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Does a stable isotopically labeled internal standard always correct analyte response? A matrix effect study on a LC/MS/MS method for the determination of carvedilol enantiomers in human plasma

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Abstract

A stable isotopically labeled (SIL) analogue is believed to be the most appropriate internal standard in a quantitative bioanalytical liquid chromatography/tandem mass spectrometry (LC/MS/MS) assay. It is assumed that a SIL internal standard always compensates for variability in chemical derivatization, sample extraction and LC/MS/MS analysis due to its nearly identical chemical and physical properties to the unlabeled analyte. Hence, the analyte to internal standard peak area ratio should be constant despite any variations in sample processing or analysis. However, in our laboratories, a deuterium labeled internal standard of carvedilol demonstrated an unexpected behavior—the analyte to internal standard peak area ratio changed with two specific lots of commercially supplied human plasma. Several experiments, including dilution of the extract with LC mobile phase and post-column infusion of the carvedilol solution followed by the injection of extracted blank plasma, have indicated that a high level of matrix suppression affected the ionization of the carvedilol-*S* enantiomer and its deuterated internal standard differently in these two lots of plasma. For the first time, it was clearly demonstrated that a slight difference in retention time between the analyte and the SIL internal standard, caused by deuterium isotope effect, has resulted in a different degree of ion suppression between these two analogues. This difference was significant enough to change the analyte to internal standard peak area ratio and affect the accuracy of the method.

Keywords: Liquid chromatography/tandem mass spectrometry (LC/MS/MS); Differential matrix effect; Stable isotopically labeled (SIL) internal standard; Deuterium isotope effect; Human plasma; Carvedilol enantiomers; Quantitative

1. Introduction

High-performance liquid chromatography coupled with atmospheric pressure ionization tandem mass spectrometry (LC/MS/MS) is widely used for the quantitative bioanalysis of small molecule drugs in the pharmaceutical industry due to its superior sensitivity and selectivity. However, the ever-increasing demands for high-throughput bioanalysis have often resulted in LC/MS/MS methods with minimum sample preparation and chromatography, where large amounts of endogenous matrix components may potentially co-elute with the target analyte. While these co-eluting components are often invisible to the MS detector when selected reaction monitoring is employed for the detection of analyte and the internal standard, they may, however, significantly affect the efficiency and reproducibility of the ionization process that occurs in the ion source [1–11]. Recently, Van Horne and Bennett have reported on phospholipids and lysophospholipids as one primary class of endogenous component causing significant ionization suppression [12].

One method of controlling the matrix effect during LC/MS/MS experiments is through the utilization of an internal standard (IS) in the form of a stable isotopically labeled (SIL) analogue because of its nearly identical chemical and physical properties to the target analyte [1–3]. Since it is processed along with the analyte, the SIL internal standard should not only help correct for sample preparation variations during extraction and chemical derivatization, but also compensate for variability in MS detection. In theory, since the SIL is almost identical in

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structure to and co-elutes with the analyte, the degree of ionization suppression or enhancement caused by the co-eluting matrix components should be the same for the IS and analyte. Therefore, while the absolute response may be affected, the analyte to IS peak area ratio should be unaffected and the bioanalytical method should be accurate, precise and rugged.

However, recent publications indicate that the utilization of an SIL does not guarantee a successful bioanalytical method. Deuterium labeled internal standards may demonstrate unexpected behavior which can compromise an accurate quantification of analyte concentration. Jemal et al. previously reported an unacceptable matrix effect in spite of utilization of a deuterated internal standard [4]. The degree of ionization suppression was found to be different between the target analyte and its SIL internal standard in one of the four batches of urine tested, corresponding to a significant decrease in analyte to IS peak area ratio. However, no detailed explanations were provided. It has been recently suggested, despite lack of directly supportive data, that one possible cause for the unexpected results of the deuterated internal standard was its partial resolution from the analyte on a reversed phased column [2]. This is thought to be due, in part, to the replacement of the carbon bound hydrogen with deuterium, which slightly alters the lipophilicity of the molecule, and hence the retention time of the deuterium labeled compound during reversed phase separations. This phenomenon is commonly known as an isotope effect [13–15].

