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Simultaneous determination of a selective adenosine 2A agonist, BMS-068645, and its acid metabolite in human plasma by liquid chromatography-tandem mass spectrometry—Evaluation of the esterase inhibitor, diisopropyl fluorophosphate, in the stabilization of a labile ester-containing drug

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Abstract

BMS-068645 is a selective adenosine 2A agonist that contains a methyl ester group which undergoes esterase hydrolysis to its acid metabolite. To permit accurate determinations of circulating BMS-068645 and its acid metabolite, blood samples must be rapidly stabilized at the time of collection. A sensitive, rapid and specific liquid chromatography-tandem mass spectrometry (LC/MS/MS) method for the simultaneous quantitation of BMS-068645 and its acid metabolite in human plasma has been developed and validated using diisopropyl fluorophosphate (DFP) as the esterase inhibitor to prevent BMS-068645 from converting to its acid metabolite. The D_5-stable isotope labeled analogs of BMS-068645 and its metabolite were used as the internal standards (IS). Analytes and IS in plasma containing 20 mM DFP were acidified and extracted into methyl *tert*-butyl ether. The liquid–liquid extraction effectively eliminated the strong matrix effect caused by the esterase inhibitor. The chromatographic separation was achieved on a Waters Atlantis C18 column with a run time of 4 min. Detection was performed on a Sciex API 4000 with positive ion electrospray mode (ESI/MS/MS), monitoring the ion transitions *m/z* 487 > 314 and 473 > 300 for BMS-068645 and its acid metabolite, respectively. The method was validated over the range from 0.020 to 10.0 ng/mL for BMS-068645 and 0.050 to 10.0 ng/mL for its acid metabolite. Inter- and intra-run precision for the quality control samples during validation were less than 8.7% and 4.0%, respectively, for the two analytes. The assay accuracy was within ±5.4% of the nominal values. The esterase inhibitor effectively stabilized BMS-068645 during blood collection and storage. Blood collection tubes containing DFP were easily prepared and used at the clinical sites and could be stored at −30 °C for 3 months. This method demonstrated adequate sensitivity, specificity, accuracy, precision, stability and ruggedness to support the analysis of human plasma samples in pharmacokinetic studies.

Keywords: BMS-068645; BMS-068645-acid; Liquid chromatography-tandem mass spectrometry; Diisopropyl fluorophosphate; DFP; Adenosine 2A agonist; Esterase inhibitor

1. Introduction

BMS-068645 is a selective adenosine 2A agonist and is being developed as a pharmacological stress agent for increasing blood flow in myocardial perfusion imaging for the evaluation of coronary artery disease [1–4]. BMS-068645 (Fig. 1) contains a methyl ester group and has been reported to undergo esterase hydrolysis to the acid metabolite, BMS-068645-acid (Fig. 1), at different rates in rodent, canine and human blood [5]. The conversion of an ester-type drug to its acid metabolite can be blocked through the use of an esterase inhibitor. However, the use of an esterase inhibitor can add to the complexity of a bio-

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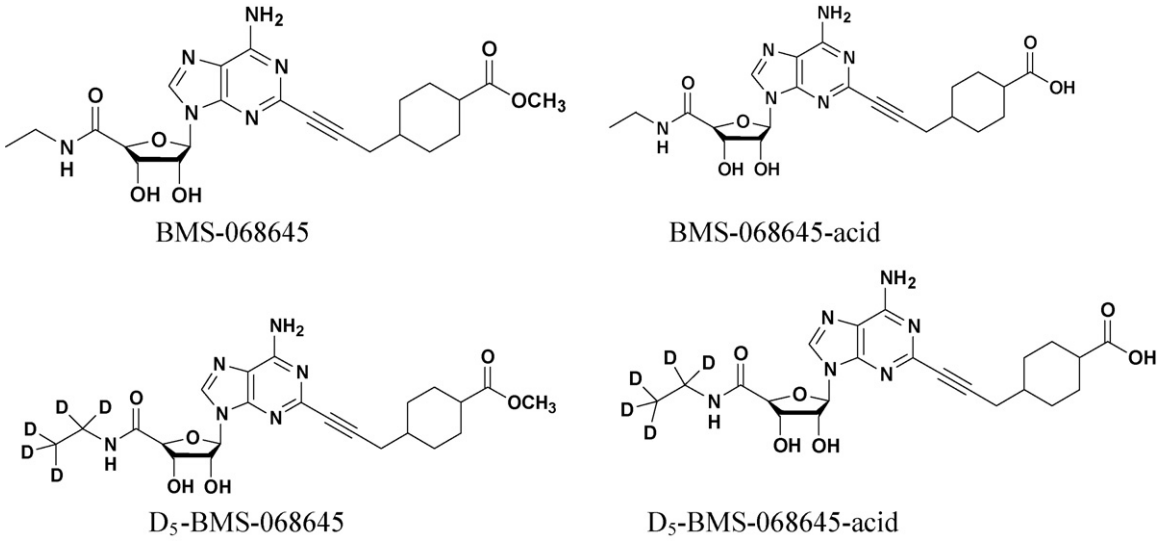

