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RESEARCH ARTICLE

Incurred sample stability of ASP3258 in the presence of its acyl glucuronide

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The acyl glucuronide (AG) of ASP3258 was abundant in incurred monkey samples, and because an AG generally back-converts to its aglycone, accurate quantification of ASP3258 in monkey plasma was a challenge. To prevent the back-conversion of ASP3258-AG, cooling and acidification were incorporated during sample collection, storage, and extraction. To demonstrate that the AG did not affect the determination of ASP3258, the present study used incurred samples to examine whole blood stability, short-term stability, freeze-thaw stability, frozen stability, and stability during extraction. The concentration changes were within −11.4% to 15.0% compared with a reference value and were therefore judged acceptable. The present study presents a detailed account of test items, a reference value, sample numbers, sample selection, and an equation for assessment in the incurred sample stability tests. This bioanalytical method was applied successfully to a study of the toxicokinetics of ASP3258 in monkeys.

Keywords: incurred sample stability, acyl glucuronide, ASP3258, animal plasma, toxicokinetics.

Introduction

The potent phosphodiesterase 4 inhibitor ASP3258 (**Figure 1**) is a novel therapeutic agent for asthma and chronic obstructive pulmonary disease [1]. We previously developed and validated a bioanalytical method to quantify ASP3258 in rat plasma [2]. Because ASP3258 metabolite was not detected in rat plasma [3], this bioanalytical method was developed without encountering issues associated with the metabolite. Subsequently, monkey toxicity studies were planned that required an appropriate bioanalytical method. When ASP3258 was orally administered to monkeys in a preliminary study, the acyl glucuronide (AG) metabolite of ASP3258 was detected in abundance in monkey plasma (data on file), in contrast to our findings using rat plasma [3]. In general, AGs readily back-convert to aglycones, causing overestimation of aglycone concentrations, and back-conversion rates vary depending on the structure of the respective aglycone [4]. It was therefore important to develop a method for quantifying ASP3258 in the presence of its AG and to confirm that conversion of the AG to ASP3258 has no effect on assay results.

To test the degradation of the AG, the only feasible option available in the present study was to use incurred samples because an authentic standard of the AG was not available, and it was necessary to quickly initiate toxicity studies using monkeys. Stability tests using incurred samples are called "incurred sample stability (ISS)" [5]. ISS is the subject of intense debate within Global Bioanalysis Consortium (GBC) [5], Global CRO Council for Bioanalysis (GCC) [6,7], European Bioanalysis Forum (EBF) [8], Workshop on Recent Issues in Bioanalysis (WRIB) [9,10], and Japan Bioanalysis Forum [11]. However, the optimum test items and study design are still obscure [12]. Given that GBC [5], the 7th WRIB [10], and 7th GCC [6] recommended evaluating ISS when metabolites are likely to affect assay results, it is important to dis-

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cuss the test items and study design of ISS experiments in the presence of AG. Unfortunately, to my knowledge, ISS in the presence of AG has been addressed by only two case studies [13, 14]. Further, both test items and the design of the two studies were inconclusive. It was therefore necessary to refine the test items and study design to confirm that ASP3258-AG does not affect quantification of ASP3258.

To achieve this goal, the present study developed and validated a method for the determination of ASP3258 in monkey plasma that precluded back-conversion of the AG to ASP3258 during sample collection, storage, and analysis. This validated method was evaluated in ISS and toxicokinetic studies. Further, the test items and study design of ISS experiments in the presence of AG are discussed.

Materials and Methods

Chemicals and reagents

ASP3258 and its internal standard (IS) (AS1406604-00) were synthesized at Astellas Pharma Inc. (Tsukuba, Japan) (**Figure 1**). Acetonitrile, acetic acid, and cyclohexane were of HPLC grade and were purchased from Fisher Scientific (Waltham, MA, USA). Ultrapure water was prepared using a Milli-Q system (EMD Millipore, Billerica, MA, USA). All other chemicals and solvents were of analytical grade.