In this paper, we report results from a systematic matrix effect study on a LC/MS/MS method for the determination of carvedilol-*S* and -*R* enantiomers in human heparinized plasma [16]. Carvedilol-*S* and carvedilol-*R* were extracted from human plasma by protein precipitation using acetonitrile containing racemic [$^2H_5$]-carvedilol as an internal standard. Extracts were then derivatized with 2,3,4,6-tetra-*o*-acetyl-beta-glucopyranosyl isothiocyanate (GITC) and the carvedilol diastereomeric derivatives were analyzed using HPLC–MS/MS with a TurboIonspray interface and selected reaction monitoring. It was observed, in this method, that two commercial lots of plasma had a profound effect on the analyte to SIL peak area ratio for the *S* enantiomer. A number of experiments were subsequently performed to understand the cause of this change in the analyte to IS peak area ratio for these lots of plasma.

2. Experimental

Details for sample preparation and LC/MS conditions along with chemicals, reagents and equipment can be found in a previously published manuscript [16].

2.1. Chemicals, reagents and equipment

Carvedilol racemate (SKF-105517, MW = 406) and [$^2H_5$]-carvedilol (MW = 411) were obtained from in-house synthesis. Control human plasma containing heparin as the anticoagulant was obtained from Biological Specialty Corp. (Colmar, PA,) and Bioreclamation, Inc. (East Meadow, NY). A Tomtec Quadra 96 model 320 automated liquid handling system (Hamden, CT, USA) and a Harvard Apparatus Model 22 infusion pump (South Natick, MA, USA) were used.

2.2. Sample preparation and dilution of the processed sample extracts

Carvedilol was dissolved in dimethyl formamide (DMF) to give a 1 mg/mL of racemic stock solution. The concentration of each enantiomer in the stock solution is 500 μg/mL. The stock solution of carvedilol was further diluted with acetonitrile/water (50/50) to make the working solution at 1 μg/mL which was used to prepare six replicates of standard at 50 ng/mL for each enantiomer in three different lots of human plasma. Internal standard solution was prepared by diluting the 1 mg/mL stock of [$^2H_5$]-carvedilol into acetonitrile to give a 10 ng/mL racemate solution.

Aliquots of 150 μL plasma samples were precipitated with 450 μL of acetonitrile containing 10 ng/mL [$^2H_5$]-carvedilol racemate. Following vortex-mixing and centrifuging for 20 min at ∼3200 × *g*, approximately 500 μL of the supernatant was transferred and evaporated to dryness under a stream of N_2 at 40 °C. Derivatization reagent (200 μL of 0.5% triethylamine (TEA) and 20 μL of 3 mg/mL 2,3,4,6-tetra-*o*-acetyl-beta-glucopyranosyl isothiocyanate in acetonitrile) was then added and the solution was held at room temperature for 20 min. The derivatized samples were again evaporated to dryness and reconstituted in 100 μL of 2 mM ammonium formate (pH 3)/acetonitrile (50/50, v/v). The reconstituted samples were either injected directly onto HPLC–MS/MS system for analysis, or were diluted 10, 20 and 50 folds, with the LC mobile phase solvent prior to injections. The injection volume was kept at 2 μL.

2.3. Post-column infusions

A continuous post-column infusion of 50 ng/mL derivatized carvedilol racemate solution at 20 μL/min by an Apparatus Model 22 infusion pump through a T-connector was introduced to the analytical LC system while an extract of derivatized drug-free blanks from three different lots of plasma were injected. The MS analysis was performed on an API-4000 triple quadrupole mass spectrometer equipped with a TurboIonSpray source (Applied Biosystem/MDS Sciex, Concord Ontario, Canada). The following precursor to product ion transitions were used for the selected reaction monitoring (SRM): diastereomeric derivatives of carvedilol (*S* and *R*) *m/z* 796–222 with the dwell time 150 ms and those of [$^2H_5$]-carvedilol (*S* and *R*) *m/z* 801–227 with the dwell time set at 100 ms. The inter-scan delay was 5 ms. The TurboIonSpray source temperature was maintained at 750 °C and the ionspray voltage was set at 5000 V. The curtain gas was set at 20, the declustering potential (DP) at 61 V and the nebulizer (GS1) and TIS (GS2) gases at 60 and 70 psi, respectively. The CID gas was set at 4, and the collision energy was set at 53 eV. Possible ion suppression of carvedilol signal by the drug-free blank matrix was examined as the "negative" chromatographic peaks from the elevated baseline. A sample volume of 2 μL of the drug-free plasma extract was injected using the CTC HTS PAL autosampler (Leap Technologies, Greensboro, NC). The LC was performed on an Ace 3

