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

linearly to 70% B over 2.0 min, and then returned to 10% B in 0.1 min. The run time was 3 min and the HPLC flow rate was 0.5 mL/min. For analysis of MA, the HPLC system employed a silica column operated under HILIC conditions. 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). The gradient was ramped linearly from 90% B to 40% B over 1.5 min. For analysis of MB1 and MB2, the HPLC system employed a silica column operated under HILIC conditions. HPLC mobile phase A was 10 mM ammonium acetate and 0.09% formic acid in water (v/v), and mobile phase B was 10 mM ammonium acetate and 0.09% formic acid in 90% acetonitrile in water (v/v/v). The gradient was held at 100% B for 1.5 min, and then ramped linearly to 35% B over 1.5 min. For analysis of MC, the HPLC system employed a C18 column operated under reversed-phase conditions. Mobile phase A was 0.1% formic acid in water (v/v), and mobile phase B was 0.1% formic acid in acetonitrile (v/v). The gradient was held at 10% B for 0.25 min, ramped linearly to 95% B in 1.5 min, and held at 95% B for 1 min before returning to starting conditions.

The HPLC systems were interfaced with an API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA). The operating parameters for analysis of propranolol and 4-hydroxypropranolol in positive Turbo Ionspray mode were: curtain gas 30 psi, GAS1 50 psi, GAS2 50 psi, Ionspray voltage 5000 V, source temperature 500 °C, and CAD gas 4 (arbitrary units). Multiple reaction monitoring (MRM) transitions were: propranolol, m/z 260 → 116; D_7-propranolol, m/z 267 → 116; 4-hydroxypropranolol, m/z 276 → 116. Declustering potential (DP) was 52 V and collision energy (CE) was 25 V for propranolol and D_7-propranolol. DP was 60 V and CE was 24 V for 4-hydroxypropranolol. Entrance potential (EP) was 10 V for all the molecular ions, and collision cell exit potential (CXP) was 5 V for all the product ions.

Mass spectrometric analysis for the other compounds in this study was carried out with an API 4000 triple quadrupole or an API 4000 Qtrap mass spectrometer operated in positive Turbo Ionspray mode. The compounds were monitored by MRM.

Integration and regression analysis for the chromatographic peaks of each analyte and internal standard were performed using Analyst® 1.4.1. software (Applied Biosystems, Foster City, CA). Linear regression with $1/x^2$ weighting was used for all compounds in this study. Area under the curve (AUC) was calculated using SigmaPlot 8.0 (Systat Software Inc., San Jose, CA).

2.5. Experiment procedures for different tests to mitigate the impact of ion suppression

2.5.1. Quantitation using APCI

To test if APCI can effectively eliminate ion suppression from propranolol to its STIL internal standard and provide better quantitation, the 4-hydroxypropranolol STD and QC samples were analyzed using an APCI method. The ion source parameters were: curtain gas 30 psi, GAS1 50 psi, ionization needle current 5 μA, source temperature 500 °C, and CAD gas 4 (arbitrary units). All the other experiment conditions were identical to those described in Sections 2.3–2.4.

2.5.2. Quantitation using increasing internal standard concentration

To test if increased internal standard concentration can mitigate the impact of ion suppression, internal standard spiking solution of D_7-propranolol in acetonitrile at concentration of 50, 500 and 1500 ng/mL, respectively, were used to extract the 4-hydroxypropranolol STD and QC samples. All the other conditions are identical as described in Sections 2.3 and 2.4.

2.5.3. Quantitation without an internal standard

The 4-hydroxypropranolol STD and QC samples were extracted and analyzed as described in Sections 2.3 and 2.4. The data were then processed using analyte/internal standard peak area ratio or analyte peak area only, to reveal if removal of internal standard from data processing can eliminate the bias caused by internal standard suppression.

2.5.4. Quantitation using a structural analog internal standard

To test if a structural analog could be free from ion suppression caused by propranolol and thereby provide better accuracy, alprenolol was evaluated as the internal standard. An internal standard working solution of alprenolol in acetonitrile (50 ng/mL) was used to extract the 4-hydroxypropranolol STD and QC samples. The MRM transition for alprenolol was m/z 250 → 116. DP, CE, EP, and CXP were 80, 24, 10, and 5 V, respectively. All other experimental conditions were identical to those described in Sections 2.3 and 2.4.

2.5.5. Sample dilution

To test if diluting the samples could alleviate ion suppression, 4-hydroxyproprolol STD and QC samples were extracted as described in Section 2.3. The samples were then diluted with 50% acetonitrile in water (v:v) either 4 times or 8 times post-extraction. All other experimental conditions were identical to those described in Section 2.4.

2.5.6. Quantitation using STIL analog of the metabolite

Internal standard working solution of D_7-4-hydroxypropranolol in acetonitrile (50 ng/mL) was used to extract the 4-hydroxypropranolol STD and QC samples. The MRM transition for D_7-4-hydroxypropranolol was m/z 283 → 123. DP, CE, EP, and CXP for were 60, 24, 10, and 5 V, respectively. All other experimental conditions were identical to those described in Sections 2.3–2.4.

3. Results and discussion

3.1. Quantitation of metabolites using the parent STIL internal standard

In support of drug development, bioanalytical assays for quantitation of drug candidates need to be validated according to FDA guidance [9]. A STIL drug as internal standard is commonly used to manage the influence of ion suppression from endogenous components such as phospholipids and exogenous additives such as dosing vehicles (polyethelyene glycol, cyclodextrin, Tween, etc.) [10]. However, several literature articles have reported that the STIL drug can also be subject to ion suppression from its non-labeled, native compound in electrospray ionization (ESI). For many compounds, it has been noted that the response for the STIL drugs decreased with increasing concentrations of the co-eluting, non-labeled analyte. This suppression phenomenon has been observed for samples extracted from biological matrices, as well as those prepared in neat organic solvent [11–13]. The mechanism of suppression can be explained by Enke's model of ESI generation, which involves competition among ions for the limited number of excess charged surface sites on generated droplets during the ESI process [14,15]. Sojo et al. showed that the response factor (ratio of analyte signal to that of the STIL parent drug normalized by analyte concentration) was constant, and thus ion suppression had no impact on the slope of the calibration curve and quantitation of the target analyte [12].

