for OAT1 and OAT3 were 21.2 ± 1.1 and 22.1 ± 0.9 (mean ± SD) in OAT1-, or OAT3-expressing cells, respectively.

We then attempted to determine the optimal experimental conditions for this uptake study. First, cells were treated with 5-CF for various periods, and it was confirmed that the accumulation of 5-CF within the cells increased linearly for up to 10 min, after which the rate of increase decreased (Fig. 2). In addition, various concentrations of 5-CF (0.1–250 μM) were added to mock, OAT1-, or OAT3-expressing cells. The uptake profiles were saturated at high concentrations in MDCKII cells expressing OAT1 or OAT3, confirming the involvement of the active transport of 5-CF into the cells. The final results were normalized by protein concentration and divided by the treatment period (10 min); the calculated K_m values of 5-CF for OAT1 or OAT3 were 49.9 and 35.1 μM, respectively. The V_max of 5-CF was 196 and 1170 pmol/mg protein/min for OAT1 and OAT3, respectively, suggesting that the maximum capacity of 5-CF transport was higher for OAT3 than for OAT1 (Fig. 3). Next, we evaluated the effect of BSA on washing buffer. The addition of BSA to the washing buffer (up to 10%) did not have a significant impact on the measured concentrations of 5-CF in the cell lysates (Fig. 4). In this optimized condition, the amount of 5-CF uptake was 2.70, 33.6, and 238 pmol/mg protein/min in mock cells, OAT1- or OAT3-expressing cells, respectively, suggesting that the condition is well established for evaluation of OAT1 or OAT3-mediated drug interactions.

### Table 2 Recovery and matrix effects of 5-CF and IS in cell lysates (mean ± SD, n = 3)

| Concentration (nM) | LQC | MQC | HQC |
|--------------------|-----|-----|-----|
| 5-CF               |     |     |     |
| Recovery (%)a      | 106 ± 7.14 | 93.2 ± 5.91 | 105 ± 3.12 |
| Matrix effect (%)b | 91.1 ± 1.84 | 101 ± 6.31 | 102 ± 2.20 |
| IS                 |     |     |     |
| Recovery (%)       | 99.5 ± 1.98 |
| Matrix effect (%)  | 95.7 ± 5.14 |

*a* Recovery (%) = mean analyte peak area of an analyte added before post-precipitation (set 3)/mean peak area of an analyte added post-precipitation (set 2) × 100  
*b* Matrix effect (%) = mean peak area of an analyte added post-precipitation (set 2)/mean peak area of an analyte in neat analyte solution (set 1) × 100

### Table 3 Stability of 5-CF in cell lysates (mean ± SD, n = 3)

| Storage condition          | Stability (%) |
|---------------------------|---------------|
|                            | LQC | HQC |
| Benchtop (room temperature for 2 h) | 97.1 ± 8.12 | 105 ± 4.14 |
| Freeze–thaw (3 cycles)     | 90.3 ± 1.10 | 101 ± 7.25 |
| Autosampler (4 °C for 24 h) | 91.2 ± 6.31 | 104 ± 3.71 |
| Long term (− 20 °C for 60 days) | 102 ± 1.05 | 97.1 ± 0.36 |

**Fig. 2** Uptake kinetics of 5-CF in MDCKII-FRT/OAT1 (A) and MDCKII-FRT/OAT3 (B) cells at various incubation time (n = 4). 5-CF at 1, 10, or 100 μM were added to the cells and incubated for various periods. The amount of 5-CF in cell lysates were measured using HPLC combined with fluorescence detection. Data are expressed as mean ± SD
Interaction studies for OAT1 and OAT3

Using this validated method, the interactions through OAT1 and OAT3 were tested for commercially available drugs or compounds (26 test articles). In the interaction studies with 5-CF (10 μM), 6 and 11 compounds inhibited OAT1 and OAT3, respectively, by more than 50% at 50 μM with statistical significance (desipramine, diclofenac, glimepiride, novobiocin, quercetin, and verapamil for OAT1, and buspirone, desipramine, diclofenac, domperidone, doxorubicin, glimepiride, novobiocin, piroxicam, prazosin, quercetin, and verapamil for OAT3; Fig. 5). In addition, diclofenac strongly inhibited both OAT1 and OAT3 at a concentration of 50 μM, showing that the remaining activity of the transporters was less than 10%. Glimepiride, novobiocin, and quercetin have also been confirmed to be strong inhibitors of OAT3.