Instruments and conditions

The instruments and conditions in the present study were essentially the same as those used in a previous rat method [2]. All analyses were performed using a Shimadzu 10A series HPLC system (Kyoto, Japan) coupled with a Jasco FP 920 fluorescence detector (Tokyo, Japan). Chromatography was performed on a Shiseido Capcell Pak C18 UG120 column (3.0 mm i.d. × 150 mm, 5 μm) (Tokyo, Japan), preceded by a Shiseido Capcell Pak C18 UG120 guard column (2.0 mm i.d. × 10 mm, 5 μm) at 45°C. The mobile phase, delivered at 0.4 mL/min, was acetonitrile-0.5% acetic acid (50:50, v/v). ASP3258 and its IS were detected using wavelengths of 315 nm for excitation and 365 nm for emission. Data were collected using Millennium32 Chromatography Manager version 3.20 (Waters, Milford, MA, USA).

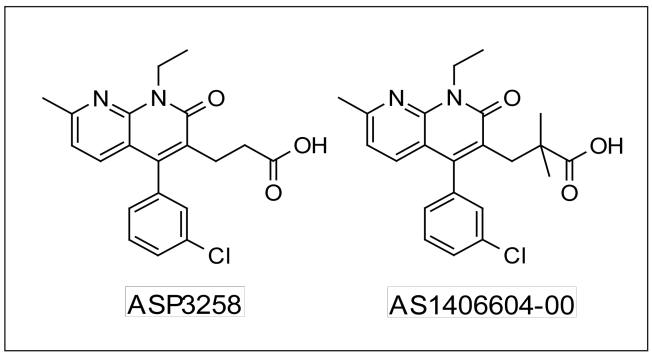

Figure 1. Chemical structures of ASP3258 and AS1406604-00 (IS).

Calibration standards and quality control samples

Stock solutions of ASP3258 (0.2 mg/mL) and AS1406604-00 (IS, 0.2 mg/mL) were prepared by dissolving the compounds in ethanol, after which the solutions were stored at 4°C. Working solutions were prepared by diluting the stock solutions with ethanol. Calibration standard samples were prepared by spiking the working solutions in blank monkey plasma at the final concentrations of 5, 10, 20, 50, 100, 200, and 500 ng/mL. Quality control (QC) samples at low (12 ng/mL), medium (120 ng/mL), and high (400 ng/mL) concentrations were prepared in monkey plasma.

Buffer preparation

Citric acid monohydrate (9.46 g [45 mmol]) and tri-sodium citrate dihydrate (1.47 g [5 mmol]) were dissolved in 25 mL of water to prepare 2 mol/L citrate buffer. A 20 fold larger volume of water was used to prepare 100 mmol/L citrate buffer. Each citrate buffer solution was approximately pH 2.6.

Sample preparation

To prevent back-conversion of AG to its aglycone, it is generally recommended that samples are handled under acidic pH at lower temperatures [15,16]. In accordance with this general recommendation, blood samples were cooled in an ice-water bath immediately after collection. After centrifugation, plasma samples were mixed with cooled 2 mol/L citrate buffer at a volume ratio of 1:3. If needed, the acidified samples were stored at −80°C and thawed in an ice-water bath. During extraction, the samples were kept in an ice-water bath. Acidified plasma samples (0.4 mL) were mixed with 100 mmol/L citrate buffer (2 mL), ethanol (0.1 mL), and IS solution (0.1 mL). Samples were extracted with cyclohexane (5 mL) for 5 min and then centrifuged at 4°C for 5 min. The organic layers were collected and evaporated to dryness at 35°C under a stream of nitrogen. The residue was reconstituted in 0.2 mL of acetonitrile-0.5% acetic acid (30:70, v/v). Because the AG was not extracted into the cyclohexane layer, it was unlikely that back-conversion of the AG affected quantification of ASP3258 during and after the evaporation of the cyclohexane layer.

Method validation
Method validation was conducted in accordance with FDA guidance [17].