Fig. 1. Chemical structures of BMS-068645, its metabolite (BMS-068645-acid) and the internal standards.

analytical assay and possesses its own distinctive challenges to the method development process.

Esterases, which are classified into cholinesterase and carboxylesterases, are widely distributed in various tissues and blood [6–8]. Several esterase inhibitors were reported to stop the activities of different esterases in stability, pharmacokinetic and metabolism studies [6–11]. Yamaori et al. [9] used phenylmethylsulfonyl fluoride (PMSF), diisopropyl fluorophosphate (DFP), bis(*p*-nitrophenyl)phosphate (BNPP), BW284C51 and ethopropazine as esterase inhibitors in their study of human blood arylesterases and liver microsomal carboxylesterases. Paraoxon and DFP, which are potent inhibitors of serine esterase, showed nearly complete inhibition of hydrolysis for ester-type pro-drugs in plasma and liver microsomes, even at low concentrations ($\sim 10^{-6}$ M) [6]. Pauletti and Yang [10,11] have used paraoxon to significantly decrease the degradation rate of the esterase-sensitive cyclic prodrugs of peptides. Besides esterase inhibitors, other reagents have also been used to stabilize different types of pro-drugs. It was reported that *in vitro* degradation of the phosphate ester pro-drug was greatly minimized in plasma containing EDTA at the concentration commonly used as anticoagulant [12]. It has also been demonstrated that different species have different type of esterases and, therefore, require different inhibitors. For example, human butyrylcholinesterase (BChE) is irreversibly inhibited by DFP, echothiophate and paraoxon, but mouse BChE spontaneously reactivates [6,13].

For the quantitation of BMS-068645 and its acid metabolite, it has been reported that the esterase activity was stopped by adding 2 mL of acetonitrile directly to 1 mL of blood samples from rodent, canine and human. An LC/MS/MS method was used for the quantitation of the analytes and had standard curve ranges from 0.12 to 91.6 ng/mL and 0.13 to 94.6 ng/mL for BMS-068645 and acid metabolite, respectively. The total run time for this method was 22 min [5]. However, in order to support an ascending single dose clinical study in which the i.v. doses started from 0.1 μg/kg, a new method with lower detection limits, utilizing less sample volume and shorter run time was needed. A new stabilization procedure for BMS-068645 that could be easily used by the clinical site was also desirable.

2. Experimental

2.1. Chemicals and reagents

BMS-068645 (96.6%), BMS-068645-acid (98.69%) and internal standards (IS) D_5-BMS-068645 (97.3%) and D_5-BMS-068645-acid (97.19%) were obtained from Bristol-Myers Squibb Company Imaging Group (Billerica, MA, USA). HPLC grade methanol, acetonitrile, DMSO and methyl *tert*-butyl ether were purchased from EM Science (Gibbstown, NJ, USA). HPLC grade water was obtained from a Millipore Milli-Q Plus Water Purification System (Bedford, MA, USA). Hydrochloric acid (36.5%), a chemical reagent grade, was purchased from EM Science (Gibbstown, NJ, USA). Formic acid, an analytical reagent grade, was purchased from J.T. Baker (Phillipsburg, NJ, USA). Diisopropyl fluorophosphate, phenylmethylsulfonyl fluoride, paraoxon and eserine, the esterase inhibitors and chemical reagents, were purchased from Sigma (Saint Louis, Missouri, USA). Control human EDTA plasma was purchased from Bioreclamation Inc. (Hicksville, NY, USA). All chemicals, solvents and reagents were of high chemical purity and used without further purification.

