Journal of Chromatography B, 878 (2010) 3267–3276

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Potential bias and mitigations when using stable isotope labeled parent drug as internal standard for LC–MS/MS quantitation of metabolites

Wenying Jian*, Richard W. Edom, Yaodong Xu, Joseph Gallagher, Naidong Weng

Bioanalysis/DMPK, Johnson and Johnson Pharmaceutical Research and Development, 1000 Route 202 South, Raritan, NJ 08869, USA

ARTICLE INFO

Article history:
Received 29 June 2010
Accepted 12 October 2010
Available online 20 October 2010

Keywords:
Stable isotope labeled internal standard
Metabolite
Quantitation
LC–MS/MS
Ion suppression
Safety assessment

ABSTRACT

In recent years, increasing emphasis has been placed on quantitative characterization of drug metabolites for better insight into the correlation between metabolite exposure and toxicological observations or pharmacological efficacy. One common strategy for metabolite quantitation is to adopt the stable isotope labeled (STIL) parent drug as the internal standard in an isotope dilution liquid chromatography–tandem mass spectrometry (LC–MS/MS) assay. In the current work, we demonstrate this strategy could have a potential pitfall resulting in quantitation bias if the internal standard is subject to ion suppression from the co-eluting parent drug in the incurred samples. Propranolol and its metabolite 4-hydroxypropranolol were used as model compounds to demonstrate this phenomenon and to systematically evaluate different approaches to mitigate the issue, including atmospheric pressure chemical ionization (APCI) mode of ionization, increased internal standard concentration, quantitation without internal standard, the use of a structural analog as internal standard, and dilution of the samples. Case studies of metabolite quantitation in nonclinical and clinical studies in drug development were also included to demonstrate the importance of using an appropriate bioanalytical strategy for metabolite quantitation in the real world. We present that bias of metabolite concentrations could pose a potential for poor estimation of safety risk. A strategy for quantitation of metabolites in support of drug safety assessment is proposed.

1. Introduction

In drug development, safety evaluation generally involves determination of parent drug plasma concentrations and calculation of exposure based on "area under the curve" (AUC). It is important to predict potential human risks based on nonclinical findings, and to control exposure levels in humans low enough to target a safety margin relative to the nonclinical species. In recent years, there is more widespread appreciation of the role of metabolites in drug toxicology evaluation due to interspecies differences in metabolism [1–4]. Concerns have been raised that certain drug metabolites could have inherent toxicity and, if they were not generated in experimental animals, such studies would not effectively assess their human risk potential. In addition, even if the same metabolites are produced in humans and experimental animal species, the exposure of a particular metabolite may vary considerably between humans and animals, a so-called disproportionate metabolite. If the metabolite is found at much higher levels in humans than in animal models, then it is argued that such a metabolite has not been appropriately assessed in preclinical toxicology studies. To address this issue, the Food and Drug Administration (FDA) published the guidance document "Guidance for Industry, Safety Testing of Drug Metabolites" (MIST) in February 2008 [5]. In June 2009, the International Conference on Harmonisation (ICH) M3 (R2) "Guidance on Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals" was published [6]. These documents stressed the need for quantitative assessment of systemic drug metabolite profiles in humans and the need for comparison of exposure levels of major metabolites with those derived in preclinical toxicology studies to avoid any potential risk associated with inadequate metabolite safety testing.

Under the ICH guideline, human metabolites that are observed at systemic exposures greater than 10% of total drug-related material at steady state should be quantified in the nonclinical toxicology species to compare the exposure. If the exposure in humans is sig-

Abbreviations: APCI, atmospheric pressure chemical ionization; AUC, area under the curve; ESI, electrospray ionization; FDA, Food and Drug Administration; HILIC, hydrophilic interaction chromatography; HQC, high QC; ICH, international conference on harmonisation; J&J PRD, Johnson and Johnson Pharmaceutical Research and Development; LC–MS/MS, liquid chromatography tandem mass spectrometry; LQC, low QC; MA, metabolite of compound A; MAD, multiple ascending dose; MB, metabolite of compound B; MC, metabolite of compound C; MQC, mid QC; MRM, multiple reaction monitoring; QC, quality control; STD, standard; STIL, stable isotope labeled.

* Corresponding author. Tel.: +1 908 927 6584; fax: +1 908 541 0422.
E-mail address: wjian@its.jnj.com (W. Jian).

1570-0232/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2010.10.008

nificantly greater than that in the nonclinical species, or if humans form a major metabolite which is unique (not observed in animal species), further evaluation may be warranted in nonclinical safety studies. This may involve the use of alternative animal species that form the metabolite at adequate exposures, or direct administration of the synthesized or isolated metabolite to animals for further safety testing. Phase II conjugate metabolites can be excused from further evaluation because they are generally considered to be pharmacologically inactive and readily excreted from the body. However, specific conjugates, such as acyl-glucuronides, may pose toxicological concerns by forming reactive intermediates and may warrant further safety assessment [7].

With the launch of regulatory guidelines for metabolite safety testing, greater emphasis has been placed on the quantitative aspects of metabolite characterization. Accurate measurement of metabolite concentration in nonclinical and clinical studies is crucial for decision-making in the scope of drug safety evaluation. Therefore, it is critical to adopt appropriate bioanalytical strategies for accurate measurement of metabolites.