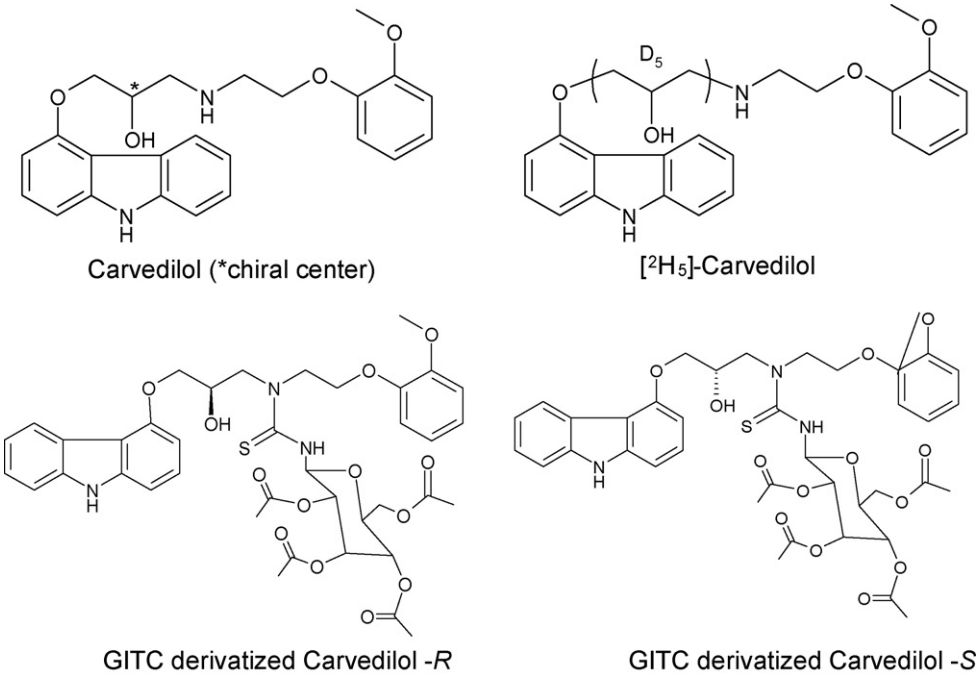

Fig. 1. Structures of carvedilol, [$^2H_5$]-carvedilol, GITC derivatized carvedilol-*R* and GITC derivatized carvedilol-*S*.

C18 column (3 μm, 50 mm × 2.1 mm, Mac Mod, Chadds Ford, PA) with an isocratic flow at 550 μL/min on a quaternary Rheous Flux 2000 pump (Leap Technologies). The mobile phase consisted of a 50:50 mixing of 2 mM ammonium formate buffer (pH 3.0) and acetonitrile.

3. Results and discussion

3.1. Observation of the matrix related problem

We have previously reported an LC/MS/MS method for the determination of carvedilol-*S* and -*R* enantiomers (structures shown in Fig. 1) in heparinized human plasma [16]. In summary, carvedilol racemate was extracted from human plasma by protein precipitation using acetonitrile containing [$^2H_5$]-carvedilol as the internal standard. Extracts were then derivatized with GITC and the resulting diastereomeric derivatives (structures also shown in Fig. 1) were analyzed by LC/MS/MS equipped with a TurboIonspray interface and selected reaction monitoring. Using 150 μL of plasma, the method was validated over a concentration range of 0.2–200 ng/mL.

However, during subsequent application of the method it was observed that calibration standards, prepared in two specific lots of commercially supplied plasma, resulted in concentrations of the carvedilol-*S* enantiomer in the QC samples that were ∼20% higher than expected. Instability was ruled out since previous experiments have established long term freezer, freeze/thaw and benchtop room temperature stability of carvedilol in human plasma throughout the studies. In addition, apparent spiking errors in calibration standard preparation were eliminated because carvedilol racemate was used for plasma spiking and there was no issue with carvedilol-*R* results. Therefore, it was suspected that matrix effects from the different lots of commercial plasma might be the cause of this inaccuracy with the *S* enantiomer despite the use of an SIL internal standard.