Selectivity and sensitivity
The selectivity of the method was assessed by ensuring there were no interference peaks around the retention times of the analyte or IS in the chromatograms of blank individual plasma samples from six monkeys. The lower limit of quantification (LLOQ) was defined as the lowest concentration with accuracy within ± 20% and precision ≤20%. The LLOQ was evaluated by analyzing six replicate samples.
Accuracy was expressed as relative error (RE) and calculated using the following equation:

$$RE(\%) = \frac{(\text{mean measured concentration} - \text{nominal concentration})}{(\text{nominal concentration})} \times 100\%$$

Precision was expressed as the coefficient of variation (CV) and calculated as follows:

$$CV(\%) = \frac{\text{standard deviation of measured concentrations}}{\text{mean measured concentration}} \times 100\%$$

Linearity
Calibration curves were constructed by analyzing spiked calibration standard samples. The peak height ratios of ASP3258 to IS were plotted against analyte concentrations, and the curves were fitted to the equations via linear regression with a weighting factor ($1/x^2$) in the range of 5 to 500 ng/mL.

Accuracy and precision
To determine intra- and inter-day accuracy and precision, low, medium, and high QC samples (n=6 each) were analyzed over three days. At these three concentrations, accuracy was required to be within ± 15% and precision ≤15%.

Extraction recovery
Low and high QC samples (n=6 each) were analyzed to evaluate the extraction recovery, and the mean peak heights of the extracted QC samples were compared with those of standard solutions that had been evaporated and reconstituted in acetonitrile-0.5% acetic acid (30:70, v/v).

Stability
Low and high QC samples (n=3 each) were analyzed to evaluate the stability of ASP3258 in acidified monkey plasma under various storage or handling conditions, and the measured concentrations were compared with nominal concentrations or mean initial concentrations. Short-term stability was determined after storing the QC samples at room temperature for 24 h. Long-term stability was assessed by storing the QC samples for 33 days in a deep freezer set at −60°C to −80°C. Freeze-thaw stability was determined after three freeze-thaw cycles. The stability of ASP3258 after sample processing was evaluated by comparing measured concentrations of low and high QC samples (n=3, each) after storage in an autosampler for 72 h with mean initial concentrations.

All stability data in this section are expressed as Stability (%) and were calculated as follows:

$$Stability\ (\%) = \frac{(\text{mean measured concentration} - \text{nominal or mean initial concentration})}{(\text{nominal or mean initial concentration})} \times 100\%$$

ISS tests
One male and one female cynomolgus monkey each received a single oral dose of ASP3258 as methyl cellulose suspension at a dose of 10 mg/kg. Two hours after drug administration, blood (approximately 9 mL per animal) was collected from a femoral vein into tubes containing sodium heparin. Blood samples were divided into 10 aliquots per animal, and labeled as A, B, C, D, E1, E2, F1, F2, G, and H (**Table 1**). Sample A served as the negative control. Immediately after blood collection, Sample A was centrifuged for 5 min at 4°C to obtain plasma. The plasma was immediately acidified, extracted, and injected into the HPLC system using the validated method. Sample H served as the positive control. Immediately after blood collection, Sample H was centrifuged for 5 min at 4°C to obtain plasma. After the plasma was incubated for 24 h at 37°C (potential extensive degradation of the AG), the plasma was acidified, extracted, and injected onto the HPLC system according to the validated method. Samples B to G were processed similarly to Sample A, but with prolonged storage (**Table 1**). The conditions were as follows: Sample B was stored in an ice-water bath for 15 min before centrifugation of whole blood. Sample C was stored in an ice-water bath for approximately 5 min between plasma separation and acidification. Sample D was stored in an ice-water bath for approximately 60 min between acidification and the initiation of extraction. Samples E1 and E2 were exposed to one and two freeze-thaw cycles, respectively, after acidification. Samples F1 and F2 were stored after acidification at approximately −80°C for 2 and 7 days, respectively. Sample G was