Isotope dilution methodology has been commonly used in quantitative liquid chromatography tandem mass spectrometry (LC–MS/MS) assays in support of drug development. The very close chemical similarity between a stable isotope labeled (STIL) analyte and the analyte itself help to ensure that variations in extraction, stability, injection, chromatography, instrument fluctuation and matrix effects are adequately compensated for. However, for metabolite quantification, especially in early stages of drug development, a STIL metabolite is usually not available. A common practice is to conveniently adopt the STIL parent drug, which is more normally available at this stage, as the internal standard for measurement of metabolites. Given the fact that parent drug and the metabolite can be quite chemically similar in some cases, this approach may be appropriate. However, caution needs to be taken because significant bias for quantitation of the metabolite could be introduced if the mass spectrometric response of the STIL parent drug is subject to ion suppression by the co-eluting parent drug, which is the subject of this report.

In the current study, we used propranolol and its metabolite, 4-hydroxypropranolol, as model compounds to systematically investigate the impact of ion suppression of the parent drug to its STIL analog on the quantitation of the metabolite when the latter is used as the internal standard. We also propose and evaluate different strategies to mitigate this issue. Real world case studies for metabolite quantitation in nonclinical and clinical studies during drug development are shown to demonstrate the importance of using an appropriate strategy to avoid introducing bias into metabolite measurement.

2. Experimental

2.1. Chemicals and reagents

Propranolol and alprenolol were purchased from Sigma–Aldrich (St. Louis, MO). 4-hydroxypropranolol, D_7-propranolol, and D_7-4-hydroxypropranolol were obtained from C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada). Ammonium hydroxide (28–30% as NH_3 in water solution), formic acid, ethyl acetate, HPLC grade acetonitrile, and trifluoroacetic acid were obtained from EMD Chemicals Inc. (Gibbstown, NJ). HPLC grade dimethyl sulfoxide was purchased from Burdick and Jackson (Morristown, NJ). Blank rat and human plasma were obtained from Bioreclamation (Hicksville, NY). Compound A, MA (metabolite of compound A), compound B, MB1 and MB2 (metabolites of compound B), compound C, and MC (metabolite of compound C), and their STIL analogs were synthesized by Johnson and Johnson Pharmaceutical Research and Development (J&J PRD).

2.2. Standard and quality control sample preparation

All stock solutions used in this study were prepared at 1.00 mg/mL in 50/50: acetonitrile/dimethyl sulfoxide (v:v) and stored under refrigerated conditions.

Calibration standard (STD) samples of 4-hydroxypropranolol were prepared at concentrations of 5, 10, 20, 50, 100, 200, 400, and 500 ng/mL fresh daily by serially diluting the stock solution in blank rat plasma. Quality control (QC) samples containing 4-hydroxypropranolol at concentrations of 15 (LQC), 250 (MQC), and 400 (HQC) ng/mL were prepared fresh daily by serially diluting a separate stock solution in blank rat plasma. QC samples containing 4-hydroxypropranolol at concentrations of 15 (LQC), 250 (MQC), and 400 (HQC) ng/mL in the presence of propranolol were prepared fresh daily by serially diluting the stock solution in rat plasma spiked with propranolol at concentrations of 1000, 4000, or 20,000 ng/mL.

Calibration curve ranges were 1–500 ng/mL for MA, 5–5000 ng/mL for MB1 and MB2, and 1–1000 ng/mL for MC.

2.3. Sample preparation procedure

For 4-hydroxypropranolol, the plasma samples were processed using protein precipitation as follows. An aliquot (25 μL) of each sample was transferred into the wells of a Strata 2 mL protein precipitation filter plate (Phenomenex, Torrance, CA). Then, 100 μL of internal standard working solution of D_7-propranolol in acetonitrile (50 ng/mL) was added to each well. The plate was vortexed and centrifuged. The filtrate was diluted with 50 μL of water and mixed well. The injection volume was 5 μL.

For extraction of MA from human plasma, the samples were processed using liquid–liquid extraction. Briefly, an aliquot (50 μL) of each plasma sample was diluted with buffer (50 μL) and internal standard working solution (20 μL of 200 ng/mL of D_4-MA in water or 200 ng/mL of D_5-compound A in water) and extracted with 500 μL of ethyl acetate. The samples were evaporated, reconstituted using 100 μL of 95% acetonitrile in water (v:v), then injected to LC system operated under hydrophilic interaction chromatography (HILIC) conditions [8].

For extraction of MB1 and MB2 from human urine, the samples were processed using protein precipitation. An aliquot (25 μL) of each urine sample was diluted with 100 μL of human plasma. After thorough mixing, an aliquot (25 μL) of the diluted samples was then further precipitated with 200 μL of internal standard working solution (6 ng/mL each of D_6-MB1, D_7-MB2, and D_4-compound B in acetonitrile) and injected.

For extraction of MC from buffered rat plasma (rat plasma added with 30% relative volume of 0.5 M ammonium formate buffer for stabilization of MC, which is an acyl-glucuronide), the samples were processed using protein precipitation: an aliquot (40 μL) of each sample was mixed with 40 μL of 0.2% formic acid in 50% acetonitrile in water (v:v) and 25 μL of internal standard working solution (500 ng/mL of $^{13}C_4$, D_3-compound C in 50% acetonitrile in water), followed by precipitation using 100 μL of 0.2% formic acid in acetonitrile and injection.

2.4. Liquid chromatography and mass spectrometry

The HPLC system consisted of Shimadzu LC20AD pumps and a SIL-HTC autosampler (Columbia, MD). For analysis of 4-hydroxypropranolol, the HPLC system employed a Zorbax Eclipse XDB C18 column (2.1 mm × 50 mm, 5 μm, Santa Clara CA). HPLC mobile phase A was 0.2% formic acid in water (v/v), and mobile phase B was 0.2% formic acid in acetonitrile (v/v). Needle rinse solvent was 0.1% trifluoroacetic acid in 50% acetonitrile in water (v/v/v). The gradient elution started at 10% mobile phase B, ramped