At early stages of drug development (nonclinical and Phase I clinical studies) when likelihood of drug success is considerably lower, and when the metabolite profile may not be completely clear [prior to the radiolabel human ADME (Absorption, Distribution, Metabolism, and Excretion) study], a STIL metabolite is usually

not prepared in trade-off for better allocation of resources. Instead, a STIL parent drug is often adopted as the internal standard for quantitation of the metabolites because it is commonly accessible at this stage of drug development. It may appear to be a scientifically sound choice because the parent drug and its metabolites, especially Phase I metabolites, can be chemically similar and are expected to display somewhat similar chromatographic properties and mass spectrometry responses. However, one needs to be cautious in taking this approach if the STIL parent drug is subject to suppression by the parent drug as described above. The metabolite is usually chromatographically separated from the parent drug, and therefore, the response of metabolite will not be affected by presence of parent drug, while that of the STIL parent drug may be suppressed when the parent drug concentration is high, resulting in increased metabolite/internal standard ratios. Thus, significant overestimation of the metabolite concentrations may result. The problem is particularly obscure in a separate metabolite assay because the STD and QC samples are prepared exclusively for the metabolite and contain no parent drug. The quantitation results of STDs and QCs may meet acceptance criteria for accuracy and precision, while the concentrations of metabolite in incurred samples, which contain both parent drug and metabolite, could be biased. It needs to be noted that separate assays are often favored over simultaneous assays for metabolite quantitation in early drug development for several reasons. These include differences between parent drug and metabolites in terms of exposure levels, sensitivity requirements, extraction procedures, regulatory rigor, as well as increased bioanalytical failure risk associated with multiple analyte assays. The examples demonstrated in this paper are all generated from separate metabolite assays.

To demonstrate this issue, an LC–MS/MS assay was developed for quantitation of 4-hydroxypropranolol, a metabolite of propranolol, using D_7-propranolol as the internal standard. STD samples (5–500 ng/mL) and QC samples (LQC at 15 ng/mL, MQC at 250 ng/mL, and HQC at 400 ng/mL) of 4-hydroxypropranolol were prepared consistent with a conventional bioanalytical protocol, containing no parent drug. As illustrated in Fig. 1A, the samples in the first oval, which are comprised of one set of STD and three replicates of each level of QC samples, show consistent internal standard peak area (the signal is normalized to that of the first sample). The standard curve is linear (data not shown) and the QC samples demonstrated acceptable accuracy and precision (Fig. 1B). To mimic incurred samples, another three sets of 4-hydroxypropranolol QC samples were prepared at the same concentrations as the first set of QCs but with propranolol spiked at 1000 ng/mL (P_1000), 4000 ng/mL (P_4000), and 20,000 ng/mL (P_20000). The second oval in Fig. 1A enclosed the 9 QC samples containing propranolol at 1000 ng/mL (3 replicates at each level). The internal standard peak area was suppressed by 20%, compared to those containing no propranolol (P_0). Accordingly, all levels of the QCs were determined to be higher than their nominal concentration by ~13–30% (Fig. 1B). Propranolol at 4000 ng/mL introduced ~30% suppression to the internal standard response and the QCs were overestimated by 30–60% (Fig. 1). When propranolol was present at a concentration of 20,000 ng/mL, internal standard peak area was suppressed by ~50%, and %bias as high as ~100% was observed for QC quantitation (Fig. 1).

The example demonstrated above shows that significant bias could be introduced by using a STIL parent drug for quantitation of a metabolite, if the STIL parent drug is subject to ion suppression from parent drug present at high concentrations. The error can occur at any concentration of metabolite because the determining factor is ion suppression to the internal standard, which is independent of metabolite concentration. The extent of bias is proportional to the parent drug concentration, and higher concentrations would introduce larger deviations. The concentration levels demonstrated

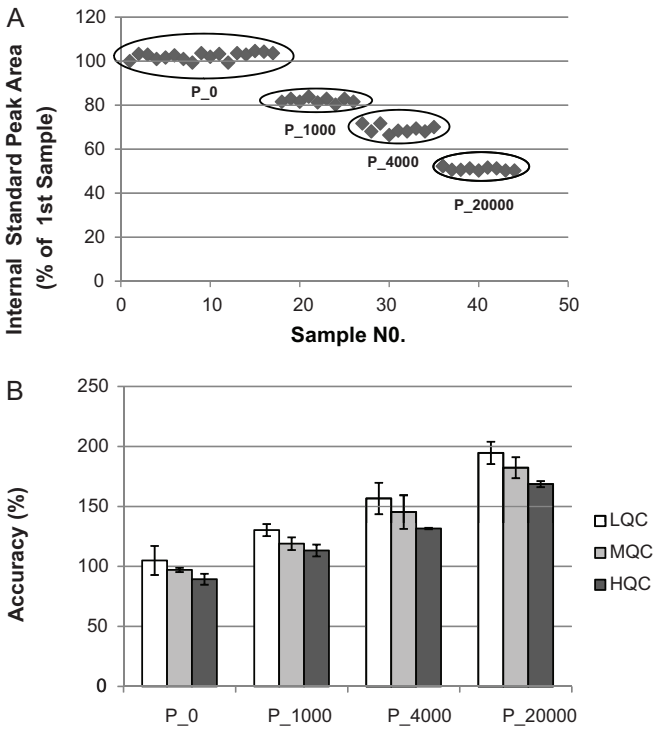

Fig. 1. Quantitative LC–MS/MS analysis of 4-hydroxypropranolol in rat plasma using D_7-propranolol as internal standard. This figure demonstrates the decrease in response of the STIL parent drug caused by different concentrations of the parent drug in the samples, and the resulting quantitation bias for the metabolite. (A) Internal standard (D_7-propranolol) peak area profile. The samples in the first oval (P_0) were STD1–STD8, LQC (3×), MQC (3×), and HQC (3×). The samples in the second oval (P_1000) were LQC (3×), MQC (3×), and HQC (3×), all with propranolol at 1000 ng/mL. The samples in the third oval (P_4000) were LQC (3×), MQC (3×), and HQC (3×), all with propranolol at 4000 ng/mL. The samples in the fourth oval (P_20000) were LQC (3×), MQC (3×), and HQC (3×), all with propranolol at 20,000 ng/mL. The signal intensity was normalized to that of the first sample on the plot (STD1, 3.04e5). (B) Quantitation results of 4-hydroxypropranolol QCs containing different concentrations of propranolol (0, 1000, 4000, 20,000 ng/mL). The data is expressed as accuracy (%) of nominal concentrations.

in this example (as high as 20,000 ng/mL), which are commonly encountered in animal toxicology studies, introduced bias up to 2-fold in the measured metabolite concentrations. It may be expected that even larger bias could occur if the parent drug was present at higher concentrations, or if the STIL parent drug was more susceptible to ion suppression, as ion suppression can be compound dependent.