3.2. Testing matrix effect in different lots of plasma

Three lots of commercial human heparin plasma were chosen for this experiment. Lot 1 (Biological Specialty Corp., PA, lot BS22-55621) was the plasma used for QC preparation, lot 2 (Biological Specialty Corp., lot BS22-55620) was a representative lot of plasma employed for the standard curve preparation that resulted in successful analyzes and lot 3 (Bioreclamation, Inc., lot BRH43113) was one of the two lots of commercially supplied plasma utilized for the standard curve preparations that resulted in inaccurate measurement of the *S* enantiomer in QC samples. Each lot of plasma was spiked with carvedilol at 50 ng/mL in replicates of six. Plasma samples were extracted and derivatized prior to LC/MS analysis following the method published previously [16].

The peak areas of carvedilol and SIL, along with the analyte/IS peak area ratio from each lot of plasma, are shown in Table 1. Results from lot 1 and lot 2 plasma were found to match well. However, the peak areas of carvedilol-*S* and its SIL in the lot 3 plasma were found to be 52.8 and 40.4% lower, respectively, than that seen in lot 1, indicating significant ionization suppression of carvedilol-*S* and its SIL in the lot 3 plasma. It was surprising, despite the utilization of the SIL carvedilol as the internal standard, that the MS response for carvedilol-*S* was suppressed more than that for the SIL internal standard. As a result, the analyte/IS peak area ratio also decreased by 18.9% compared with the lot 1 plasma. On the other hand, it was interesting to note that the results for carvedilol-*R* and its internal

Table 1
Mean peak areas and analyte-to-IS peak area ratios of carvedilol-*S* and -*R* in three different lots of human heparin plasma

Plasma lot	Carvedilol-*S*			Carvedilol-*R*		
	Mean peak area		Analyte/IS ratio	Mean peak area		Analyte/IS ratio
	Analyte	IS		Analyte	IS	
Lot 1 (%CV, $n=6$)	1583443 (2.2%)	347746 (3.1%)	4.55	1565029 (2.3%)	337718 (2.8%)	4.64
Lot 2 (%CV, $n=6$)	1629480 (4.5%)	361143 (5.2%)	4.51	1542086 (4.4%)	338136 (3.9%)	4.56
%Difference from Lot 1	2.9%	3.9%	−0.9%	−1.5%	0.1%	−1.7%
Lot 3 (%CV, $n=6$)	746331 (4.3%)	207184 (1.8%)	3.69	1689956 (4.0%)	358941 (3.1%)	4.71
%Difference from Lot 1	−52.8%	−40.4%	−18.9%	8.0%	6.3%	1.5%

standard were very similar in all three lots of plasma, and there were negligible difference in analyte/IS peak area ratios when compared with lot 1. Therefore, unlike carvedilol-*S*, lot 3 plasma had no effect on the carvedilol-*R* determination. Also worthy of note is the excellent coefficient of variation (CV%) observed for the analyte and IS peak areas among six replicates of samples in each of these three lots of plasma (the maximum CV% observed is 5.2%).

Matrix suppression in lot 3 plasma was also clearly illustrated in the LC/MS/MS chromatograms shown in Fig. 2. Since there are equal amounts of carvedilol-*S* and -*R* in the racemate and they were eluted off the LC column isocratically, the carvedilol-*S* peak was expected to be slightly sharper in its shape and slightly higher in intensity. This was indeed the case in lot 1 and 2 (Fig. 2A and B). However, the pattern was different in lot 3 plasma (Fig. 2C). The carvedilol-*S* peak was found to be much smaller than expected, indicating the presence of co-eluting matrix and subsequent strong matrix suppression on carvedilol-*S*.

