Introduction

Optically active pharmaceuticals are known to have different efficacies and metabolic fates for each isomer. Therefore, the development of highly sensitive chiral analytical methods for accurate drug evaluation and differential analysis of drug metabolism is required. For example, R-warfarin is metabolized mainly by cytochrome P450 1A2 (CYP1A2) to 6- and 8-hydroxywarfarin, by CYP3A4 to 10-hydroxywarfarin, and by carbonyl reductases to diastereoisomeric alcohols. On the other hand, S-warfarin is primarily metabolized by CYP2C9 to 7-hydroxywarfarin. Therefore, quantifying various trace metabolites of chiral pharmaceuticals for evaluating the metabolic activity of P450 enzymes and predicting the side effects of drugs has become increasingly important.

To date, more than 100 chiral stationary phases (CSPs) for liquid chromatography (LC) columns are commercially available. Polysaccharide-type CSPs are used most frequently, followed by macrocyclic antibiotic and cyclodextrin-type CSPs. The separation mechanism underlying most chiral columns is still unknown, and applicable analyses for these columns are limited. Thus, column-screening approaches are widely used. Our group reported novel dress-up columns featuring reproducibly removable and replaceable chiral stationary phases. Our group reported novel dress-up columns featuring reproducibly removable and replaceable chiral stationary phases.

A sensitive analytical method was developed for the simultaneous detection of 11 chiral pharmaceuticals and their hepatic metabolites by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using an ovomucoid chiral column. After optimization of the LC conditions, all pharmaceuticals examined were enantio-separated with Rs of >0.82 in LC-MS/MS analysis. The limit of detections of all pharmaceuticals by MS/MS detection ranged from 1.2 to 92.3 nM, which is approximately 1000–25000 times lower than those obtained by UV detection. From hepatic metabolite analyses in P450-expressing cells, metabolites of three pharmaceuticals were detected and enantio-separated. By using the proposed method, changes in the optical isomer ratio of the hepatic metabolites chlorpheniramine and verapamil caused by differential cytochrome P450 enzyme expression for each isomer, could be successfully traced.

Keywords Chiral pharmaceuticals, LC-MS/MS analysis, ovomucoid column, hepatic metabolites, CYP

(Received May 13, 2018; Accepted July 9, 2018; Advance Publication Released Online by J-STAGE July 20, 2018)
Herein, we report on a novel sensitive and simultaneous analytical method for the detection of chiral pharmaceuticals and their hepatic metabolites by LC-MS/MS using the ULTRON ES-OVM-3 column as a CSP. We selected 11 optically active pharmaceuticals and their hepatic metabolites by LC-MS/MS using the ULTRON ES-OVM-3 column as a CSP. We selected 11 optically active pharmaceuticals and their hepatic metabolites by LC-MS/MS using the ULTRON ES-OVM-3 column as a CSP. We selected 11 optically active pharmaceuticals and their hepatic metabolites by LC-MS/MS using the ULTRON ES-OVM-3 column as a CSP. We selected 11 optically active pharmaceuticals and their hepatic metabolites by LC-MS/MS using the ULTRON ES-OVM-3 column as a CSP. We selected 11 optically active pharmaceuticals and their hepatic metabolites by LC-MS/MS using the ULTRON ES-OVM-3 column as a CSP. We selected 11 optically active pharmaceuticals and their hepatic metabolites by LC-MS/MS using the ULTRON ES-OVM-3 column as a CSP. We selected 11 optically active pharmaceuticals and their hepatic metabolites by LC-MS/MS using the ULTRON ES-OVM-3 column as a CSP. We selected 11 optically active pharmaceuticals and their hepatic metabolites by LC-MS/MS using the ULTRON ES-OVM-3 column as a CSP. We selected 11 optically active pharmaceuticals and their hepatic metabolites by LC-MS/MS using the ULTRON ES-OVM-3 column as a CSP. We selected 11 optically active pharmaceuticals and their hepatic metabolites by LC-MS/MS using the ULTRON ES-OVM-3 column as a CSP.

First, the LC separation and MS/MS detection conditions for these pharmaceuticals were optimized. Subsequently, a detection sensitivity enhancement of the LC-MS/MS was performed and compared to LC-UV. The developed method was then applied to the chiral analysis of pharmaceutical metabolites produced in HepG2 cells expressing P450.

This method enabled highly sensitive LC-MS/MS analyses of pharmaceuticals and their hepatic metabolites, which is necessary for evaluating and predicting both drug metabolism and toxicity. The developed method used only one column and simple mobile phases. This study provides useful and practical analytical methods for researchers interested in drug discovery, toxicology, and pharmacology.

