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Development and validation of sensitive and selective LC–MS/MS methods for the determination of BMS-708163, a γ-secretase inhibitor, in plasma and cerebrospinal fluid using deprotonated or formate adduct ions as precursor ions

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ABSTRACT

BMS-708163 is a γ-secretase inhibitor that is being developed for the treatment of Alzheimer's disease. Several LC–MS/MS methods have been developed for the determination of BMS-708163 in both plasma and cerebrospinal fluid in support of dog, rat, mouse and human studies. To support non-clinical studies, an LC–MS/MS method with a lower limit of quantitation (LLOQ) of 5 ng/mL, was developed and validated in dog, rat, and mouse plasma by using the deprotonated ion as the precursor ion. To support clinical studies, an LC–MS/MS method with LLOQ of 0.1 ng/mL, was developed and validated in human plasma by using the formate adduct as the precursor ion. Formic acid (0.01%) in water and acetonitrile was found to be the most favorable mobile phases for both deprotonated and formate adduct ions in negative electrospray ionization mode. A combination of a 3M Empore™ C18 plate for SPE and a Waters Atlantis dC18 analytical column for separation was used to achieve a highly selective solid phase extraction and chromatographic procedure from plasma without dry down and reconstitution steps. In the development of an assay for BMS-708163 in cerebral spinal fluid (CSF), significant non-specific binding of BMS-708163 was observed and resolved with pre- or post-spike of 0.2% Tween 20 into CSF samples. A dilute-and-shoot LC–MS/MS method with LLOQ of 0.1 ng/mL was developed and validated to assess BMS-708163 exposure in human CSF.

1. Introduction

Alzheimer's disease (AD) is the most common form of dementia, and is an irreversible, progressive and fatal brain disease. There are an estimated more than 35 million people with AD worldwide [1]. People with AD live an average of eight years after the AD symptoms become noticeable to others [2]. AD was first described in 1906 by German physician Alois Alzheimer, but the cause of AD is still not fully understood today [3]. However, many studies support the hypothesis that the increased formation of oligomeric species of β-amyloid (Aβ) peptides may be the cause of AD [4–7]. Aβ peptides are the metabolites of the transmembrance amyloid precursor protein (APP), and they are generated by the cleavages of APP by different enzymes. γ-Secretase is an enzyme involved in the last step of the cleavage generating Aβ peptides [8]. As a result, compounds that can reduce the formation of Aβ peptides by inhibiting the γ-secretase enzyme are being investigated as potential drugs to help AD patients.

In addition to APP, there are several other substrates for γ-secretase, including Notch protein, a single-pass transmembrane receptor protein, which is involved in cell growth, gene expression and cell–cell interactions. γ-Secretase activity is required for signaling by the Notch-family of transmembrane receptors. Inhibition of Notch signaling could cause gastrointestinal toxicity and mechanism-based side effects [9,10]. Therefore, the selectivity for inhibition is critical for the drug efficacy and safety. BMS-708163 is a potent and selective γ-secretase inhibitor being developed for the treatment of AD [11–14]. *In vitro* pharmacology studies indicate that BMS-708163 is about 193-fold more selective for APP than Notch [11]. *In vivo* studies in rats and dogs show that BMS-708163 reduces brain Aβ in both rats and dogs without Notch-related side effects [14]. Similar Aβ reduction effect in human cerebral spinal fluid (CSF) is observed in phase I clinical studies. BMS-708613 is currently in phase II clinical trials.

Several liquid chromatography–mass spectrometry/mass spectrometry (LC–MS/MS) methods have been developed and validated for the determination of BMS-708163 in plasma and CSF in support of dog, rat, mouse and human *in vivo* studies. To support the safety

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evaluation of BMS-708163 in rodent and non-rodent animals, an LC–MS/MS method with a lower limit of quantitation (LLOQ) of 5 ng/mL was developed and validated in dog, rat and mouse plasma. To support the safety, tolerability and pharmacokinetics (PK) evaluation of BMS-708163 in human and capture the day one (0–24 h) exposure for the very low starting dose of 0.3 mg in the first-in-human study, an LC–MS/MS method with a LLOQ of 0.1 ng/mL was developed and validated in human plasma. A selective solid phase extraction (SPE) procedure without dry down and reconstitution steps was developed for both methods. To assess exposure of BMS-708163 in human CSF, a dilute-and-shoot LC–MS/MS method in 0.2% Tween 20 treated human CSF was developed and validated. The assay was designed to overcome non-specific binding of BMS-708163 during sample collection and processing.