3.3. Matrix effect investigations

The matrix effect from different lots of plasma was further investigated through dilution of the processed sample extracts and also with post-column infusion of analyte, coupled with injection of the treated blank plasma extract. After derivatization, the samples were evaporated to dryness, and then reconstituted in 100 μL of LC mobile phase prior to LC/MS/MS

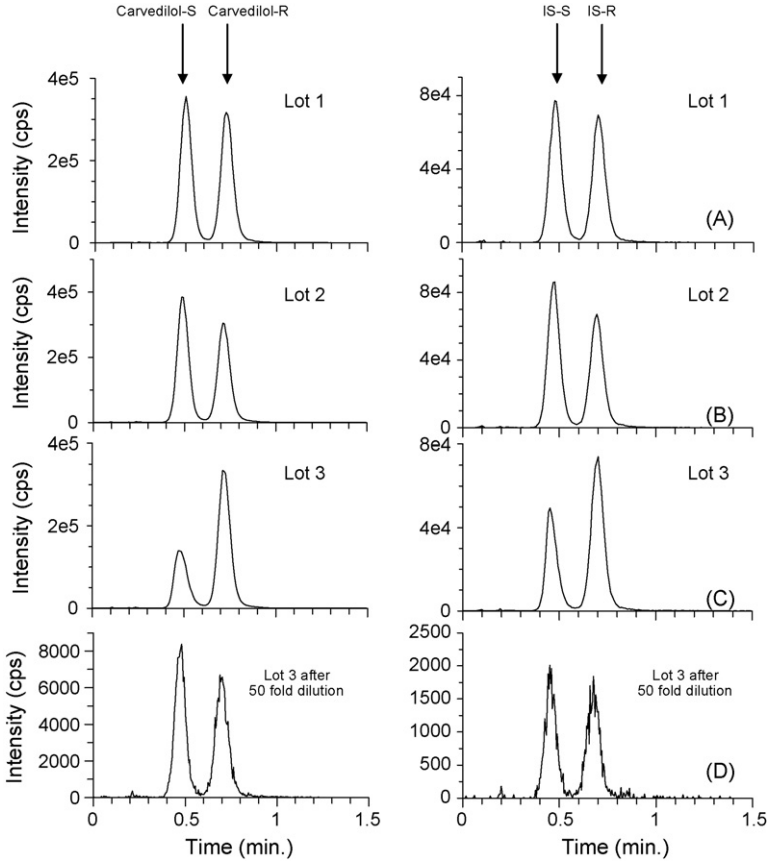

Fig. 2. HPLC/MS/MS chromatograms of a processed plasma sample spiked at 50 ng/mL in: lot 1 (A), lot 2 (B), lot 3 (C) and the 50-fold dilution of the processed extract from lot 3 (D). MS data acquisition starts at 1.4 min.

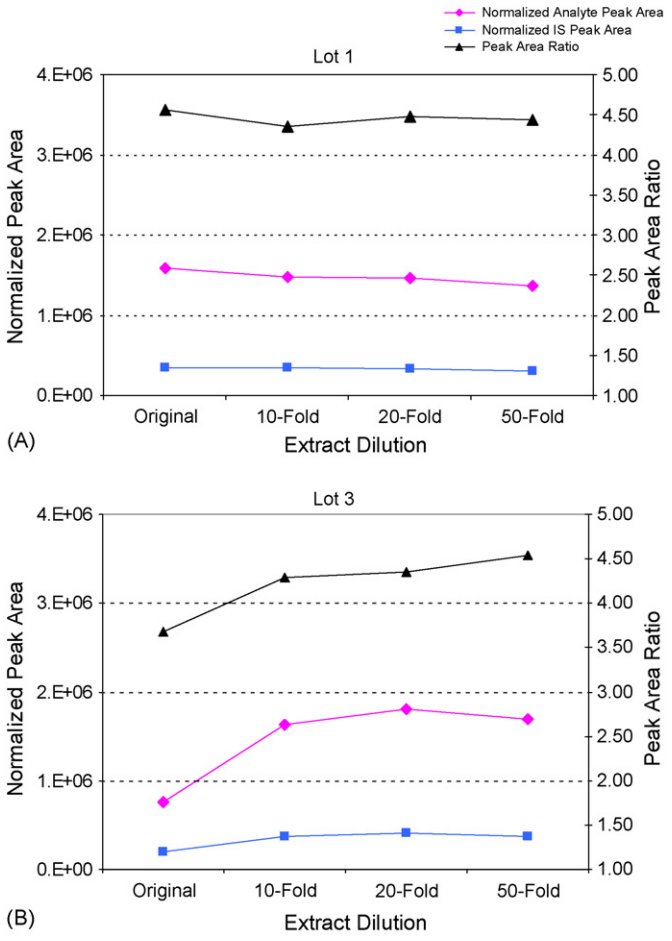

Fig. 3. Normalized peak areas and peak area ratios of carvedilol-*S* in the original, 10-, 20- and 50-fold mobile phase diluted processed sample extracts. Carvedilol-*S* was spiked in plasma lot 1 and lot 3 at a concentration of 50 ng/mL.