3.2. Strategies to mitigate the impact of ion suppression

There are several possible strategies that can be used to alleviate or eliminate the impact of ion suppression from a parent drug, leading to improved accuracy for quantitation of metabolites. In this study, we systematically explored these approaches and evaluated their effectiveness and feasibility.

3.2.1. APCI

APCI as an alternative ionization technique has been shown to be generally much less prone to ion suppression than ESI [16,17]. The same set of samples demonstrated in Section 3.1 (Fig. 1) was re-injected using an APCI method. As shown in Fig. 2A, the STIL propranolol in the propranolol-containing QC samples that was subjected to ion suppression using ESI was free from suppression under APCI mode. As a result, the QCs demonstrated excellent accuracy and precision at all levels, independent of parent concentrations (LQC result as a representative was shown in Fig. 2B).

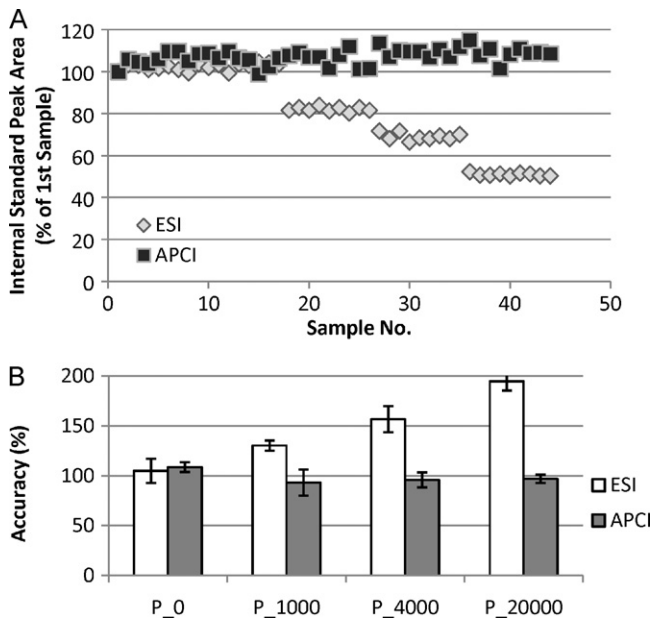

Fig. 2. Quantitative LC–MS/MS analysis of 4-hydroxypropranolol in rat plasma using D_7-propranolol as internal standard. Comparison between ESI and APCI. APCI effectively eliminated the ion suppression and quantitation bias observed under ESI mode. (A) Internal standard (D_7-propranolol) peak area profile using either ESI or APCI. The sample sequence is same as that of Fig. 1. The signal intensity was normalized to that of the first samples on the plot (STD1, ESI, 3.04e5; APCI, 3.40e4). (B) Quantitation results for 4-hydroxypropranolol LQC containing different concentrations of propranolol (0, 1000, 4000, 20,000 ng/mL) using either ESI or APCI. The data is expressed as accuracy (%) of the nominal concentrations.

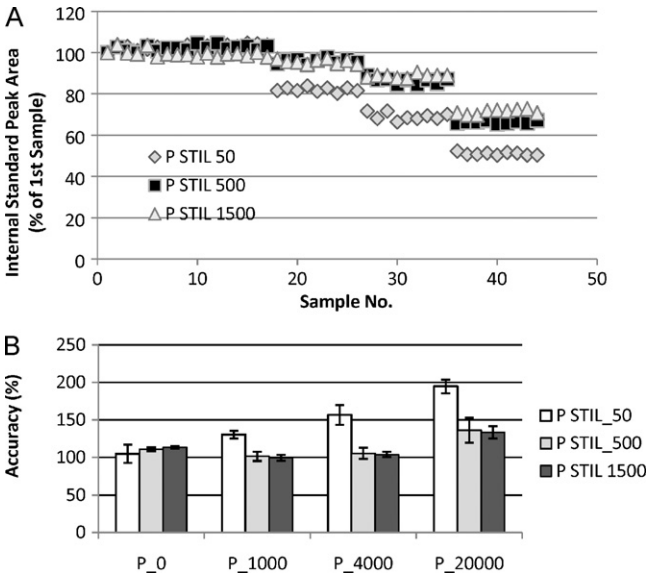

Fig. 3. Quantitative LC–MS/MS analysis of 4-hydroxypropranolol in rat plasma using different concentrations of D_7-propranolol as internal standard. Increasing internal standard concentration partially mitigated the effect of ion suppression and quantitation bias observed under ESI mode. (A) Internal standard (D_7-propranolol) peak area profile using internal standard working solution at concentrations of 50, 500, and 1500 ng/mL. The sample sequence is same as that of Figure 1. The signal intensity was normalized to that of the first samples on the plot (STD1, 50 ng/mL, 3.04e5; 500 ng/mL, 2.19e6; 1500 ng/mL, 5.45e6). (B) Quantitation result of 4-hydroxypropranolol LQC containing different concentrations of propranolol (0, 1000, 4000, 20,000 ng/mL) using internal standard working solution at concentrations of 50, 500, and 1500 ng/mL. The data is expressed as accuracy (%) of nominal concentrations.

Therefore, APCI can be used for quantitation of metabolites for better accuracy, if ion suppression from the parent drug to its internal standard is a known issue. However, one needs to keep in mind that APCI is not feasible for thermally liable compounds or metabolites that break-down or convert to the parent drug in the ion source, such as glucuronides, *N*-oxide metabolites, disulfides, and hydroxy acids, etc. [18–20]. In addition, APCI is often less sensitive than ESI and may not be an optimal choice for assays requiring high sensitivity.