### Experimental

#### Reagents and solutions

Deionized and distilled water, purified using a PURELAB flex system (ELGA, Marlow, UK), was used to prepare all aqueous solutions. Ibuprofen, propranolol hydrochloride and trihexyphenidyl hydrochloride were obtained from Sigma-Aldrich (St. Louis, MO, USA). (±)-2-(6-Methoxy-2-naphthyl) propionic acid [(±)-naproxen], ketoprofen, pindolol, propafenone hydrochloride, chlorthpheniramine maleate, warfarin, verapamil hydrochloride, and ammonium formate were purchased from Wako Pure Chemical (Osaka, Japan). (R,S)-Loxoprofen was kindly donated by Daiichi-Sankyo Co Ltd. (Tokyo, Japan). HPLC-grade acetonitrile (MeCN) and ethyl acetate were obtained from Kanto Chemicals (Tokyo, Japan). All other chemicals were used as received in the highest purity available. Each stock solution of chiral pharmaceuticals (10 mmol/L) except for NSAIDs (ibuprofen, loxoprofen, naproxen, ketoprofen) was prepared in water. Stock solutions of the NSAIDs (10 mmol/L) were prepared in methanol. These solutions were stored at 4°C and diluted with water to the desired concentration immediately before use. The pH (3.0, 4.6, 6.0) of 10 mmol/L aqueous ammonium formate solution used in mobile phase was adjusted by the addition of 1 mol/L hydrochloric acid.

#### LC-ESI-MS/MS system and conditions

The liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESIMS/MS) analysis was performed using an ACQUITY ultra-performance liquid chromatograph (UPLC H-class, Waters) connected to a photodiode array detector (Waters, Milford, MA, USA) and an Xevo TQ-D triple quadrupole-mass spectrometer (Waters). An ULTRON ES-OVM-3 column (3 mm, 150 mm × 2.1 mm i.d.; Shinwa Chemical Industries Ltd., Kyoto, Japan) was used at a flow rate of 0.2 mL/min and 25°C. All pharmaceuticals and their metabolites were ionized in the ESI positive ion mode. The separation and detection conditions were as follows: mobile phase, acetonitrile-10 mmol/L aqueous ammonium formate; capillary voltage, 3.00 kV; cone voltage, 50 V; desolvation gas flow, 1000 L/h; cone gas flow, 150 L/h; nebulizer gas flow, 7.0 L/h; collision gas (Ar) flow, 0.15 mL/min; collision energy, 20 eV; collision cell exit potential, 3 V; and desolvation temperature, 500°C. Analytical software (MassLynx, Ver. 4.1) was used for system control and data processing. When conducting a high-sensitivity analyses of hepatic metabolites, another UPLC pump was connected to the LC-MS/MS system, and an acetonitrile was delivered to the column eluate. The limit of detections (LOD) of 11 pharmaceuticals using UV and MS/MS detections were determined from a signal-to-noise ratio of 3.

#### Preparation of HepG2 cells mimicking the activity levels of CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 in human hepatocytes (Ad-P450 Cells)

HepG2 cells were purchased from RIKEN Cell Bank (Tsukuba, Japan) and were cultured in DMEM (Wako Pure Chemical Industries), containing 10% fetal bovine serum (Biowest, Miami, FL, USA), nonessential amino acids (Thermo Fisher Scientific, Waltham, MA, USA), and Antibiotic-Antimycotic (Thermo Fisher Scientific) under 5% CO2 and 95% air at 37°C. The cells were seeded in a 48-well tissue culture plate (Becton Dickinson) at 5.0 × 10^4 cells/well. After 48 h, the cells were simultaneously infected with CYP1A2-expressing adenoviruses (Ad-CYP1A2) at five multiplicity of infections (MOIs): Ad-CYP2C9 at 1 MOI, Ad-CYP2C19 at 2 MOI, Ad-CYP2D6 at 0.05 MOI, and Ad-CYP3A4 at 10 MOI. The Ad-P450 cells were cultured for 72 h, incubated in culture medium containing each pharmaceutical (10 μmol/L) for 5 h, and subsequently extracted with ethyl acetate (1 mL). After evaporation of the organic layer, the residue was dissolved in the mobile phase, and an aliquot (0.4 μL) of the solution was subjected to LC-MS/MS.