analysis. For this experiment, the reconstituted samples of carvedilol at 50 ng/mL for each enantiomer were further diluted 10, 20 and 50 folds, respectively, with the LC mobile phase while keeping the injection volume at 2 μL. As a result, smaller amounts of matrix components were injected onto the LC column and the matrix effect observed previously in the lot 3 plasma was therefore reduced. The HPLC–MS/MS chromatogram of a 50-fold diluted sample extract prepared in the lot 3 plasma is shown in Fig. 2D. Unlike the chromatogram from the original reconstituted sample (Fig. 2C), the carvedilol-*S* peak in Fig. 2D was expectedly higher than carvedilol-*R* peak, displaying a similar pattern to that observed in lot 1 and 2 plasma (Fig. 2A and B). This has therefore further confirmed the presence of strong ionization suppression of carvedilol-*S* by the co-eluting matrix components in the lot 3 plasma.

A summary of carvedilol-*S* and its SIL peak areas and the analyte-to-IS peak area ratios in lot 1 and 3 plasma obtained from the original reconstituted samples and after 10-, 20- and 50-fold dilutions of extracts with mobile phase is shown in Fig. 3. The results for the lot 2 plasma were similar to the lot 1 and are therefore not discussed further. The peak areas of the diluted samples were normalized by the corresponding dilution factor so that they could be directly compared with the originals. Since the analyte and internal standard were diluted concurrently, the analyte-to-IS peak area ratio should remain constant between the original and the diluted samples if the suppression by matrix components affects the analyte and IS equally. In plasma lot 1, the normalized peak areas of 10-, 20- and 50-fold dilution samples were similar to the original result for both analyte and SIL, and there were no changes in the peak area ratios, indicating the absence of obvious matrix suppression for carvedilol-*S* in the lot 1 plasma (Fig. 3A). On the other hand, as a result of strong matrix suppression in the lot 3 plasma, the normalized peak areas of 10-, 20- and 50-fold dilution samples were significantly higher than the original result for both analyte and IS. Also, despite the presence of SIL internal standard, the analyte-to-IS peak area ratios of lot 3 went up by ~20% compared to the undiluted sample extract (Fig. 3B). The peak area ratios in the diluted sample extracts in lot 3 closely matched the ones in the lot 1 plasma.

An LC/MS/MS post-column infusion experiment was performed to investigate the cause of differential matrix effect in the lot 3 plasma. In this experiment, a continuous infusion of 50 ng/mL solution of derivatized carvedilol was introduced into the post-LC column flow while the extracts of the derivatized drug-free plasma were injected. As previously reported [6], the post-column infusion experiment provided a direct visualization

