Journal of Pharmaceutical and Biomedical Analysis 155 (2018) 194–201

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

LC–MS/MS method for the simultaneous quantification of intestinal CYP and UGT activity

Diana Busch [a], Anja Fritz [a], Lars Ivo Partecke [b], Claus-Dieter Heidecke [b], Stefan Oswald [a,*]

[a] Department of Clinical Pharmacology, University Medicine Greifswald, Greifswald, Germany
[b] Department of General Surgery, Visceral, Thoracic and Vascular Surgery, University Medicine Greifswald, Greifswald, Germany

ARTICLE INFO

Article history:
Received 29 December 2017
Received in revised form 2 April 2018
Accepted 3 April 2018
Available online 5 April 2018

Keywords:
LC–MS/MS
Intestinal metabolism
CYP enzymes
UGT

ABSTRACT

Many orally administered drugs are subject to first-pass metabolism by cytochrome P450 (CYP) enzymes and uridine 5′-diphospho-glucuronosyltransferases (UGT). While their hepatic activity is well characterized, respective information about the intestine are very scare due to limited availability of tissue, very low microsomal protein content and the heterogeneity of the individual segments. As a consequence, determination of enzyme kinetic parameters is challenging. It was therefore the aim of this study to develop a sensitive liquid chromatography tandem mass spectrometry method for the simultaneous quantification of CYP and UGT metabolites formed by clinically relevant intestinal biotransformation enzymes: 4-hydroxydiclofenac (CYP2C9), 5-hydroxyomeprazole (CYP2C19), dextrorphan (CYP2D6), 1-hydroxymidazolam (CYP3A), ezetimibe glucuronide (UGT1A) and naloxone glucuronide (UGT2B7). After precipitation of microsomal protein with acetonitrile, analytes were chromatographically separated on a C18 column with gradient elution using acetonitrile and water, both containing 0.1% formic acid and detected with a tandem mass spectrometer operating in positive mode with electron spray ionization. The assay was validated according to current bioanalytical guidelines regarding linearity, accuracy, precision, stability, recovery and matrix effects spanning an analytical range from 1 to 200 nmol/L for each analyte. The developed method was successfully applied to a proof of concept experiment using pooled human jejunal microsomes (50 μg protein/mL) in order to determine enzyme kinetic parameters. Formation of all monitored metabolites followed Michaelis-Menten kinetics and allowed calculation of K_M and V_{max} values. The developed method may be useful for characterization of enzymatic activity in the human intestine which may allow more precise insights into the intestinal contribution to first pass metabolism of drugs.

1. Introduction

Drug metabolizing enzymes, categorized into phase I and phase II, play an important role for the oral absorption and the elimination of several compounds. Phase I and II metabolism increase the water solubility of the respective substrates by introducing/modifying functional groups or conjugating the substrate to endogenous substances and therefore facilitate their biliary and renal excretion. Approximately 75% of the drugs on the market undergo phase I metabolism by cytochrome P450 (CYP) enzymes, while uridine 5′-diphospho-glucuronosyltransferases (UGT), belonging to phase II enzymes, catalyze the turnover of nearly one third of the approved drugs [1]. Clinically relevant enzymes include CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5, UGT1A1, UGT1A3, UGT2B7 and UGT2B15 [2]. While their hepatic activity was studied extensively [3,4], investigations regarding their activity in the intestine are very limited [5–10] despite its high contribution to the first pass metabolism of many orally administered drugs [5]. Due to the limited availability of intestinal tissue and the very low microsomal protein amount (2–3 mg microsomal protein per mg intestinal tissue [11] compared to 30–40 mg microsomal protein per mg hepatic tissue [12]), studies were mainly

Abbreviations: 1-OH-MDZ, 1-hydroxymidazolam; 4-OH-DCL, 4-hydroxydiclofenac; 5-OH-OME, 5-hydroxyomeprazole; CYP, cytochrome P450 enzymes; DXP, dextrorphan; ESI, electrospray ionization; EZE-Glc, ezetimibe glucuronide; LC–MS/MS, liquid chromatography tandem mass spectrometry; NAL-Glc, naloxone glucuronide; UGT, uridine 5'-diphospho-glucuronosyltransferases.