3.2.2. Increasing internal standard concentration

In the present study, it was speculated that higher internal standard concentrations could lessen the impact of ion suppression because there would be more internal standard molecules to compete for ionization, thus decreasing the percentage of internal standard suppression. To test this hypothesis, internal standard working solutions at concentrations of 50 ng/mL (1×), 500 ng/mL (10×) and 1500 ng/mL (30×) for D_7-propranolol were used to extract the plasma samples and to compare the internal standard response profile and QC quantitation results. As expected, increasing the internal standard concentration reduced the relative suppression of the internal standard signal intensity. At internal standard concentrations of 500 ng/mL and 1500 ng/mL, the suppression for QC samples containing propranolol at 1000 ng/mL and 4000 ng/mL was effectively mitigated and the accuracy of quantitation was excellent (Fig. 3). However, in the presence of higher levels of propranolol, i.e. 20,000 ng/mL, increased internal standard concentration did not effectively mitigate the impact of ion suppression to acceptable extent, and the QCs were still biased by 20–40% (Fig. 3). In addition, minimal difference was observed between the results from internal standard concentration of 500 ng/mL and those from 1500 ng/mL, implying the potential for improvement by further increasing the internal standard concentration is limited.

From the above experiment, it can be inferred that increasing internal standard concentration might be a feasible way to mitigate the current issue. To adopt this strategy, metabolite QC samples containing parent drug at the highest level observed in the incurred samples need to be used to test for an internal standard concentration that can effectively eliminate the impact of suppression. However, this approach does have limitations. As shown in the example of propranolol, if the parent concentration is too high, the effect from suppression cannot be completely eliminated.

3.2.3. Quantitation without an internal standard

The quantitation error discussed in the present study was attributed to internal standard response suppression, and therefore, removal of internal standard from the assay may eliminate the problem. The same set of data was processed either by peak area ratio (4-hydroxypropranolol/D_7-propranolol) or peak area alone (4-hydroxypropranolol) to establish the calibration curve and to calculate concentrations. The quantitation results were then compared. Quantitation using peak area alone was clearly free from the bias, and all the QC concentrations calculated close to their nominal concentrations (Data not shown).

Therefore, quantitation without internal standard can be a choice for quantitation of metabolites if suppression from parent drug to its internal standard is observed. The process needs to be tightly controlled, though, due to the lack of internal standard to compensate for extraction recovery, analyte stability, injection volume variations, matrix effects, and instrument response drifting. Importantly, for metabolites that are subject to significant matrix effects, such as those caused by the presence of an intravenous dosing vehicle, quantification without an internal standard might not be a feasible choice. Post-column infusion experiments and phospholipid profiling with incurred samples may be needed to ensure that analytes elute away from any suppression or enhance-

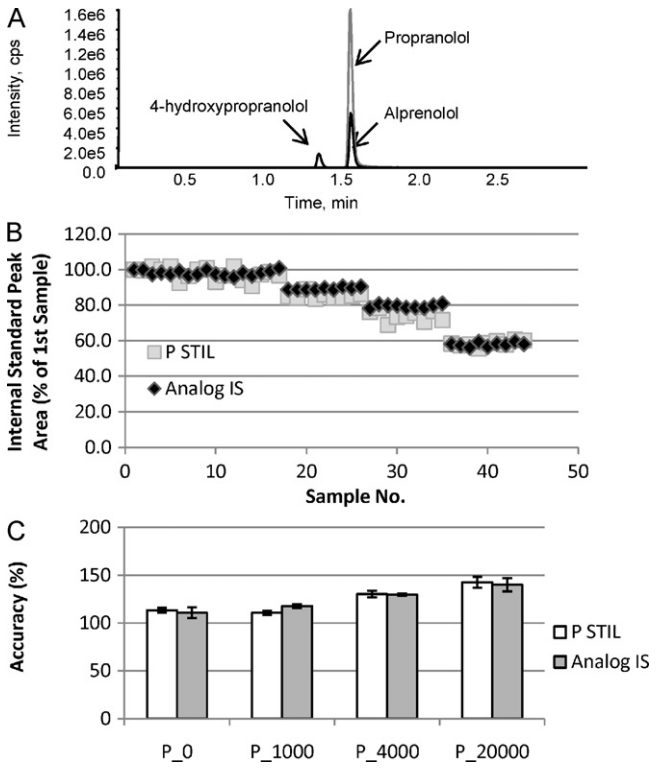

Fig. 4. Quantitative LC–MS/MS analysis of 4-hydroxypropranolol in rat plasma. Comparison between D_7-propranolol (P STIL) and alprenolol (Analog IS) as internal standard. The analog internal standard, alprenolol, was subject to ion suppression by co-eluting propranolol and therefore exhibited similar quantitation bias as using D_7-propranolol. (A) LC–MS/MS chromatogram of a 4-hydroxypropranolol HQC (400 ng/mL) containing propranolol at 1000 ng/mL using alprenolol as internal standard. (B) Internal standard (D_7-propranolol or alprenolol) peak area profile. The sample sequence is same as that of Fig. 1. The signal intensity was normalized to that of the first samples on the plot (STD1, D_7-propranolol, 2.18e5; alprenolol, 1.16e6). (C) Quantitation result of 4-hydroxypropranolol LQC containing different concentrations of propranolol (0, 1000, 4000, 20,000 ng/mL) using D_7-propranolol or alprenolol as internal standard. The data is expressed as accuracy (%) of nominal concentrations.

ment zones [21–23]. In addition, human plasma samples would require more diligent method development because there may be larger inter-subject variation in the matrix components compared to experimental animals due to larger variability in diet, environment and genetic factors.

3.2.4. Structural analog internal standard

It was questioned whether a structural analog would be free from the ion suppression effects of the parent drug and therefore provide better accuracy for metabolite quantitation than a STIL parent drug. Therefore, alprenolol, a structural analog of propranolol, was used as internal standard and tested for its effectiveness in avoiding quantitation error. Alprenolol was found to co-elute with propranolol (Fig. 4A). As it is highly structurally similar to propranolol, it was subjected to ion suppression from propranolol to almost the same extent as D_7-propranolol (Fig. 4B). As a result, there was no significant difference between the quantitation data obtained using alprenolol as internal standard versus that from D_7-propranolol, especially when propranolol was present at 20,000 ng/mL (LQC result as a representative was shown in Fig. 4C).

This case demonstrated a worst case scenario when using a structural analog as internal standard for metabolite quantitation. If a structural analog is to be used as internal standard for metabolite quantitation, it is important to choose one that does not co-elute with the parent drug in order to avoid potential ion suppression. Preferably, the internal standard would co-elute with the metabolite so that the chance for it to experience similar suppression or enhancement as the metabolite is larger. Regardless whether the structural analog co-elutes with the metabolite or not, it is important to thoroughly evaluate its effectiveness as an internal standard, because it may experience different matrix effects as the targeted analyte.