2. Experimental

2.1. Materials and reagents

BMS-708163 and its stable labeled internal standard (IS) $^{13}C_6$-BMS-708163 (Fig. 1) were synthesized at Bristol-Myers Squibb. Control K_2EDTA plasma for different species and control human CSF were purchased from Bioreclamation Inc. (Hicksville, NY). Ammonium formate (>96%) was purchased from J.T. Baker (Phillipsburg, NJ). Formic acid (>98%) and dimethylsulfoxide (DMSO) were purchased from EMD (Darmstadt, Germany). Acetonitrile and methanol, ACS HPLC grade, were purchased from EM Sciences (Gibbstown, NJ). Polyoxyethylene (20) sorbitan monolaurate solution (10% Tween 20 in water) was purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Sample preparation for LC–MS/MS analysis

BMS-708163 stock solutions for standard (STD), quality control (QC) and its IS, at a nominal concentration of 0.2 mg/mL, were prepared in acetonitrile/DMSO (1:1, v/v). A working solution of 20,000 ng/mL of BMS-708163 was prepared by appropriate dilution of a 0.2 mg/mL stock solution with control matrix. For the assay in dog plasma, standards at nominal concentrations of 5, 10, 20, 100, 500, 2500, 4000 and 5000 ng/mL were prepared daily by serial dilution of the 20,000 ng/mL working solution. QC samples at nominal concentrations of 5, 15, 200, 2500 and 4000 ng/mL were prepared by serial dilution of the 20,000 ng/mL working solution prepared from the QC stock solution. For the assays in human plasma or Tween 20 treated human CSF, standards at nominal concentrations of 0.1, 0.2, 0.4, 2, 10, 50, 80 and 100 ng/mL and QC samples at 0.1, 0.3, 4, 50 and 80 ng/mL were prepared similarly as those in the animal assay. Tween 20 treated human CSF was prepared by adding 1 mL of the purchased Tween 20 in water (10%) to 49 mL of human CSF.

After plasma samples (50 μL for the animal plasma assay and 100 μL for human plasma assay) and 50 μL of IS (200 ng/mL for the animal assay and 2 ng/mL for the human assay) were manually transferred into Costar 96-well plates (VWR Scientific Products, Bridgeport, NJ), a Tecan Genesis RSP 150 liquid handling system (Tecan US, Durham, NC) with fixed tips was used for all steps of SPE process, except the step to load samples onto SPE plates, where a Tomtec QUADRA 96-320 system (Hamden, CT) with disposable tips was used to avoid carryover. The detailed SPE procedure is described here: 200 μL of 50 mM ammonium formate in water was added to each well, and then the 96-well plates were vortexed for 1 min to mix the buffer with plasma samples. 3M Empore™ C18 High Performance Extraction Disk plates (3M Bioanalytical Technologies, St. Paul, MN) were conditioned with 400 μL of methanol, followed by 400 μL of 50 mM ammonium formate in water. After all samples were loaded onto the conditioned SPE plates, the SPE plates were washed with 400 μL of 50 mM ammonium formate in water, followed by 400 μL of 20% methanol in water containing 50 mM ammonium formate. Finally, the SPE plates were eluted twice with 100 μL of 40% acetonitrile, 40% methanol, 20% water containing 1% formic acid, and 10 μL of the eluant was injected for LC–MS/MS analysis.

For the human CSF assay, 200 μL of IS in acetonitrile (1 ng/mL) was added into 100 μL 0.2% Tween 20 treated human CSF samples. After mixing and centrifugation, 200 μL of supernatant was transferred with a Tomtec liquid handler and 10 μL was injected into the LC–MS/MS system.

2.3. LC–MS/MS instrumentation

LC–MS/MS data were acquired by Analyst® software for LC–MS (version 1.4) on a Sciex API 4000 tandem mass spectrometer (Applied Biosystems, Foster City, CA) in negative electrospray ionization (ESI) mode. The separation was achieved on a Waters (Milford, MA) Atlantis dC18 analytical column (2.1 mm × 50 mm, particle size 5 μm) with gradient elution using mobile phases of 0.01% formic acid in water and 0.01% formic acid in acetonitrile. Two Shimadzu (Tokyo, Japan) LC-10ADvp pumps and a Shimadzu SIL-HTC autosampler were used as the delivery pumps and autosampler, respectively. A linear gradient was used as follows: increase linearly from 40% to 95% B from 0 to 1.5 min; hold for 0.6 min; decrease linearly from 95% to 40% from 2.1 to 2.2 min; hold for 0.8 min. The flow rate was 0.4 mL/min.