Table 1. Study design of ISS

Sample name	Duration and condition	Timing
Sample A	NA (Negative control)	NA
Sample B	15 min under cool condition	Between blood collection and centrifugation
Sample C	5 min under cool condition	Between plasma harvest and citrate buffer addition
Sample D	60 min under cool condition	Between citrate buffer addition and initiation of extraction
Sample E1	One freeze-thaw cycle[a]	Between citrate buffer addition and initiation of extraction
Sample E2	Two freeze-thaw cycles[a]	Between citrate buffer addition and initiation of extraction
Sample F1	2 days in a freezer	Between citrate buffer addition and initiation of extraction
Sample F2	7 days in a freezer	Between citrate buffer addition and initiation of extraction
Sample G	45 min at room temperature	During extraction
Sample H	24 h at 37°C (Positive control)	Between plasma harvest and citrate buffer addition

[a]Samples were frozen in liquid nitrogen and stored in a freezer. Samples were thawed in an ice-water bath. NA; Not applicable

stored during extraction (total duration: approximately 45 min). Specifically, after mixing with cyclohexane, Sample G was stored at room temperature for approximately 15 min, and centrifuged. After centrifugation, Sample G was stored at room temperature for approximately 30 min, and the organic layer was collected. All samples were determined in triplicate.

All ISS data are expressed as the Difference (%), which was calculated as follows:

$$Difference\ (\%) = \frac{(Conc_{stability} - Conc_{control})}{(Conc_{control})} \times 100\%$$

where $Conc_{stability}$ is mean measured concentrations of stability samples, and $Conc_{control}$ is mean measured concentrations of negative control samples.

Toxicokinetic study

To assess whether the validated method could be applied to a toxicokinetic study, plasma concentrations of ASP3258 in study samples were determined. Male and female cynomolgus monkeys aged approximately two to five years were obtained from Covance Research Products Inc (Alice, TX, USA). The monkeys were allowed free access to water. Food was withdrawn from at least 1 h before dosing to at least 4 h after dosing. Following a single oral administration of ASP3258 at a dose of 0.3, 3, or 30 mg/kg, blood samples were collected at 0.5, 1, 2, 4, 8, and 24 h post-dose. Blood samples were collected from a femoral vein and sodium heparin was used as the anticoagulant. The samples were cooled, centrifuged to obtain plasma, and mixed with 2 mol/L citrate buffer at a volumetric ratio of 1:3. The acidified plasma samples were flash-frozen in liquid nitrogen and stored in a deep freezer set at −60°C to −80°C until analysis. Blood and plasma samples were handled within the demonstrated stability period. Plasma concentration-time data were analyzed using Pharsight WinNonlin version 3.1 (Mountain View, CA, USA).

Results

Method validation

Specificity and sensitivity. Representative chromatograms of the blank and spiked plasma samples are shown in **Figure 2**. No interfering peaks were observed at the retention times of the analyte or IS. Given that CV was 10.8% and RE 1.8%, the LLOQ for ASP3258 in plasma was 5 ng/mL and met the acceptance criteria stated above.

Linearity and calibration curve

Calibration curves for monkey plasma were linear over the concentration range of 5 to 500 ng/mL with a correlation coefficient (r) of ≥0.997. A typical equation of the calibration curve is as follows: $y=0.0194x+0.00505$, where y represents the peak height ratio of ASP3258 to IS and x the plasma concentration.

Accuracy and precision

The results of accuracy and precision are summarized in **Table 2**. The inter-day accuracies in RE ranged from −9.2% to −4.0%, whereas the intra-day accuracies ranged from −7.5% to 4.2%. The inter-day precision in CV was ≤8.9%, whereas the intra-day precision was ≤4.0%. These results met the acceptance criteria.