3.2.5. Sample dilution

It was speculated that dilution of the samples would decrease the concentration of ions present in the ion source and therefore reduce suppression. To test this hypothesis, the same set of samples as presented in Section 3.1 was diluted either 4 times or 8 times post-extraction, and the quantitation results were compared to that of non-diluted samples (data not shown). It was found there was slight alleviation of ion suppression and the quantitation results for the QC samples containing relatively low parent concentration (propranolol at 1000 ng/mL) were brought into an acceptable range. However, when higher propranolol concentrations were present in the samples, the effect of dilution was marginal. In addition, 8 times dilution was slightly more effective than 4 times dilution. However, further dilution would not be feasible because signal intensity would be too low. An alternative way of conducting dilution is to dilute the incurred samples before extraction to reduce the concentration of the parent drug. However, this approach may only have limited application because metabolite concentrations can be significantly lower than the parent, and thus many fold of dilution cannot be afforded.

Overall, diluting the samples may be a remedy for the current issue only in limited scenarios when metabolite concentrations are relatively high, parent concentration is relatively low, and the instrument sensitivity is sufficient. Systematic investigation prior to sample analysis would need to be conducted if this approach was to be taken.

3.2.6. STIL metabolite as the internal standard

Unquestionably, the most suitable internal standard for metabolite quantitation is the dedicated STIL metabolite. As shown in Fig. 5, D_7-4-hydroxypropranolol is free from ion suppression of propranolol and the quantitation result for 4-hydroxypropranolol met the acceptance criteria regardless of the presence of propranolol.

However, one needs to be aware of the expense and time associated with synthesis of a dedicated STIL metabolite, especially in cases when multiple metabolites need to be measured in a study. At early stages of drug development, when the chance for drug success is lower, it may be wasteful to prepare a dedicated STIL metabolite. Nevertheless, when there is ion suppression from parent drug to its internal standard, analytical methods using a STIL metabolite as the internal standard provide the most accurate quantitation results compared to other approaches discussed above. A dedicated STIL metabolite should be considered to ensure data quality whenever metabolite data is considered critical to the development for a drug.

3.3. Case studies

3.3.1. Quantitation of a metabolite in human plasma

Compound A is a drug candidate being developed at J&J PRD. Its metabolite, MA, has been identified as a major metabolite in human plasma in a Phase I clinical study. In order to compare exposure levels of MA in humans to those determined in toxicology studies, selected samples from the multiple ascending dose (MAD) phase of a clinical study were analyzed for concentrations of MA. The assay employed liquid–liquid extraction as the sample clean-up method, and a STIL MA (D_4-MA) as internal standard. To investigate the impact of using the STIL parent drug for metabolite quantitation, the same set of samples were reanalyzed using STIL

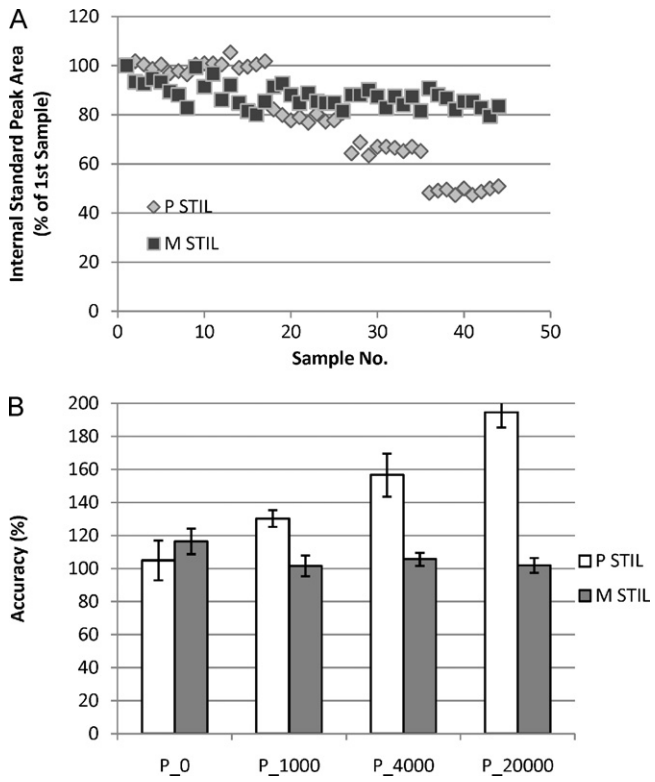

Fig. 5. Quantitative LC–MS/MS analysis of 4-hydroxypropranolol in rat plasma using D_7-hydroxypropranolol (M STIL) or D_7-propranolol (P STIL) as internal standard. The dedicated STIL metabolite is free from ion suppression by parent drug and it therefore achieved unbiased quantitation results. (A) Internal standard peak area profile using either D_7-hydroxypropranolol (M STIL) or D_7-propranolol (P STIL). The sample sequence is same as that of Fig. 1. The signal intensity was normalized to that of the first samples on the plot (STD1, M STIL, 1.51e5; P STIL, 3.04e5). (B) Quantitation result of 4-hydroxypropranolol LQC containing different concentrations of propranolol (0, 1000, 4000, 20,000 ng/mL) using D_7-hydroxypropranolol (M STIL) or D_7-propranolol (P STIL) as internal standard. The data is expressed as accuracy (%) of nominal concentrations.