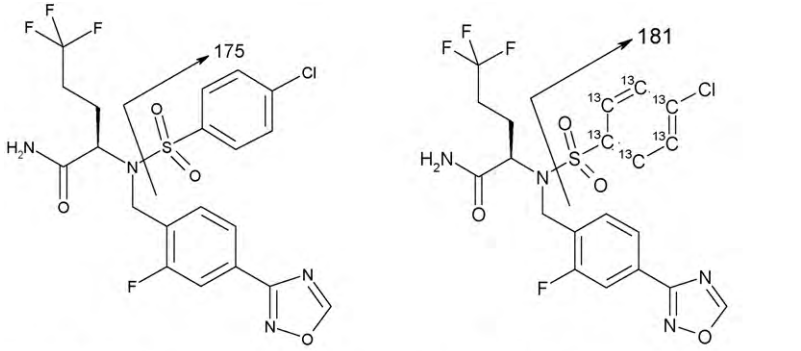

Fig. 1. Chemical structures of BMS-708163 and it stable labeled IS, ^{13}C6-BMS-708163.

2.4. Method validation

For both animal and human assays, linearity, accuracy and precision, specificity, recovery, matrix effect, stability and re-injection integrity were evaluated following the FDA Guidance for Industry—Bioanalytical Method Validation (2001) [15].

Accuracy and precision were evaluated using QC samples prepared at five different concentration levels covering the STD curve ranges. In addition, a dilution QC was used to evaluate dilution linearity. Three accuracy and precision runs were conducted for full validations in dog plasma, human plasma and human CSF assays, respectively. The interferences from endogenous materials and IS were tested by extracting six different lots of blank matrices with and without IS, and six LLOQ samples prepared in the corresponding six different lots of blank matrices. The interference was evaluated by comparing the SRM chromatograms among the three types of samples. The extraction recovery for BMS-708163 was evaluated by comparing the responses of the pre-extraction spiked samples to those of post-extraction spiked samples. The matrix effect for BMS-708163 was evaluated by comparing the responses of the post-extraction spiked samples to those of samples prepared in neat solution.

3. Results and discussion

3.1. Mobile phases and MS conditions

BMS-708163 is a hydrophobic basic compound. The aqueous solubility is less than 1 μg/mL and measured log *P* and log *D* (pH 7.4) are 4.6 and 3.52, respectively. During the method development, both positive and negative ESI modes were evaluated by post-column infusion of BMS-708163 (5 μg/mL) at 10 μL/min while the flow rate for mobile phases (20% water and 80% acetonitrile containing 0.1% formic acid) was 0.4 mL/min. The most abundant precursor ion is the protonated ion $[M+H]^+$ (*m*/*z* at 521) for BMS-708163 under the positive ESI mode. Better sensitivity could be achieved by using the deprotonated ion $[M-H]^-$ (*m*/*z* at 519) ion in negative ESI mode even with the presence of 0.1% formic acid in the mobile phases, which is thought to be an unfavorable environment for the ESI negative mode [16]. In addition to the deprotonated ion, the formate adduct $[M+HCOO]^-$ ion (*m*/*z* at 565) was also observed and its abundance was more than 10-fold higher than that of the deprotonated ion. The negative ESI MS and MSMS mass spectra for BMS-708163 are shown in Fig. 2a–c. The most abundant product ion was the same (*m*/*z* at 175) for the deprotonated and formate adduct precursor ions.

Selected reaction monitoring (SRM) was used for ion detection. The MS/MS parameters of SRM transition of *m*/*z* 519 → 175 were fine tuned in the different mobile phases described in Table 1. BMS-708163 at 10 ng/mL in acetonitrile and water (1:1) neat solution was then injected using the corresponding optimized SRM conditions for the different mobile phases and the results are shown in Table 1. Similar background noise (~100 cps) was observed in all cases.

Weak organic acids, such as acetic acid or formic acid, are commonly used additives for the ESI positive mode since an acidic environment is favorable to the protonation of basic analytes [16,17]. Similarly, the use of base additive in the ESI negative mode favors the deprotonation of acidic analytes. However, this general rule is not always true and the effect of mobile phase additives on the MS response is largely compound dependent, in particular in regard to ion suppression/enhancement effects [16–19]. In our study, as shown in Table 1, compared to the result obtained with no additive in mobile phases, addition of 0.01% ammonium hydroxide caused significant deprotonated ion suppression, while the addition of 5 mM ammonium acetate or 5 mM ammonium formate showed mild deprotonated ion suppression. It was also observed, the deprotonated ion suppression decreased gradually with decreasing formic acid concentration from 0.1% (~26 mM), to 0.05% (~13 mM) to 0.02% (~5.2 mM), and finally to 0.01% (~2.6 mM), where 150% of ion enhancement was observed.