### Table 1 Physical and chemical properties of the 11 chiral pharmaceuticals examined

<table>
<thead>
<tr>
<th>Name (abbreviation)</th>
<th>Category</th>
<th>Molecular weight</th>
<th>pKₐ</th>
<th>Acidic or basic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ibuprofen (IBU)</td>
<td>NSAIDs</td>
<td>206.3</td>
<td>4.41</td>
<td>Acidic</td>
</tr>
<tr>
<td>Loxoprofen (LOX)</td>
<td></td>
<td>246.3</td>
<td>4.39</td>
<td>Acidic</td>
</tr>
<tr>
<td>Naproxen (NAP)</td>
<td></td>
<td>230.3</td>
<td>4.84</td>
<td>Acidic</td>
</tr>
<tr>
<td>Ketoprofen (KET)</td>
<td>β-Blocker</td>
<td>254.3</td>
<td>4.23</td>
<td>Basic</td>
</tr>
<tr>
<td>Pindolol (PIN)</td>
<td></td>
<td>248.3</td>
<td>9.54, 13.94</td>
<td>Basic</td>
</tr>
<tr>
<td>Propranolol (PRO)</td>
<td></td>
<td>259.3</td>
<td>9.50, 13.84</td>
<td>Basic</td>
</tr>
<tr>
<td>Propafenone (PFE)</td>
<td>Na channel blocker</td>
<td>341.4</td>
<td>9.31, 13.82</td>
<td>Basic</td>
</tr>
<tr>
<td>Chlorpheniramine</td>
<td>Antihistamine</td>
<td>274.8</td>
<td>9.33</td>
<td>Basic</td>
</tr>
<tr>
<td>Warfarin (WAR)</td>
<td>Anticoagulant</td>
<td>308.3</td>
<td>4.50</td>
<td>Acidic</td>
</tr>
<tr>
<td>Trihexyphenidyl</td>
<td>Anticholinergic agent</td>
<td>301.5</td>
<td>9.31, 14.31</td>
<td>Basic</td>
</tr>
<tr>
<td>Verapamil (VER)</td>
<td>Ca channel blocker</td>
<td>454.6</td>
<td>8.97</td>
<td>Basic</td>
</tr>
</tbody>
</table>

- Ibuprofen, propranolol hydrochloride and trihexyphenidyl hydrochloride were obtained from Sigma-Aldrich (St. Louis, MO, USA).
- (±)-2-(6-Methoxy-2-naphthyl) propionic acid [(±)-naproxen], ketoprofen, pindolol, propafenone hydrochloride, chlorthpheniramine maleate, warfarin, verapamil hydrochloride, and ammonium formate were purchased from

Wako Pure Chemical (Osaka, Japan). (R,S)-Loxoprofen was kindly donated by Daiichi-Sankyo Co Ltd. (Tokyo, Japan). HPLC-grade acetonitrile (MeCN) and ethyl acetate were obtained from Kanto Chemicals (Tokyo, Japan). All other chemicals were used as received in the highest purity available.
Results and Discussion

Optimized chiral separation of the 11 pharmaceuticals

Figure 1 shows MRM chromatograms of 11 chiral pharmaceuticals and their MRM transitions. The optimized mobile phase (pH, acetonitrile content) for the separation and retention times of both enantiomers ($t_{R1}$ and $t_{R2}$) and their resolutions ($R_s$) are listed in Table 2. By using a pH-adjusted mixed solution of ammonium formate and acetonitrile as a mobile phase, all pharmaceuticals were enantio-separated with a $R_s$ of $>0.82$ in the LC-MS/MS analysis. Acidic pharmaceuticals, such as non-steroidal anti-inflammatory drugs (NSAIDs), were successfully enantio-separated under the acidic elution conditions.
Comparison of detection sensitivity of UV and MS/MS methods

In the ESI positive ion mode, acidic pharmaceuticals were detected in their proton adduct forms. The LODs of all 11 pharmaceuticals by MS/MS detection ranged from 1.2 to 92.3 nM (0.48 - 36.9 fmol per 0.4 μL injection), approximately 1000 - 25000 times lower than those obtained by UV detection (Table 3). Depending on the analyte, the LOD values at nM levels were comparable to those obtained by previously reported derivatization-LC-MS/MS methods. Thus, the present method easily affords sufficient sensitivity for analytes without derivatization.

Chiral LC-MS/MS analysis of pharmaceutical metabolites produced in cultured human hepatic cells

Recently, we developed hepatocellular carcinoma cells...
(HepG2 cells) that mimicked the activities of CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4, which strongly contribute to drug metabolism in human comparable to those in human hepatocytes, termed Ad-P450 cells. The activities of the five P450s expressed and their equivalence with hepatocytes were confirmed by measuring the unmetabolized drugs by MS. The LODs of all 11 pharmaceuticals by MS/MS detection were achieved after analytical validation using stable isotope labeled internal standards, and could be used for the metabolic variation analysis of various pharmaceuticals in pharmacokinetics and toxicokinetic studies.

**Conclusions**

In this study, a sensitive and analytical method was developed for the simultaneous detection of 11 chiral pharmaceuticals and their hepatic metabolites by LC-MS/MS using an OVM column. After optimization of the LC conditions, all pharmaceuticals examined were enantiomer-separated with R of >0.82 using LC-MS/MS. The LODs of all 11 pharmaceuticals by MS/MS detection ranged from 1.2 to 92.3 nM, approximately 1000 - 25000 times lower than those obtained by UV detection. From hepatic metabolite analyses in Ad-P450 cells, the metabolites of three pharmaceuticals (CLP, VER, PFE) and their metabolizing levels differ depending on each isomer.29,30 The results obtained in this study further supported these findings.

**Acknowledgements**

This study was supported in part by a JSPS KAKENHI Grant Number JP16K08200.