To further investigate the potency of the identified strong inhibitors (diclofenac, glimepiride, novobiocin, and quercetin) for OAT1 and OAT3, various concentrations of the test article were incubated with 5-CF in MDCKII/FRT cells expressing OAT1 or OAT3 (Fig. 6). The calculated IC50 values of diclofenac for OAT1 was 3.73 μM and those of diclofenac, glimepiride, novobiocin, and quercetin for OAT3 were 11.2, 1.87, 4.86, and 7.69 μM, respectively.

In all analytical runs for drug interaction studies, it was confirmed that ≥ 67% of the QC samples and ≥ 50% of QCs per level were within ± 15% of the nominal concentrations in accordance with international guidelines [17, 18].

Discussion

Drug interactions can induce significant clinical impacts, either by increasing adverse effects or by decreasing the therapeutic effect of drugs, and thus, need to be explored thoroughly. Recent trends in drug approvals reflect the importance of drug interactions; therefore, drug developers attempt to determine the potential impact of drug interactions in the drug development process. In terms of pharmacokinetics, drug transporters are one of the main components of the interactions, as they are involved in the absorption, disposition, and excretion processes of drugs [27, 28].

5-CF, which contains a green fluorophore, can be quantified using a fluorescence detector. A simple fluorometer that measures the fluorescence intensity without any separation of samples can be used to quantify the amount of 5-CF in the samples when no interfering compounds exist in the samples. However, drug interaction studies may be vulnerable to interference from other compounds in the measurement of
fluorescent intensity of 5-CF, and a more cautious approach is necessary, especially when high concentrations of test articles are used in the study. In our preliminary study, some of the commercially available test compounds (such as FITC and quercetin) provoked interference of the fluorescence intensity of 5-CF when measured using a simple fluorometer, leading to the false conclusion of potential inhibitors or inducers for the function of OAT1 or OAT3. Thus, we attempted to develop an analytical method without interference from coexisting compounds within samples using chromatographic separation. Symmetric peak shapes without interference were achieved after evaluation of various columns and the composition of the mobile phase. In addition, it is critical to quantify the amount of 5-CF accurately within a reliable quantification range, showing an adequate concentration–response relationship for the determination of inhibitor activity such as IC₅₀ values. The method developed in this study showed a linear correlation between 5-CF concentration and response within the range of 1–1000 nM. LLOQ was set at 1 nM, which was enough to quantify 5-CF within the cells, suggesting that this analysis method is sensitive to be used in this interaction study. Dilution integrity was also confirmed to be reliable for samples at ULOQ concentrations. The accuracy and precision of the developed method were proven using the intra- and inter-assay test, which showed results satisfying the criteria defined in the guidance. In addition, the matrix effect in the cell lysates was confirmed to be negligible, and recovery with the suggested

Fig. 5 Interaction study with 26 test articles using 5-CF as a substrate in MDCKII-FRT/OAT1 (A) and MDCKII-FRT/OAT3 (B) cells (n=4). Data are expressed as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001
Semi-sensitive and valid assay for reliable evaluation of drug interactions mediated by human organic...

Pre-treatment method was nearly perfect, suggesting that the sample preparation method in this study is appropriate for use. The samples were stable under various handling and storage conditions, and the carry-over effect was negligible. Overall, the method developed in this study satisfied the requirements of the US FDA and EMA guidelines and was thus considered appropriate for use in drug interaction studies.

We next attempted to optimize the various conditions of the drug interaction study with 5-CF. The optimal incubation period was determined to be 10 min, because 5-CF uptake velocity remained linear for 10 min and thereafter showed a non-linear uptake rate over time. The 5-CF concentration for the interaction study was set at 10 μM considering the calculated $K_m$ values for OAT1 and OAT3 (49.9 and 35.1 μM, respectively) and the usual variability of the experiments. The non-specific binding remaining after washing was confirmed to be negligible when washing with only HBSS or various concentrations of BSA in HBSS. This may be because of the hydrophilic properties of 5-CF, which can be sufficiently removed by washing without a competitor. Therefore, we selected HBSS as the only washing solution in the subsequent drug interaction study. The final optimized conditions of the study showed a 12.2- or 88.1-fold increase in 5-CF uptake into cells expressing OAT1 or OAT3 compared to the mock cells, and this seems to be the appropriate setting for sensitive and reliable drug interaction studies.