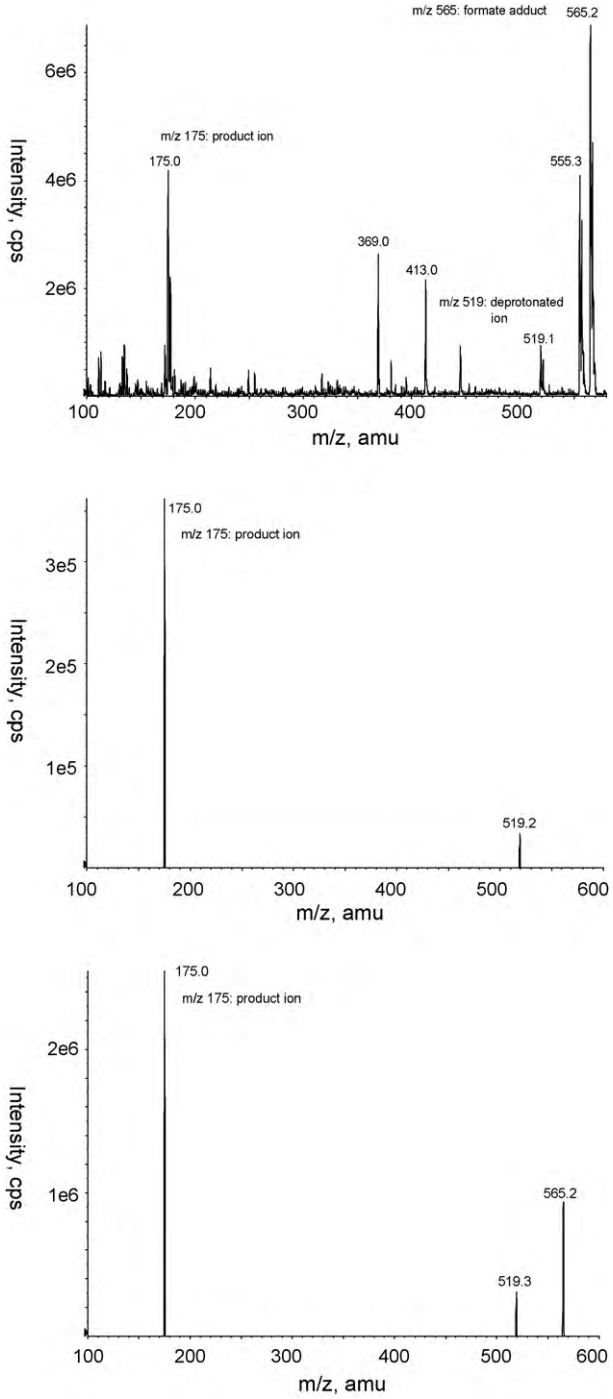

Fig. 2. (a) Electrospray negative ion Q1 mass spectrum for BMS-708163 with *m*/*z* 519 for $[M-H]^-$ and *m*/*z* 565 for $[M+HCOO]^-$; (b) electrospray negative ion MS/MS product ion spectrum of precursor ion of *m*/*z* 519 for BMS-708163; (c) electrospray negative ion MS/MS product ion spectrum of precursor ion of *m*/*z* 565 for BMS-708163.

The SRM conditions were also fine tuned with the formate or acetate adduct as the precursor ion when ammonium acetate, ammonium formate or formic acid were used as additive in the mobile phase. As shown in Table 1, compared to the responses

Table 1
LC–MS/MS chromatogram peak heights for SRM transitions using deprotonated or adduct ion as precursor ion.

Additive in mobile phase A (water) and B (acetonitrile)	Adduct observed	SRM (m/z 519–175) (cps)	SRM (using adduct as precursor ion) (cps)
0.01% ammonium hydroxide	No	750	NA
5 mM ammonium acetate	Acetate adduct	2000	12,000
5 mM ammonium formate	Formate adduct	2000	33,000
0.1% formic acid	Formate adduct	1000	15,000
0.01% formic acid	Formate adduct	6000	100,000
No additive	No	4000	NA

obtained for the deprotonated ions, the adduct ion responses increased by 6–16-fold, and the greatest increase was achieved for the formate adduct when 0.01% formic acid was used. The reason might be 2-fold: a formic acid concentration of 2 mM (0.01%) in the mobile phase was high enough to favor formate adduct formation but low enough to avoid ion suppression for ESI in the negative mode. This is similar to the data reported by Quang et al. [20], in that a very significant sensitivity increase was achieved by monitoring formate adduct for two neutral compounds with 2 mM of formic acid in mobile phase.