Table 2. Accuracy and precision of an HPLC method for determining the ASP3258 concentration in monkey plasma

QC Sample	Nominal concentration (ng/mL)	Intra-day (n=6) Measured concentration (ng/mL)[a]	RE (%)	CV (%)	Inter-day (n=18 [n=6 on 3 days]) Measured concentration (ng/mL)[a]	RE (%)	CV (%)
Low	12	12.5 ± 0.408	4.2	3.3	11.4 ± 1.01	-5.0	8.9
Medium	120	111 ± 4.43	-7.5	4.0	109 ± 4.57	-9.2	4.2
High	40	380 ± 13.5	-5.0	3.6	384 ± 15.0	-4.0	3.9

[a]Mean ± SD

Table 3. Stability of ASP3258 in monkey plasma or extracts using spiked samples

Storage or handling	QC Sample	Nominal concentration (ng/mL)	Measured concentration (ng/mL)[f]	Stability (%)	CV (%)
Short-term[a]	Low	12.0	10.5 ± 0.943	-12.5	9.0
	High	400	383 ± 8.72	-4.3	2.3
Long-term[b]	Low	12.0	10.6 ± 0.462	-11.7	4.4
	High	400	379 ± 2.65	-5.3	0.7
Freeze-thaw[c]	Low	12.0	11.8 ± 0.361	-1.7	3.1
	High	400	395 ± 5.13	-1.3	1.3
Post-preparative[d]	Low	11.1[e]	11.2 ± 0.700	-6.7	6.3
	High	365[e]	359 ± 13.0	-10.3	3.6

[a]24 h at room temperature, [b]33 days in a deep freezer set to maintain −60°C to −80°C, [c]three cycles, [d]72 h at 4°C, [e]mean initial concentration, [f]mean ± SD

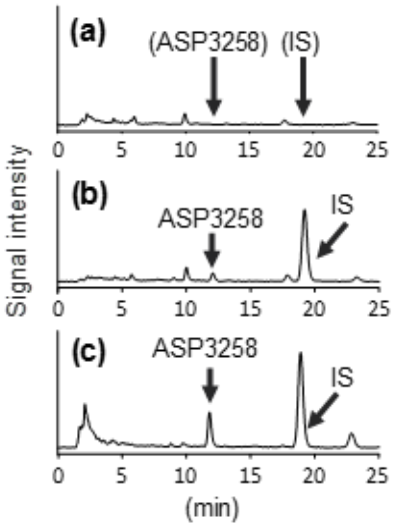

Figure 2. Representative HPLC chromatograms of (a) blank monkey plasma, (b) blank monkey plasma spiked with ASP3258 (5 ng/mL) and IS, and (c) a monkey plasma sample obtained at 2 h after single oral administration of ASP3258 at 0.3 mg/kg.

Extraction recovery

The extraction recoveries of ASP3258 at 12 ng/mL and 400 ng/mL were 50.3% and 49.6%, respectively, indicating consistent and reproducible recovery. The recovery of IS was 54.4%, which was similar to that of ASP3258.

Stability

Results of stability using spiked samples are shown in **Table 3**. The changes after storage or handling were within −12.5% and −1.3%, respectively, and were judged acceptable. ASP3258 was stable in monkey plasma at room temperature for 24 h, in a deep freezer for 33 days as well as after three freeze-thaw cycles. After sample processing, ASP3258 was stable for 72 h.

ISS

The results of ISS are shown in **Table 4.** The changes in concentrations of Samples B to G were within −11.4% to 15.0% compared to those of Sample A and were therefore judged acceptable. The concentrations in Sample H were 17.3% to 25.0% greater than those of

Table 4. ISS of ASP3258

Sample name	Stability item	ASP3258 Concentration (ng/mL)		Difference (%)	
		Male	Female	Male	Female
Sample A	Negative control	1110	535	NA	NA
Sample B	Whole blood stability (intact whole blood, 15 min, cool)	1290	570	15.0	6.3
Sample C	Short-term stability (intact plasma, 5 min, cool)	1230	579	10.3	7.9
Sample D	Short-term stability (acidified plasma, 60 min, cool)	1070	587	-3.7	9.3
Sample E1	Freeze-thaw stability (acidified plasma, one cycle)[a]	1090	512	-1.8	-4.4
Sample E2	Freeze-thaw stability (acidified plasma, two cycles)[a]	990	554	-11.4	3.5
Sample F1	Frozen stability (acidified plasma, two days)	1050	485	-5.6	-9.8
Sample F2	Frozen stability (acidified plasma, seven days)	1110	552	0.0	3.1
Sample G	Stability during extraction (45 min, room temperature)	1140	491	2.7	-8.6
Sample H	Positive control (24 h at 37°C)	1320	688	17.3	25.0