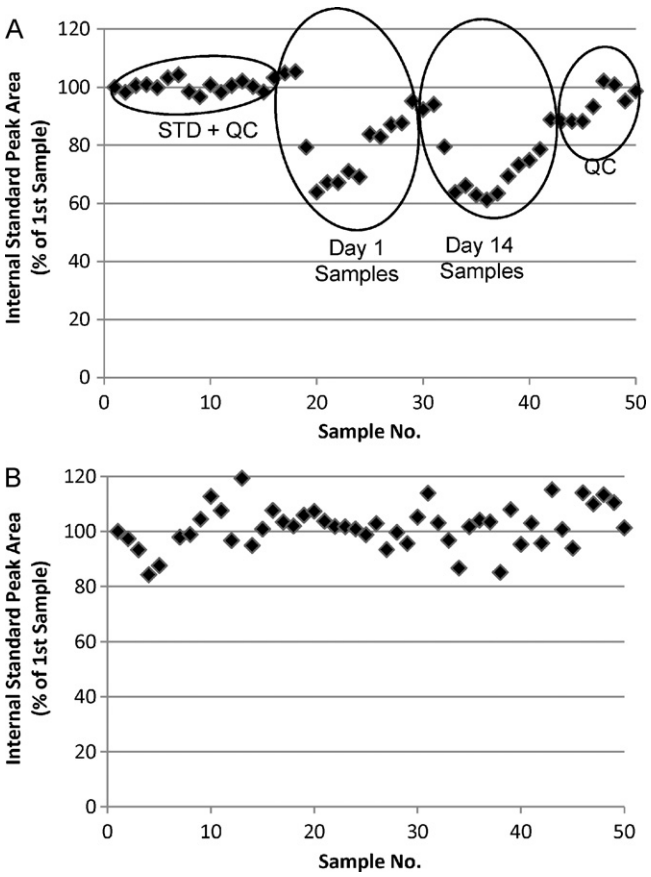

Fig. 6. Quantitative LC–MS/MS analysis of MA in human plasma in a MAD study of compound A using either D_5-compound A (P STIL, A) or D_4-MA (M STIL, B) as internal standard. The STIL parent drug was subject to ion suppression from the parent drug in the incurred samples while the STIL metabolite was free from suppression. (A) Internal standard (D_5-compound A) peak area profile. The samples in the first oval were STD1–STD8, LQC (2×), MQC2 (2×), MQC1 (2×), and HQC (2×). The samples in the second oval and the third oval were incurred samples from one subject on Day 1 and Day 14, respectively. The samples in the fourth oval were LQC (2×), MQC2 (2×), MQC1 (2×), and HQC (2×). The signal intensity was normalized to that of the first sample on the plot (STD1, 6.73e5). (B) Internal standard (D_4-MA) peak area profile. The sample sequence is same as that of (A). The signal intensity was normalized to that of the first sample on the plot (STD1, 5.39e5).

compound A (D_5-compound A) as internal standard and the results were compared with those obtained using D_4-MA.

Fig. 6A shows the internal standard response profile for analysis of Day 1 and Day 14 samples from one subject using D_5-compound A as internal standard. The internal standard response for STD and QC samples was consistently 90–110% of the first sample, as shown in the first and fourth ovals. In comparison, the internal standard response of the incurred samples showed time-point dependent suppression, which was proportional to the parent drug concentration in the samples (time points and parent drug concentrations for Day 14 samples are shown in Fig. 7). As these samples are from human oral solid-dose administration, ion suppression caused by a dosing vehicle [24] was ruled out. This was further confirmed by the observation that the response of D_4-MA as internal standard was relatively consistent (Fig. 6B). Even though the STD and QC samples met acceptance criteria using either STIL parent drug or STIL metabolite as internal standard, the quantitation results were significantly different between these two approaches due to the presence of compound A in the incurred samples and its ion suppression to the STIL parent drug. Using Day 14 samples from one of the subjects as an example, MA concentrations measured using the STIL parent drug were higher than those measured using the STIL MA (Fig. 7). As a result, the % AUC of MA to that of parent drug was calculated to be 27.5% using STIL parent drug, instead of 22.9% as determined using STIL MA, an overestimation of 20.2%.

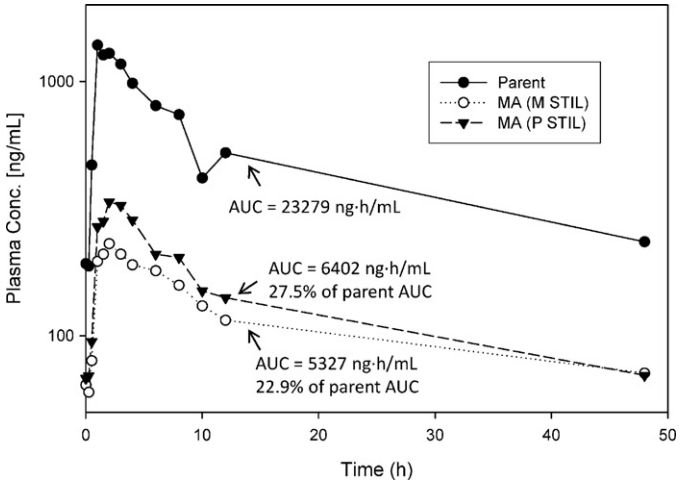

Fig. 7. Plasma concentration–time course of compound A (parent drug), and MA (metabolite A), following repeated dosing of compound A for 14 days, determined using either D_4-MA (M STIL) or D_5-compound A (P STIL) as internal standard. AUC of MA was overestimated by 20.2% by using the STIL Compound A.

The data demonstrated in this example imply that a potential issue for safety evaluation could arise by using STIL parent drug for quantitation of metabolites. In some cases, overestimation of metabolite concentrations could make the exposure appear to reach the threshold (10% of total drug-related exposure according to ICH guidance) [6] when it actually does not. This may trigger a commitment to quantify metabolite concentrations in animals and humans without there being a truly justified reason to do so. Conversely, the overestimation of metabolite concentrations in animals, which is more prone to occur in animal studies than those conducted in humans due to the higher parent drug concentrations often observed in toxicology studies, may pose a more serious problem. Overestimation of metabolite exposure in animals may make it appear that appropriate safety margins have been achieved when in actual fact, they have not.

3.3.2. Quantitation of metabolites in human urine

Compound B is also a drug candidate being developed at J&J PRD. A combined assay for metabolite B1 (MB1) and metabolite B2 (MB2) in human urine was developed which employed a dedicated STIL analog as the internal standard for each metabolite. To investigate the impact of using the STIL parent drug for metabolite quantitation, it was added together with the STIL metabolites during the sample extraction. There was base-line chromatographic separation between MB1, MB2, and Compound B, and the STIL compounds almost co-eluted with their corresponding natural compounds. The metabolite data was processed using their respective STIL metabolite as the internal standard, and then again using the STIL parent drug as internal standard to compare the calculated concentrations. The STD and QC samples met acceptance criteria using either approach. However, for the incurred samples, there was a significant difference between the calculated metabolite concentrations. Table 1 shows representative data from MB1 in one of the subjects. The %Bias between the two approaches ranged from 33.9% to 214.4%, largely proportional to the parent drug concentration in the samples. As expected, the difference was due to presence of very high concentrations of compound B in the incurred samples and the suppression from compound B to its STIL analog. The STIL parent drug response was suppressed as much as 70% while that of the metabolites was not affected (internal standard response profiles not shown). Bias of a similar magnitude was also observed for MB2 (data not shown), which was expected because the error was introduced by STIL parent drug suppression which was identical for both metabolites. Additionally, because the two sets of concentrations were determined in a single run, inter-assay variability can be excluded as a source of bias.