Although a significant sensitivity increase was observed by monitoring adducts in the negative ion mode, there is still one major concern in using anionic adducts for quantitative LC–MS/MS methods. Cai et al. [21] investigated the formation and stability of anionic adducts in the negative ESI mode. The anionic adduct normally is formed due to the solution-phase affinity between the analyte molecule and the anion before the electrospray process, but the stability of anionic adduct is mainly determined by factors related to the gas-phase basicity, which is difficult to predict. For these reasons, the deprotonated ion was selected as the precursor ion for the animal plasma assays with LLOQ of 5 ng/mL. Formic acid, 0.01% in water (mobile phase A) and acetonitrile (mobile phase B) were used in the animal plasma assays. However, since a much lower LLOQ could be easily achieved by using the formate adduct as the precursor ion without any other modifications for sample preparation and LC conditions, this was the approach used for the human plasma assay, in which an LLOQ of 0.1 ng/mL was desirable to capture the full PK profile in the first-in-human studies. It was observed that similar accuracy and precision were achieved for both assays, which are presented in the validation results. This result indicates that either the formate adduct ions for BMS-708163 are stable in the gas-phase or the stability issue is canceled out by using the C^{13}-labeled IS.

3.2. Solid phase extraction

Because of the hydrophobic nature of BMS-708163, a selective reversed phase SPE method was relative easy to develop. However, to skip the dry down and reconstitution steps, one aspect had to be taken into consideration. The organic strength of the SPE elution should be compatible with the organic strength in mobile phases when the analyte elutes out on a reversed phase analytical column. Both Waters Oasis® HLB (10 mg, Waters Corporation, Miford, MA) SPE plate (containing a polymeric water-wettable reversed phase sorbent), and 3M C18 (15 mg) SPE plate (containing an octadecyl functional group bonded to the endcapped silica surface to provide non-polar interactions and minimize the polar interactions) were screened and produced clean extract with high recovery. Screening results showed that BMS-708163 was well retained on both reversed phase SPE plates and polar endogenous compounds could be washed out using a wash solution of relative high eluting strength (30% for the 3M plate and 40% for the HLB plate) without affecting recovery. Almost 100% recovery could be achieved with a 90% organic eluant for the HLB plate and 70% organic eluant for the 3M plate. Since BMS-708163 was well retained on a Waters Atlantis column (dC18 5 μm, 2.1 mm × 50 mm) with less than 80% acetonitrile in mobile phases, plasma samples were processed with 3M plate in order to skip the dry down and reconstitution steps. In addition, to have consistent SPE recovery from run to run, a 20% organic wash and 80% organic eluant were used in the final method to accommodate any variability from SPE plates or SPE solutions. This sample preparation procedure was used in all the plasma assays.

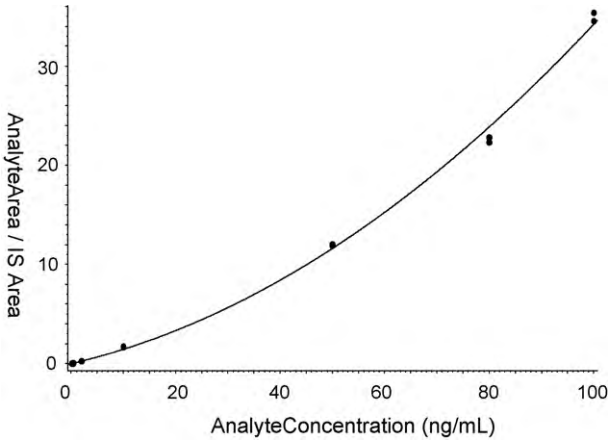

Fig. 3. STD curve (0.1–100 ng/mL) for BMS-708163 in human CSF prepared with multi-step serial dilution.

3.3. Non-specific binding in human CSF

Very significant non-specific binding losses were observed in the development of the assay in human CSF, possibly due to the hydrophobicity of BMS-708163. Fig. 3 shows the STD curve for BMS-708163 in human CSF exhibiting curvature indicative of loss due to adsorption. The adsorption loss from each step of serial dilution in human CSF was quantified by a BMS-708163 STD curve prepared in 1:1 acetonitrile/water from 0.1 to 100 ng/mL, and the results are shown in Table 2. It was observed that the adsorption loss occurred across the entire concentration range from 0.2 mg/mL to 0.1 ng/mL. As shown in Table 2, more than 50% of the compound had already been lost during the first three steps of serial dilution

Table 2
Adsorption loss from each step of serial dilution of BMS-708163 in human CSF.

Nominal conc. for CSF samples (ng/mL)	Predicted conc. (ng/mL)	Loss %	Serial dilution step from 0.2 mg/mL stock solution
100	47.56	52.4	3
80.0	30.69	61.6	3
50.0	16.27	67.5	4
10.0	2.33	76.7	5
2.00	0.35	82.5	6
0.40	<0.10		7
0.20	<0.10		8
0.10	<0.10		9

Table 3
Adsorption loss on different types of vials for BMS-708163 in human CSF.