[a]Samples were frozen in liquid nitrogen and stored in a freezer. Samples were thawed in an ice-water bath. [b]Mean (n=3)
NA; Not applicable

Table 5. Toxicokinetic parameters of ASP3258 after single oral administration to monkeys (0.3, 3, and 30 mg/kg)

Dose (mg/kg)	Sex	Cmax (ng/mL)	Tmax (h)	AUC_{24h} (ng.h/mL)
0.3	M	34.2	2.00	306
	F	19.8	4.00	157
3	M	541	4.00	5459
	F	384	4.00	2819
30	M	1410	4.00	11415
	F	1670	4.00	18207

AUC_{24h}, area under the plasma concentration-time curve from time 0 to 24 h; Cmax, maximum plasma concentration; Tmax, time to reach maximum plasma concentration. Note: One animal per sex per dose

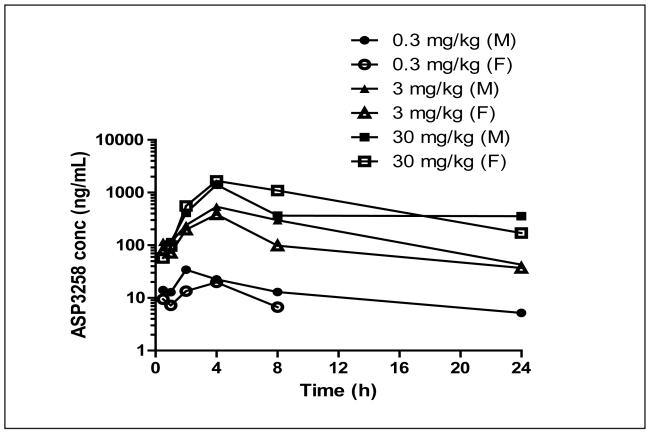

Figure 3. Plasma concentrations of ASP3258 after single oral administration of ASP3258 at 0.3, 3 or 30 mg/kg to monkeys (n=1 per sex per dose). M, male; F, female.

Sample A, indicating that the incurred samples contained a substantial quantity of the AG. These data demonstrate ISS in chilled blood for 15 min, in chilled intact plasma for 5 min, in chilled acidified plasma for 60 min, in acidified plasma for two freeze thaw cycles, in frozen acidified plasma for 7 days, and during the extraction process.

Application to a toxicokinetic study

The method validated above was used to determine the plasma concentrations of ASP3258 in monkeys.

Figure 3 shows the plasma concentration-time profiles of ASP3258 following a single oral administration.

The toxicokinetic parameters are shown in **Table 5**. These results suggest that the validated method is applicable to toxicokinetic studies in monkeys. The toxi-

cokinetic parameters reveal the following properties of ASP3258 in monkeys: (1) maximum plasma concentration was observed at 2–4 h after a single oral dose, indicating rapid absorption; and (2) plasma concentration of ASP3258 increased as a function of increasing oral dose.

Discussion

To my knowledge, few studies [13,14] have considered ISS in the presence of AG. Studies of ISS under general situation (i.e. there is no concern associated with AG) appear to assume that an interfering metabolite, if present, is moderately stable, because a typical reference value is established from a sample that is collected without special care, shipped in a frozen state, and measured at a bioanalytical laboratory. A number of case studies typically determined frozen long-term stability [18–26], some of which also examined other test items. Test items and study design were surveyed by EBF [27] and GCC [7] and extensively discussed by Yadav et al. [28]. Although the information contributed by these reports is informative for the general ISS evaluation, they are insufficient to address ISS in the presence of AG. Moreover, the regulatory guidelines of the United States [17], European Union [29], and Japan [30] do not address important aspects of ISS as follows: 1) test items, 2) a reference value, 3) sample numbers and selection, and 4) an equation for assessment. The narrative that follows considers these items from scientific and practical perspectives.