Accumulative excretion of MB1 from urine in the subject shown in Table 1 was calculated from the respective urine volumes. There was a compelling difference between the data obtained by the two approaches. The amount excreted at end of 120 h was calculated to be 44.4 mg using data obtained with the STIL parent drug, which was 2.7-fold of that determined by the STIL metabolite (16.4 mg). This example further demonstrated that a significant error in estimation of metabolite exposure/excretion could be introduced if the STIL parent drug is inappropriately used as the internal standard when ion suppression occurs.

3.3.3. Quantitation of a metabolite in rat plasma

MC was identified as an acyl-glucuronide metabolite of compound C, a drug candidate currently being developed at J&J PRD. An acyl-glucuronide metabolite is considered a potentially reactive metabolite, which may have toxicity implications, and therefore, it was deemed necessary to quantify MC in rats in a one-month repeated-dose toxicology study. The concentration of parent drug, compound C, in the samples was found to range from ~ 8 to 400 μg/mL, raising the concern of ion suppression and quantitation bias if the STIL parent drug was to be used as internal standard. A STIL metabolite would ideally eliminate this issue and provide accurate quantitation, however, it was not available at the time of this experiment. Analysis using APCI ionization mode was excluded due to the thermal liability of acyl-glucuronides. Instead, it was decided to use peak area alone (no internal standard) to conduct the quantitation in order to avoid any potential bias introduced by using parent internal standard. Since analysis without an internal standard would reduce our ability to ascertain the performance of the assay, the STIL parent drug, $^{13}C_4$, D_3-compound C, was added in every sample. Its presence was used to monitor some key aspects of the analysis, such as chromatographic performance, but it was not used directly as an internal standard to construct the peak area ratio since its response would not necessarily track that of the analyte, MC. In addition, post-column infusion and phospholipid profiling experiments were conducted to confirm there was no significant ion suppression for MC in the incurred samples. The assay performed well, even without an internal standard. It was also decided to evaluate the impact had the STIL parent drug actually been used as the internal standard. Data was re-processed using the peak area ratio of MC to that of $^{13}C_4$, D_3-compound C. As expected, the internal standard response in the incurred samples dropped as much as ~10-fold, compared to that of the STDs and QCs due to the high concentrations of parent drug present in the incurred samples. As a result, the plasma concentration of MC calculated using peak area ratio was higher than determined using peak area alone by as much as ~10-fold. When the peak area ratio was used, AUC calculated using the mean concentration from four male rats on Day 24 was overestimated by 676% (7.76-fold) from 6731 ng h/mL to 52,199 ng h/mL (Fig. 8).

This example demonstrated the potential risk of using the STIL parent drug as the internal standard for quantitation of metabolites in nonclinical studies. The higher dose levels of nonclinical

Table 1
Comparison of urine concentrations of MB1 determined using D_6-MB1 or D_4-compound B from one subject following repeated dosing of compound B for 14 days.

Time (h)	Compound B conc. [ng/mL]	MB1 conc. (D_6-MB1) [ng/mL]	MB1 conc. (D_4-compound B) [ng/mL]	% Bias caused by using D_4-compound B
0–4	227,000	8270	26,000	214.4
4–8	120,000	6770	19,900	193.9
8–12	110,000	9150	23,700	159.0
12–24	143,000	12,900	36,000	179.1
24–48	37,600	3680	6650	80.7
48–72	14,300	1680	4200	150.0
72–96	7420	930	1920	106.5
96–120	4240	334	682	104.2
120–312	2600	203	338	66.5
312–480	1240	125	183	46.4
480–648	783	81.1	109	34.4
648–816	447	50.4	67.5	33.9

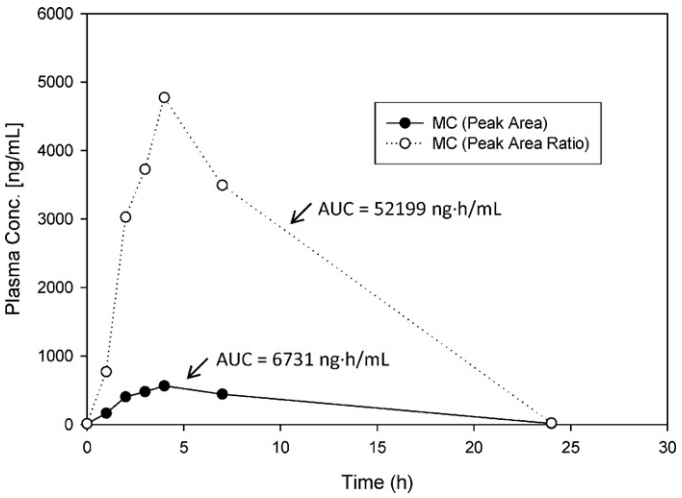

Fig. 8. Mean plasma concentration–time course of MC (metabolite C) in rats ($n = 4$) determined using either peak area or peak area ratio (MC to $^{13}C_4$, D_3-compound C) following repeated dosing of compound C for 24 days. A % Bias of 676% (7.76-fold) in plasma AUC of MC was introduced by using the STIL compound C as internal standard.

safety studies will likely have many fold higher exposure of parent drug than those in human studies conducted at an efficacious dose. Therefore, metabolite concentration may more likely be affected in nonclinical than clinical studies, leading to potential mistakes in safety margin judgments, or to the false appearance that toxicology of the metabolite has been adequately assessed in the nonclinical studies.

4. Conclusions

In this work, we demonstrated that potential quantitation bias for metabolites could be caused by using the STIL parent drug as the internal standard in an LC–MS/MS assay. Ion suppression of the parent drug to its co-eluting STIL parent drug could result in overestimation of metabolite concentrations in the incurred samples, giving rise to misleading information.