Transfer step	Polypropylene vial		Glass vial		Silanized glass vial	
	Pred. conc. (ng/mL)	Loss %[a]	Pred. conc. (ng/mL)	Loss %[a]	Pred. conc. (ng/mL)	Loss %[a]
Original	39.55		43.40		46.90	
1st transfer	25.85	34.6	35.40	18.4	41.20	12.2
2nd transfer	15.40	40.4	27.45	22.5	33.75	18.1
3rd transfer	8.05	47.8	17.50	36.2	25.90	23.3

[a] Loss on each sequential step.

from 0.2 mg/mL to 100 ng/mL or 80 ng/mL. More than 80% loss was observed with 6 steps of dilution.

Adsorption losses are a common issue for hydrophobic compounds in low-protein biological fluids such as urine or CSF [22]. Additions of plasma, bovine serum albumin [23,24] and surfactants, such as Tween 20 [22] or 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) [25], or changing the type of container or vial [22] have been shown to be effective to resolve or minimize the adsorption loss, although the effectiveness of each solution is compound dependent. Three different types of vials, i.e. polypropylene, glass and silanized glass vials, were evaluated for the adsorption loss. To this end, three aliquots of 1 mL CSF sample from the same source were transferred into 2 mL polypropylene, glass and silanized glass vials. After a brief vortex, one aliquot of 50 μL CSF sample (original) from each type of vial was transferred into a 96-well plate for sample preparation. The remaining CSF samples were transferred into a corresponding new vial and vortexed. Again, one aliquot of 50 μL CSF sample (1st transfer) was transferred into the 96-well plate for analysis. The same procedure was repeated for the 2nd and 3rd transfer. Polypropylene disposable tips (1 mL) were used for the transfer from vial to vial, and 250 μL polypropylene disposable tips were used for the transfer from vial to 96-well plate. As shown in Table 3, silanized glass vials showed the best result in terms of reducing adsorption losses. However, that level of the adsorption loss was still not satisfactory for an accurate LC–MS/MS assay. It should be pointed out that the adsorption loss from each step represents the combined losses to the surfaces of the vial and the pipette tip used to transfer the solution. About 10% loss to the surface of 1 mL polypropylene tip was observed at 30 ng/mL concentration level when 1 mL human CSF was transferred.

To prevent the non-specific binding of BMS-708163 in human CSF sample, the addition of the non-ionic surfactant, Tween 20, at different concentrations (0.01–0.5%) was evaluated. Test results showed that adsorption to surfaces could be totally eliminated in human CSF containing 0.1–0.5% of Tween 20. Table 4 shows the results with 0.2% Tween 20 in human CSF, and no adsorption loss for BMS-708163 was observed. Based on the test results, 0.2% Tween 20 was pre-spiked into human CSF before the preparation of STD and QC samples in the method validation and sample analysis. Unlike for STD and QC samples, Tween 20 could only be spiked into CSF incurred samples post-collection. Therefore, the recovery of BMS-708163 from QC samples in CSF post-spiked with 0.2% of Tween 20 was evaluated, and no significant difference was observed between pre- and post-spiking of 0.2% Tween 20 into human CSF, suggesting that Tween 20 could be added into human CSF incurred samples after sample collection or before sample preparation. However, it must be noted that the addition of 0.2% of Tween 20 only addresses adsorption losses in the collection vial and during sample preparation, but not the potential losses to the needle and tubing during sample collection. To minimize these losses or at least to get uniform loss among subjects, a standardized collection procedure, including the type of needle and vial, was developed. CSF samples were collected through the needle directly, without any tubing. The first portion of the CSF sample (~1 mL) was discarded and the second portion of the CSF sample was collected for other purposes. The third portion of the CSF sample (1 ± 0.2 mL) was collected for the LC–MS/MS analysis of BMS-708163. A volume of 20 μL of 10% Tween 20 solution in water was added into each vial before the sample preparation.

Table 4
Serial dilution of BMS-708163 in 0.2% Tween 20 treated human CSF.

Nominal conc. BMS-708163 in 0.2% Tween 20 treated CSF samples (ng/mL)	Predicted conc.[a] (ng/mL)	% Dev.	Serial dilution step from 0.2 mg/mL stock solution
100	102.802	2.8	3
20.0	20.421	2.1	4
4.00	3.911	−2.2	5
0.40	0.393	−1.8	6
0.20	0.194	−3.0	7
0.10	0.102	2.0	8

[a] Predicted concentration was calculated based on a STD curve prepared in 1:1 acetonitrile/water.

3.4. Validation results

After the method optimization, full validations were conducted to assess the linearity, accuracy and precision, specificity, recovery, matrix effect, stability, re-injection integrity and carryover for plasma and CSF in the different animal species and in human. Only the validations for the dog plasma assay, human plasma assay and 0.2% Tween 20 treated human CSF assay are reported here but similar performance was obtained for the other assays.