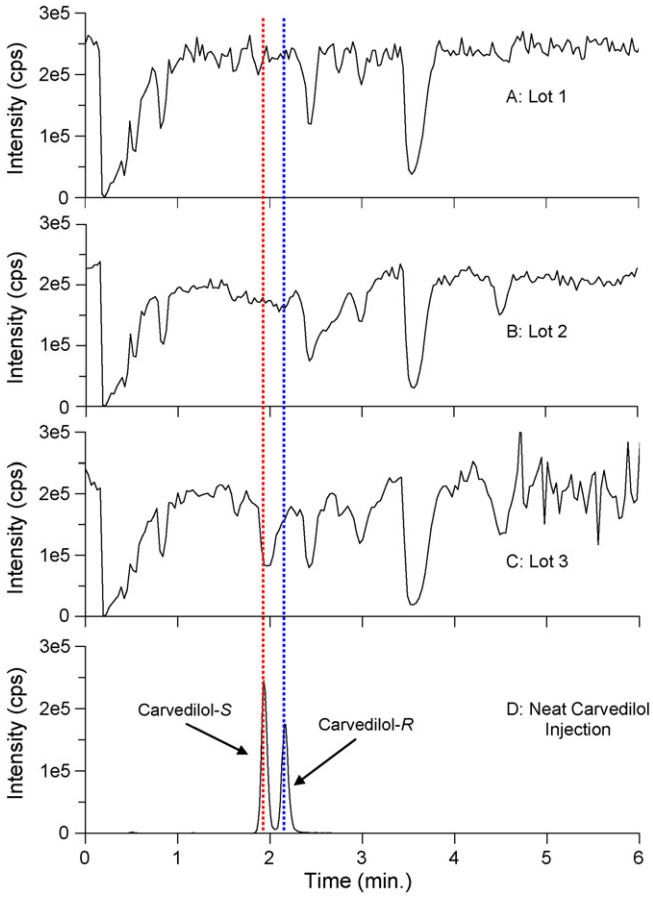

Fig. 4. Post-column infusion of carvedilol with injection of the extracts of the drug-free plasma: lot 1 (A), lot 2 (B) and lot 3 (C) and LC/MS/MS chromatogram of carvedilol. Matrix effects are observed as the troughs on the elevated baseline.

of the matrix effect on the analyte of interest by monitoring the negative peaks on the elevated baseline throughout the LC/MS/MS run. Post-column infusion chromatograms from the lot 1, 2 and 3 plasma and the chromatogram from the direct injection of a derivatized carvedilol sample are shown in Fig. 4A–D, respectively. There was no suppression trough around the retention time of either carvedilol-*S* or -*R* from lot 1 and 2. However, the lot 3 drug-free plasma extract showed a significant trough at the retention time of carvedilol-*S* while no obvious suppression trough near the retention time of carvedilol-*R* was found (Fig. 4C). This clearly demonstrates that some interfering endogenous matrix components co-eluted with carvedilol-*S* and suppressed the ionization considerably in the lot 3 plasma. Furthermore, by monitoring the characteristic SRM transitions from the precursor ions of *m/z* 496, 524, 704, 758, 786, 806 to the product ion at *m/z* 184 in positive ion mode, we have confirmed that the suppression troughs around 2.5 and 3.6 min found in all three lots of plasma (Fig. 4A–C) were caused by the late eluting phospholipids [12]. However, with a cycle time of 2.7 min or longer, these late suppression troughs should not co-elute with either carvedilol-*S* or -*R* in the next injection.

Fig. 5 is an expanded version of Fig. 4C and D along with the LC/MS/MS chromatogram for the SIL internal standard, and therefore provides a "close-up" examination on the matrix suppression for carvedilol and its SIL in the lot 3 plasma. It was noticed that carvedilol-*S* and -*R* eluted slightly later than their respective internal standards (retention time of 1.93 min for carvedilol-*S* versus 1.91 min for its SIL, and retention time of 2.16 min for carvedilol-*R* versus 2.14 min for its SIL). As stated before [14,15], it is believed that the deuterium isotope effect caused the slight separation between the analyte and its deuterated analogue in the reversed phase chromatography. The deuterium atoms were found to be less lipophilic and the deuterated IS thus eluted slightly earlier on a reversed phase chromatography system. As a result, carvedilol-*S* eluted closer to the peak of matrix suppression than [$^2H_5$]-carvedilol-*S*. With 0.02 min (or 1.2 s) separation between them, the amount of ionization suppression shown at the trough valley increased by as much as 25% from [$^2H_5$]-carvedilol-*S* to carvedilol-*S*, leading to greater ionization suppression in carvedilol-*S* and a lower analyte-to-IS peak area ratio in the lot 3 plasma.