* Corresponding author at: University Medicine Greifswald, Center of Drug Absorption and Transport (C_DAT), Department of Clinical Pharmacology, Felix-Hausdorff-Str. 3, D-17489 Greifswald, Germany.
E-mail address: stefan.oswald@uni-greifswald.de (S. Oswald).

https://doi.org/10.1016/j.jpba.2018.04.003
0731-7085/

conducted with pooled microsomes [7–10]. However, the intestine is not as homogenous as the liver, but shows a substantially different enzyme distribution in duodenum, jejunum, ileum and colon [13]. A study addressing this issue was conducted by Paine et al. [5] who assessed the intra-subject CYP3A metabolism in different intestinal segments [5]. According activity data on other drug metabolizing enzymes along the intestine are not yet available and cannot be derived from protein abundance due to various factors such as the heterogeneity of intestinal segments and procedural differences in the isolation of microsomal fractions [8]. In order to obtain data on intestinal drug metabolism and the respective enzyme kinetic parameters, sensitive and specific analytical methods are necessary.

Published assays usually focus either on CYP [3] or UGT metabolism [14–16], although both enzyme classes should be investigated simultaneously. Performing those reactions in the same microsomal preparation bears the risk of depleting CYP metabolites that might be substrates for subsequent phase II reactions. Despite the separate incubations, corresponding samples could be merged prior analysis to save time and resources [17]. Additionally, the so far available assays consider predominately hepatic phase I or phase II enzyme activity [3,14–16], but were not specifically tailored for the intestinal enzyme set, namely CYP2C9, CYP2C19, CYP2D6, CYP3A4, CYP3A5, UGT1A1, UGT1A3 and UGT2B7 [13,18].

From the methodical point of view, current state-of-the-art analysis of so called cocktail studies are done with liquid chromatography tandem mass spectrometry (LC–MS/MS) which enables specific and sensitive quantification of the respective metabolites [3,14–17].

It was therefore the aim of this study to develop and validate a LC–MS/MS method for the simultaneous quantification of 4-hydroxydiclofenac (CYP2C9), 5-hydroxyomeprazole (CYP2C19), dextrorphan (CYP2D6), 1-hydroxymidazolam (CYP3A4/5), ezetimibe glucuronide (UGT1A1/3) as well as naloxone glucuronide (UGT2B7) which considers both, the intestinal enzyme pattern and the very low microsomal protein content in order to enable the determination of enzyme kinetic parameters from small tissue amounts.

2. Materials and methods

2.1. Reagents and consumables

Acetonitrile and methanol were purchased in LC–MS quality (Chromasolv®, Sigma–Aldrich, Taufkirchen, Germany). Deionized water (conductance: ≤0.055 μS/cm, pH 5.0–6.0) was generated with the system Astacus (membrapure, Hennigsdorf, Germany). 4′-hydroxydiclofenac-$^{13}C_6$ solution, 4-hydroxydiclofenac, diclofenac sodium salt, 5-hydroxyomeprazole, omeprazole, dextrorphan-d_3 solution, dextrorphan tartrate, dextromethorphan hydrobromid, 1-hydroxymidazolam-d_4 solution, 1-hydroxymidazolam, midazolam hydrochloride, ezetimibe, naloxone-d_5-β-D-glucuronide solution as well as naloxone hydrochloride dihydrate were obtained from Sigma Aldrich (St. Louis, Missouri, USA), naloxone-3-β-D-glucuronide hydrate from Lipomed (Weil am Rhein, Germany) and ezetimibe phenoxy-β-D-glucuronide from Alsachim (Illkirch-Graffenstaden, France). With the exception of omeprazole and ezetimibe, solutions were prepared in deionized water. Omeprazole was dissolved in acetonitrile and ezetimibe in methanol. All solutions were stored at −80 °C until use. Pooled human liver microsomes, potassium dihydrogen phosphate, Gentest™ NADPH Regenerating System Solution A, Gentest™ NADPH Regenerating System Solution B, UGT Reaction Mix Solution A and UGT Reaction Mix Solution B were purchased from Corning (Corning, Amsterdam, The Netherlands). The Microsome Isolation Kit was ordered from Abcam (Cambridge, UK) and the Protease Inhibitor Cocktail Set III from Merck (Darmstadt, Germany). Isolation of the microsomal fraction and activity assays were conducted in Protein LoBind Tubes (Eppendorf, Hamburg, Germany).