3.5. STD curve linearity and QC accuracy and precision

Both linear and quadratic regression models were evaluated for all three types of assays. A linear regression provided the best fit of the STD curves for all assays and with the different curve ranges. The accuracy and precision results are listed in Tables 5 and 6. The results for the STD curves and QCs demonstrated that all assays were precise and accurate for the analysis of BMS-708163 in non-clinical and clinical studies.

3.6. Specificity and LLOQ

For all matrices tested, no significant interfering peaks from endogenous materials and the isotopic labeled IS were observed at the retention time and in the ion channel of either the analyte or the IS. In addition, for the six pairs of LLOQ and blank matrix samples with IS, the ratio of the LLOQ signal over the blank sample noise was evaluated. All of the 6 lots tested for 3 assays (total $N = 18$) showed a ratio value > 5. The specificity test results indicated that the interferences from endogenous materials or IS were minimal. Typical SRM chromatograms for the blank with and without IS, and the LLOQ for dog and human plasma assays are shown in Figs. 4 and 5.

Table 5
Accuracy and precision for BMS-708163 in dog plasma.

Assay	Nominal conc. (ng/mL)	LLOQ (5.00 ng/mL)	Low QC (15.0 ng/mL)	GM QC (200 ng/mL)	Mid QC (2500 ng/mL)	High QC (4000 ng/mL)	Dilution (20,000 ng/mL)
Dog plasma	Mean conc.	5.00	14.93	204.83	2623.52	4111.57	20050.18
	% Dev.	0.0	−0.5	2.4	4.9	2.8	0.3
	Inter-assay precision (%CV)	9.8	4.4	0.0	4.9	1.9	0.0
	Intra-assay precision (%CV)	6.0	10.4	2.0	4.5	2.3	2.7

Table 6
Accuracy and precision for BMS-708163 in human plasma and human CSF.

Assay	Nominal conc. (ng/mL)	LLOQ (0.100 ng/mL)	Low QC (0.300 ng/mL)	GM QC (4.00 ng/mL)	Mid QC (50.0 ng/mL)	High QC (80.0 ng/mL)	Dilution (10,000 ng/mL)
Human plasma	Mean conc.	0.107	0.283	3.922	48.444	77.720	9606.666
	% Dev.	7.0	−5.7	−2.0	−3.1	−2.9	−3.9
	Inter-assay precision (%CV)	10.1	0.0	0.7	3.8	3.6	4.5
	Intra-assay precision (%CV)	8.7	5.9	5.5	2.4	1.7	2.0
Human CSF	Mean conc.	0.102	0.305	3.954	50.371	78.708	993.293
	% Dev.	2.0	1.7	−1.2	0.7	−1.6	−0.7
	Inter-assay precision (%CV)	3.8	1.3	0.8	2.9	1.4	2.4
	Intra-assay precision (%CV)	4.3	4.0	3.1	3.4	2.4	3.6

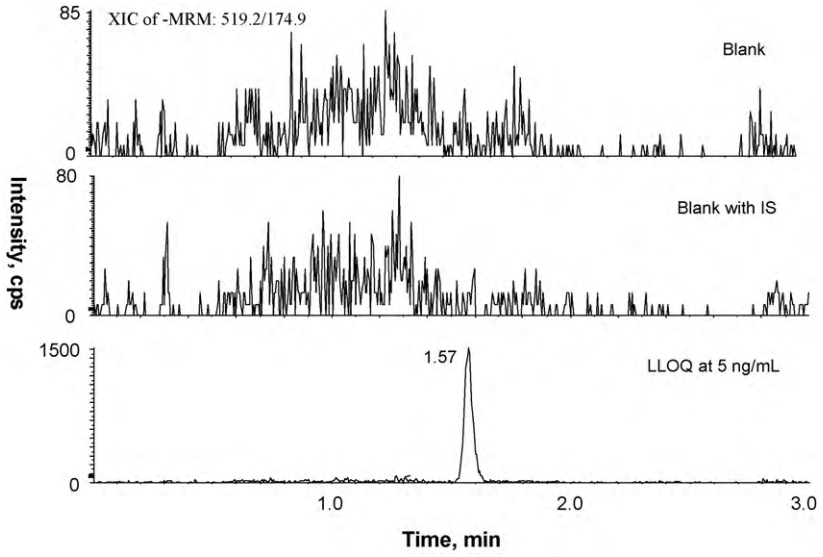

Fig. 4. SRM chromatogram for BMS-708163 in dog plasma.