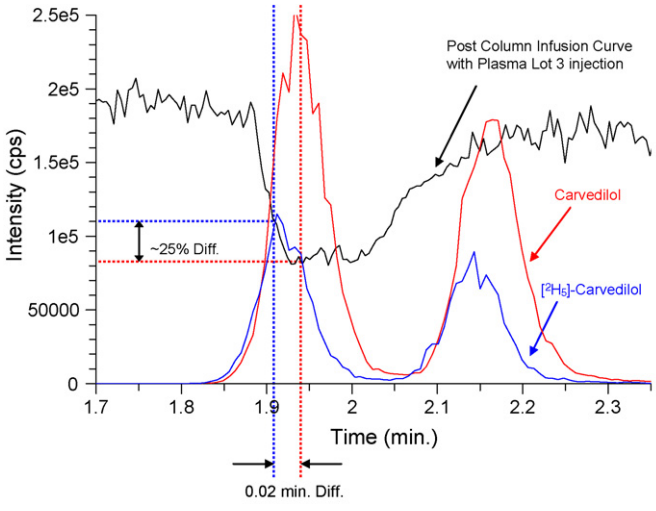

Fig. 5. A "close-up" examination on the matrix suppression for carvedilol and the deuterated IS in the lot 3 plasma. The post-column infusion of carvedilol with injection of an extract of the derivatized drug-free plasma lot 3 (as shown in Fig. 4C) was overlaid with the LC/MS/MS chromatograms of carvedilol and its SIL. A slight difference in retention time between carvedilol and [$^2H_5$]-carvedilol was observed.

The nature of co-eluting matrix components that caused the strong ionization suppression to carvedilol-*S* and [$^2H_5$]-carvedilol-*S* in the lot 3 plasma was unknown. A generic solid phase extraction (SPE) method recommended by the vendor in the product manual was proven to be inadequate in the removal of the co-eluting matrix components, likely due to their similar lipophilicities to the analyte. After optimizing the washing conditions on a mixed mode SPE 96-well plate, we were able to remove the interfering matrix components in the lot 3 while maintaining good analyte recovery.

In conclusion, it has been clearly demonstrated, with the matrix effect as shown in the lot 3 plasma, that a slight difference in retention time between analyte and its deuterated IS could result in a different degree of ionization suppression between the two analogues, and this could have a profound affect on the analyte to IS peak area ratio consistency and method ruggedness.

3.4. Method modifications

The matrix effect problem observed in the carvedilol assay appeared to be only related to two lots of plasma purchased from one vendor. Following this investigation, 18 more lots of commercial plasma from several vendors and the pre-dose samples from several pharmacokinetic studies were processed. The post-column infusion experiments performed with these samples did not show the matrix suppression problem similar to the lot 3 plasma, indicating that this phenomenon may be limited to these two specific lots of commercially supplied plasma.

Nevertheless, two minor modifications were made to the current method in order to minimize the matrix effect. Less plasma (100 μL versus 150 μL) was processed for each analysis, and the derivatized samples were reconstituted with 200 μL, instead of 100 μL mobile phase solvent. While keeping the injection volume at 2 μL and maintaining a signal-to-noise ratio above five at the lower limit of quantification (LLQ) concentration, these minor modifications resulted in approximately a three-fold smaller amount of sample (and endogenous components) being loaded onto the column with each injection. The modified method was demonstrated to be amenable to all lots of plasma tested so far, including the lot 3 plasma.

4. Conclusions

From this study, matrix effects may not be entirely compensated for even if a stable isotopically labeled internal standard is utilized. The deuterium isotope effect may cause the partial resolution of analyte and its deuterated IS in reversed phase LC. If a large and sharp matrix suppression peak elutes at around the retention time of analyte and IS as observed in this study, the slight difference of retention time may cause differential matrix

effects and affect the accuracy and precision of the quantitative bioanalytical analysis. For the first time, experimental data are available to directly support a previously proposed matrix effect theory [2].

SIL internal standards are still preferred for an accurate and robust LC/MS/MS assay. The ^{13}C-, ^{15}N- or ^{17}O-labeled SIL analogue may be more ideal than the ^{2}H-labeled compounds. However, synthesizing exclusively the non-deuterium labeled SIL may be expensive and impractical. Synthetic strategies in which the stable isotope labels are introduced late in the synthesis using the unlabeled substrate already available are obviously an attractive option. In particular, the *O*- and *N*-alkylations of amines, phenols, alcohols and thiols with, for example, [^{13}C, $^{2}H_3$]-iodomethane, have allowed chemists to generate some SIL standards very quickly. While incorporation of ^{13}C or ^{15}N only into the "backbone" of a molecule is possible, this approach, however, often involves *de novo* synthesis and is potentially much more time consuming.

Assessment of matrix effects in different batches of biological matrix has been included as part of method development and validation in many bioanalytical labs. However, the method performance still needs to be closely monitored throughout the sample analysis runs since only limited lots of biological matrix are tested during method validation.

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