Test items of ISS in the present study were whole blood stability, short-term stability (before and after acidification), freeze-thaw stability, frozen stability, and stability during extraction. Tan et al. determined short-term stability, frozen long-term stability, freeze-thaw stability, and autosampler stability for ramipril and ramiprilat using incurred samples [13]. To investigate the remaining test items (i.e. whole blood stability and stability during extraction), Tan et al. used blank plasma spiked with authentic AG. Nouman et al. investigated only short-term stability and frozen long-term stability in their study of dabigatran etexilate and its active metabolite [14]. Because sample collection and extraction can lead to the degradation of AG [31, 32], stability during these phases should be tested using incurred samples. Further, because AG is more stable after acidification [33], it is important to determine ISS before the acidification step. Because ASP3258-AG is discarded during extraction in the present method, the AG will not affect autosampler stability, which is why autosampler stability was not examined in the ISS test presented here. However, autosampler stability should be determined if scientifically required.

A reference value is needed to assess stability. In the present study, the reference value was the mean measured concentration of samples that were processed without a delay from blood collection to extraction completion (Sample A). Because the test items in the present study included stability during sample collection and extraction, the reference value was acquired from samples for which the times required for sample collection and extraction were minimized as much as possible. This experimental procedure was not employed in studies by either Tan et al. [13] or Nouman et al [14], possibly because human incurred samples were used, and because it is generally difficult in a clinical setting to start an analysis immediately after blood collection. Sample H (positive control) helped qualify the use of Sample A as a reference value, because the ASP3258 concentration of Sample H was higher than that of Sample A, suggesting that ASP3258-AG was not completely converted to ASP3258 in Sample A. Therefore, Sample A can serve as a negative control.

In the present study, a limited number of samples were used for the ISS test, because Sample A had to be processed quickly, and Samples B, C, D, and G required intensive and punctual laboratory work. Furthermore, relatively few samples were necessary, because samples from experimental animals are more homogeneous compared to samples acquired from humans. Because maximum plasma concentration (Cmax) was important for the planned toxicity studies, samples around Cmax were used for determining ISS. In contrast to the present study, Tan et al. used nine individual samples for frozen long-term stability, 15 individual samples for short-term stability, and three pooled samples for short-term stability, autosampler stability, and freeze-thaw stability [13]. Nouman et al. used one pooled sample that was prepared by pooling samples from a subject at multiple sampling time points [14]. Because an acceptable number of samples for ISS tests can vary for practical reasons, it is advisable to determine the number on a case-by-case basis. Tan et al. and Nouman et al. chose samples to cover multiple sampling time points [13,14], likely because they planned to use their methods to evaluate full pharmacokinetic profiles. When both Cmax and elimination phase are important in a pharmacokinetic study, it is reasonable to select samples accordingly, because the abundance of AG relative to the analyte can vary depending on the sampling times and the ISS results therefore can change as well.

The present study used an equation to compare the mean of triplicate determinations of stability samples (Samples B to G) with that of a negative control (Sample A). This equation is used frequently in whole blood stability assessment where spiked whole blood samples are centri-

fuged, and the harvested plasma samples are assayed in triplicate. Tan et al. and Nouman et al. used the same approach for pooled sample(s) [13,14]. An alternative equation would be one used for incurred sample reanalysis (ISR), where the denominator is the mean of a test sample and a negative control sample. This equation serves as a good option for studies of ISS, particularly when the ISS study design is similar to that of ISR. For example, Tan et al. employed this equation when they analyzed individual samples [13].

Conclusions

The presence of ASP3258-AG in incurred samples has been a major obstacle to reporting the accurate concentrations of ASP3258 for submission to regulatory agencies. The present study successfully developed and validated a method in accordance with FDA guidance [17], and conducted an ISS test to demonstrate that AG did not affect the quantification of ASP3258. This method was successfully applied to a toxicokinetic study in monkeys.

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