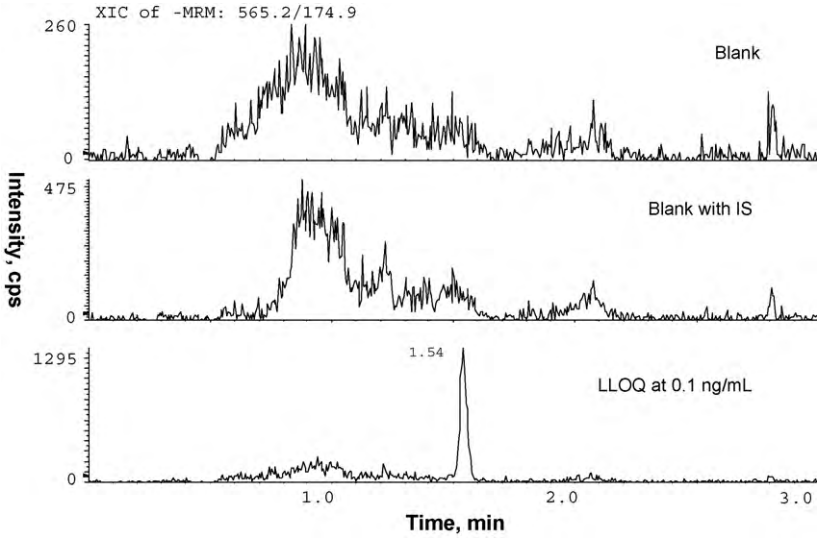

Fig. 5. SRM chromatogram for BMS-708163 in human plasma.

Table 7
Stability data for BMS-708163.

Stability type	Room temperature	Freeze–thaw at −20 °C	Frozen storage at −20°	Re-injection integrity at 5 °C
Dog plasma	24 h	4 cycles	433 days	7 days
Human plasma	24 h	5 cycles	374 days	3 days
Human CSF	NA	NA	196 days	NA
0.2% Tween 20 treated human CSF	24 h	5 cycles	295 days	8 days
Human blood	2 h	NA	NA	NA
DMSO/ACN (1:1)	6 h	NA	323 days	NA

NA, not applicable.

Table 8
Recovery of BMS-708163 in human CSF by post-spike 0.2% Tween 20.

Sample type	Nominal conc. (ng/mL)	Mean predicated conc. (ng/mL)	% Dev.
Low QC in human CSF[a]	0.3	0.25	−16.7
High QC in human CSF[a]	80	64.20	−19.8
Dilution QC in human CSF[a]	1000	798.60	−20.1
Low QC in 0.2% Tween 20 treated human CSF	0.3	0.30	0.0
High QC in 0.2% Tween 20 treated human CSF	80	76.52	−4.4
Dilution QC in 0.2% Tween 20 treated human CSF	1000	935.60	−6.4

[a] Containing less than 0.02% of Tween 20.

3.7. Extraction recovery and matrix effect

The SPE recoveries for dog and human plasma assays were 94.7% and 99.0%, respectively. The extraction recovery for the dilute-and-shoot human CSF assay was 96.0%. The matrix effect for the dog and human plasma assays was 1.18 and 0.92, respectively. The matrix effect for the dilute-and-shoot human CSF assay was 0.70.

3.8. Stability

Stability for BMS-708163, including room temperature, freeze–thaw, and long term storage in matrix, was evaluated in triplicate. The re-injection integrity was evaluated by re-injecting an entire run. To establish the stability for BMS-708163, the deviations of the mean measured concentrations of the test samples from the nominal concentrations have to be within 15%. All established stabilities for BMS-708163 are shown in Table 7.

3.9. Recovery of BMS-708163 in human CSF post-spiked with 0.2% Tween 20

BMS-708163 was recovered for incurred samples in human CSF (~1 mL) by post-spiking 20 μL of Tween 20 solution (10% Tween 20 in water). The recovery of BMS-708163 was evaluated and is listed in Table 8. Almost 100% recovery for BMS-708163 in human CSF was demonstrated by post-spiking 0.2% Tween 20 over the concentration range of 0.3–1000 ng/mL.

4. Conclusion

Sensitive and selective LC–MS/MS methods for the quantitation of BMS-708163 in dog and human plasma, and human cerebrospinal fluid were developed and validated using the deprotonated or formate adduct ion as the precursor ion, depending on the required sensitivity. Formic acid (0.01%) in mobile phase was found to be the most favorable mobile phases for both deprotonated and formate adduct ion in the ESI negative mode. A simple SPE procedure was developed for plasma assays by a combination of 3M Empore™ C18 SPE plate for SPE and Waters Atlantis dC18 column for separation. The issue of the non-specific binding of BMS-708163 in human CSF was addressed by spiking 0.2% Tween 20 into human CSF. These methods were successfully used to support non-clinical and clinical studies.

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