Overestimation of metabolite concentrations in clinical studies could make the exposure in human to appear to reach the 10% threshold, when it actually does not, thereby triggering further quantitation work and potential evaluation in additional toxicology studies. Conversely, overestimation of the metabolite in nonclinical studies poses a greater concern since there is a potential to artificially increase the safety margin or give the false impression that the toxicology of the metabolite has been adequately assessed in the nonclinical studies. Since the exposure of parent drug in animal toxicology studies is usually much higher than in humans, the chance and magnitude of metabolite overestimation in nonclinical studies is expected to be much higher than in clinical studies. Therefore, it is critical to take an appropriate approach for accurate measurement of metabolites as early as possible in drug development.

Based on the discussion in this current study, a recommended strategy for quantitation of metabolites in support of drug safety assessment is proposed. Ideally, the best internal standard for metabolite quantitation is a dedicated STIL metabolite, the signal intensity of which will closely track that of the metabolite but be independent from the parent drug. However, the availability of a STIL metabolite is always limited by cost and time, especially at the early stages of drug development.

If a STIL metabolite is not available, a good alternative could be a structural analog of the metabolite which preferably co-elutes with the metabolite, or at least does not co-elute with the parent drug. However, if there is no other choice than using the STIL parent drug, an investigation needs to be conducted to find out if there is ion suppression from the parent drug to its internal standard, which does not always happen. One way of elucidating ion suppression is to inspect the internal standard response profile in the parent drug assay to visualize if there is any concentration-dependent ion suppression. However, one needs to be aware that any sample containing parent drug concentrations above the upper limit of quantitation will have been diluted in the parent drug assay, while those same samples may be reanalyzed for the metabolite using a full aliquot if the metabolite is present in much lower concentrations than the parent drug. Therefore, a visual inspection of the internal standard response in the parent drug assay may not clearly elucidate a trend toward concentration-dependent suppression of the internal standard. Metabolite QC samples containing the parent drug at the highest levels observed in the incurred samples need to be prepared to test if the internal standard is free from suppression. If ion suppression is present, APCI could be adopted if there are no issues with thermal liability or in-source degradation and if the sensitivity of APCI satisfies the requirement of the assay. Alternatively, an increase to the internal standard concentration, or dilution of the incurred samples, may also be explored as ways of mitigating the quantitation bias. Regardless of the specific approach, the internal standard response profile in the incurred samples needs to be closely monitored to reveal any abnormal variations. Finally, in the absence of other viable alternatives, metabolite quantitation could be conducted with no internal standard. Careful attention is always required when using this approach. The metabolite ionization must be free from significant matrix effects and the sample extraction procedure must not be too complicated. Otherwise, without internal standard to compensate for variability, the performance of the assay could be compromised. We have successfully used this approach on a few occasions such as the quantification of acyl-glucuronide conjugates where finding an appropriate internal standard is particularly challenging.

In summary, quantitative metabolite data is considered important to the development plan for new drug candidates, and there are many known pitfalls which can complicate the acquisition of accurate data. We have investigated ion suppression from a parent drug to its stable isotope labeled parent drug as internal standard, which could cause inaccuracy in data when this internal standard is also applied for quantitation of metabolites.

References

[1] T.A. Baillie, M.N. Cayen, H. Fouda, R.J. Gerson, J.D. Green, S.J. Grossman, L.J. Klunk, B. LeBlanc, D.G. Perkins, L.A. Shipley, Toxicol. Appl. Pharmacol. 182 (2002) 188.
[2] D.C. Evans, A.P. Watt, D.A. Nicoll-Griffith, T.A. Baillie, Chem. Res. Toxicol. 17 (2004) 3.
[3] D.C. Liebler, F.P. Guengerich, Nat. Rev. Drug Discov. 4 (2005) 410.
[4] L. Leclercq, F. Cuyckens, G.S. Mannens, R. de Vries, P. Timmerman, D.C. Evans, Chem. Res. Toxicol. 22 (2009) 280.
[5] FDA, Guidance for Industry: Safety Testing of Drug Metabolites (2008).
[6] ICH, CPMP/ICH/286/95 ICH Topic M3 (R2) Non-clinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals (2009).
[7] E.M. Faed, Drug Metab. Rev. 15 (1984) 1213.
[8] W. Jian, R.W. Edom, Y. Xu, N. Weng, J. Sep. Sci. 33 (2010) 681.
[9] FDA, Guidance for Industry: Bioanalytical Validation (2001).
[10] X.S. Tong, J. Wang, S. Zeng, J.V. Pivnichny, P.R. Griffin, X. Shen, M. Donnelly, K. Cakerich, C. Nunes, J. Fenyk-Melody, Anal. Chem. 74 (2002) 6305.
[11] H.R. Liang, R.L. Foltz, M. Meng, P. Bennett, Rapid Commun. Mass Spectrom. 17 (2003) 2815.
[12] L.E. Sojo, G. Lum, P. Chee, Analyst 128 (2003) 51.
[13] D. Remane, D.K. Wissenbach, M.R. Meyer, H.H. Maurer, Rapid Commun. Mass Spectrom. 24 (2010) 859.
[14] N.B. Cech, C.G. Enke, Anal. Chem. 73 (2001) 4632.
[15] C.G. Enke, Anal. Chem. 69 (1997) 4885.
[16] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 70 (1998) 882.

[17] J. Schuhmacher, D. Zimmer, F. Tesche, V. Pickard, Rapid Commun. Mass Spectrom. 17 (2003) 1950.
[18] M. Jemal, Y.Q. Xia, Rapid Commun. Mass Spectrom. 13 (1999) 97.
[19] M. Jemal, Z. Ouyang, Y.Q. Xia, Biomed. Chromatogr. 24 (2009) 2.
[20] R. Ramanathan, A.D. Su, N. Alvarez, N. Blumenkrantz, S.K. Chowdhury, K. Alton, J. Patrick, Anal. Chem. 72 (2000) 1352.
[21] J.L. Little, M.F. Wempe, C.M. Buchanan, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 833 (2006) 219.
[22] W.Z. Shou, W. Naidong, Rapid Commun. Mass Spectrom. 17 (2003) 589.
[23] M. Jemal, Z. Ouyang, Y.Q. Xia, Biomed. Chromatogr. 24 (2010) 2.
[24] X. Xu, H. Mei, S. Wang, Q. Zhou, G. Wang, L. Broske, A. Pena, W.A. Korfmacher, Rapid Commun. Mass Spectrom. 19 (2005) 2643.