2.2. Isolation of microsomal fraction

Jejunal tissue was collected from 11 patients undergoing intestinal surgery with approval by the local Ethics Committee of the University Medicine Greifswald. The collected samples were immediately snap frozen in liquid nitrogen and grinded in a stainless steel mortar prior to pooling them in equal shares. In triplicate, approximately 800 mg of the resulting tissue powder were placed in prechilled Douncers and homogenized with 1.6 mL homogenization buffer (Microsome Isolation Kit, Abcam) containing 5 μL/mL protease inhibitor. Homogenates were placed in Protein LoBind tubes on a vertical shaker for 30 min at 4 °C and centrifuged at 600*g* for 5 min at 4 °C. Subsequently, the supernatant was transferred to new Protein LoBind tubes for centrifugation at 100,000*g* for 70 min at 4 °C. Resulting pellets were washed with 300 μL homogenization buffer containing 5 μL/mL protease inhibitor and afterwards centrifuged at 100,000*g* for 70 min at 4 °C. The pellets were finally resuspended in 300 μL storage buffer (Microsome Isolation Kit, Abcam) containing 5 μL/mL protease inhibitor and stored at −80 °C until further use.

2.3. Determination of CYP and UGT activity

The protein content in the microsomal fractions was determined by the bicinchoninic acid assay (Thermo Fisher Scientific, Waltham, USA). To investigate the enzyme activity, the Gentest™ NADPH Regenerating System and UGT Reaction Mix from Corning® were used according to the manufacturer's instructions in a final reaction volume of 100 μL. The CYP approach contained the substrates diclofenac (CYP2C9), omeprazole (CYP2C19), dextromethorphan (CYP2D6) and midazolam (CYP3A) while the UGT approach included the substrates ezetimibe (UGT1A) and naloxone (UGT2B7) in six concentrations (1.0, 2.0, 3.0, 6.0, 12.0 and 25.0 μmol/L). Monitored biotransformation reactions are shown in Fig. 1. In addition, the CYP approach comprised of 50 μg/mL microsomal protein, 100 mmol/L potassium dihydrogen phosphate (pH 7.4), 3.3 mmol/L magnesium chloride, 1.3 mmol/L $NADP^+$, 3.3 mmol/L glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase and 0.15% acetonitrile. Further components of the UGT approach were 50 μg/mL microsomal protein, 50 mmol/L Tris-HCl (pH 7.5), 8 mmol/L magnesium chloride, 2 mmol/L uridine 5′-diphospho-glucuronic acid, 25 μg/mL alamethicin and 0.25% methanol. CYP and UGT reaction mixtures were incubated separately in a heating block at 37 °C. Substrate conversion was stopped after 30 min by precipitating the protein with 200 μL ice-cold acetonitrile. Reaction tubes were centrifuged at 21,100*g* at 4 °C for 10 min and the supernatants of the CYP and UGT metabolism from the same microsomal preparation were merged equally and stored at −80 °C until LC–MS/MS analysis.

2.4. LC–MS/MS analysis

The sample analysis was conducted with the Agilent 1260 Infinity Binary HPLC system (Agilent Technologies, Waldbronn, Germany) coupled to a QTRAP5500 mass spectrometer controlled by the Analyst® 1.6.3 software (Sciex, Darmstadt, Germany). For chromatographic separation, a XTerra® MS C18 column (2.1 × 100 mm, 3.5 μm, Waters Corporation, Milford, USA) was used with acetonitrile containing 0.1% formic acid (solvent A) and distilled water containing 0.1% formic acid (solvent B) applying a