The drug interaction potentials were evaluated for 26 test articles using a validated analytical method and optimized experimental conditions. In overall, the inhibitory profiles of the test compounds were similar between OAT1 and OAT3, except for buspirone and prazosin that showed more than 60% inhibition for OAT3 whereas no statistically significant inhibition was observed for OAT1. The different effect of prazosin in OAT1 and OAT3 has been already reported [29]. For the case of buspirone, different affinity for OAT1 and OAT3 has been reported for mouse [30], but that for human is the first observation in this study to the best of our knowledge. Although overlapping substrate specificity of OAT1 and OAT3 has been reported in several studies [2, 23], different inhibitory profiles or potency need to be investigated further to better understand the transporter-mediated drug interactions as similarity in amino acid sequence of OAT1 and OAT3 is limited to 51% [6].

Novobiocin is an acidic drug (pKa 4.3 [31]) and a well-known inhibitor of OAT1 and OAT3. This study showed strong inhibition of OAT1 and OAT3 function through novobiocin, proving that the experimental systems are reliable. Among the test articles, some showed strong inhibition with IC$_{50}$ values ≤ 10 μM, suggesting potential drug interactions through OAT1 and OAT3. The IC$_{50}$ values obtained for diclofenac and novobiocin, well-known inhibitors for OATs, were similar to the values reported in previous studies [32, 33], proving the reliability of this study. Quercetin, the most abundant dietary flavonoid, strongly inhibited OAT3 with an IC$_{50}$ of 7.69 μM. In addition to food intake, dietary supplements containing quercetin are being widely taken without any consideration of drug interactions owing to its well-known bioactivity, such as antioxidant effect, control of glucose, and prevention of inflammation [34–36]. This may induce unexpected adverse effects of OAT3 substrate drugs by decreasing their renal clearance, thereby increasing systemic exposure. Quercetin is also an inhibitor for organic anion transporting polypeptide (OATP)1B1, OATP1B3, OATP2B1, breast cancer resistance protein (BCRP) [37], and p-glycoprotein (p-gp) [38]. Thus, the administration of quercetin with drugs should be carefully investigated from the perspective of drug interactions.

In general, OATs transport anionic compounds; however, they are also reported to interact with some cationic compounds [30]. In this study, we observed that basic drugs, such as desipramine [39] and verapamil [40] inhibit OAT1

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**Fig. 6** Inhibitory potency of test articles for 5-CF uptake in MDCKII-FRT/OAT1 (A) and MDCKII-FRT/OAT3 (B) cells (n=4). Various concentrations of test articles were added with 5-CF to the cells for the calculation of IC$_{50}$ values. Data are expressed as mean ± SD.
and OAT3. The newly reported inhibitor for OAT3, buspirone, is also a weak basic drug [41], supporting the claim for the interactions between OATs and basic drugs. Thus, OAT1- and OAT3-mediated drug interactions need to be evaluated not only for acidic drugs but also for basic drugs for more successful tailored medications.

Even though high throughput screening method using simple fluorometer without chromatographic separation can be used at early drug development stage, more elaborated and valid experimental and analysis method is necessary at later stage to obtain accurate inhibitory potency such as IC_{50} that is directly used for prediction of clinical drug interactions using static or dynamic model. The separation method such as chromatographic technique allows prevention from any interferences from coexisting compounds that mask or induce fluorescence intensity of a fluorescent substrate. Thus, the valid analysis method and optimized systems developed in this study can be used to improve the predictability of drug interactions in humans and consequently aid in successful disease treatment by maintaining appropriate systemic exposures.

**Conclusion**

In this study, we developed and validated a sensitive and reproducible analytical method for 5-CF using a chromatographic technique combined with a fluorescent detection system. In addition, we optimized the experimental conditions to evaluate drug interactions through OAT1 and OAT3, using 5-CF as a substrate. A combination of the validated analytical method and optimized experimental conditions was successfully applied to evaluate OAT1- and OAT3-mediated drug interactions. The utilization of this combined method can help to provide more useful and accurate information to predict clinical drug interactions for safer and more effective drug regimens.

**Author contributions** All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Y-J C, K-R L, and J-E C. The first draft of the manuscript was written by Y-J C and K-R L and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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**Declarations**

**Conflict of interest** The authors declare no conflicts of interest.

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