2.2. Instrumentation

The LC/ESI/MS/MS system consisted of Shimadzu LC-10AD pumps (Columbia, MD, USA), a Perkin-Elmer Series 200 LC autosampler (Norwalk, CT, USA) and a Sciex API 4000 mass spectrometry (Foster City, CA, USA) connected to an Atlantis dC18 analytical column (3 μm; 50 mm × 2.1 mm i.d.) from Waters Corporation (Milford, MA, USA). The injection volume was 10 μL and the run time was 4 min. A mixture of 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) was used with a gradient program

at a flow rate of 0.3 mL/min at ambient temperature. The gradient program started with 20% mobile phase B for 0.4 min, then a linear increase to 70% B within 1.5 min, remained at 70% B for one minute and changed to 20% B with a linear gradient in 0.1 min for re-equilibration.

Detection was performed by selected reaction monitoring (SRM) from the protonated molecular ion ($[M+H]^+$) of the analytes to its product ion using positive ion electrospray tandem mass spectrometry. The product ion efficiency of the $[M+H]^+$ was optimized by varying all the ion source parameters. The final settings were: ionspray voltage (IS) 4.0 kV, declustering potential (DP) 81 V, collision energies (CE) 33 eV, collision cell exit potential (CXP) 18 V, entrance potential (EP) −10 V, and source temperature 400 °C. Nebuliser gas (GS1 and GS2), curtain gas (CUR) and collision gas (CAD) were set to 40, 40, 20 and 6, respectively. The monitoring ions for SRM analysis were set to *m/z* 487 > 314 for BMS-068645, *m/z* 473 > 300 for BMS-068645-acid, *m/z* 492 > 314 for D_5-BMS-068645 and *m/z* 478 > 300 for D_5-BMS-068645-acid. The scan dwell time was set to 0.3 s.

2.3. Stability evaluation

BMS-068645 stability in human blood (with and without esterase inhibitors) was evaluated. Human urine and methanol, with no or minimum esterase activity, were used as control in the stability test. The esterase inhibitors, DFP and paraoxon in water, PMSF and eserine in DMSO, were added to 1 mL fresh human blood (heparinized) to a final concentration of 10 mM of each inhibitor. BMS-068645 was added to blood, blood with 10 mM inhibitors, human urine and methanol to a final concentration of 25.0 ng/mL. The blood and methanol samples were maintained for 4 h at room temperature and the urine samples were maintained at room temperature for 96 h. Two milliliters of acetonitrile and internal standard were added to completely quench any esterase activity and end the incubation. The samples were vortexed, centrifuged and the supernatant was dried under nitrogen at 37 °C. The samples were reconstituted with 0.1% formic acid in water and analyzed by LC/ESI/MS/MS.

After DFP was chosen to be used as the stabilizer for BMS-068645, extensive evaluation was done to establish the stability of BMS-068645 and its acid metabolite in blood and plasma containing 20 mM DFP (3.5 μL/mL of blood or plasma). Also, the stability of DFP in the vacutainer tube was evaluated. The plastic vacutainer tubes (6 mL, K_2EDTA), which contain 21 μL of DFP, were prepared by the injection of DFP with a syringe into commercially available K_2EDTA vacutainer tubes. To avoid losing vacuum in the tubes, a 25 μL syringe with a sharp, thin needle (needle gauge around 22–26) was needed to pierce through the septa of the vacutainer tubes. After the addition of the inhibitor, the tubes were stored at −30 °C and warmed to room temperature less than half an hour prior to use. Blood samples (approximately 6 mL per sample) were collected in the tubes resulting in a final DFP concentration in blood of 20 mM. Immediately after collection, each blood sample was gently inverted several times to ensure complete mixing with the anticoagulant (K_2EDTA) and DFP and then placed in chipped ice. Twelve microliter of stock solutions, at concentrations of 4.0 and 0.03 μg/mL for BMS-068645 in methanol or 4.0 and 0.075 μg/mL for BMS-068645-acid in methanol, were spiked individually into the blood collected with the vacutainer tubes. Each condition was tested in triplicate. After 1 h on ice, the blood samples were centrifuged for 15 min at 1000 × *g* at 4 °C to obtain plasma. The separated plasma samples were stored at −30 °C until analyzed for BMS-068645 and its acid metabolite with the method described below. The DFP vacutainer tubes prepared and stored at −30 °C for 3 months were compared with freshly prepared tubes to evaluate the stability of DFP in the vacutainer tubes.

2.4. Preparation of standard and quality control (QC) samples in the human plasma

Two sets of primary stock solutions of BMS-068645 and BMS-068645-acid for standard and quality control samples were prepared from separate weighings by dissolving the analytes into methanol and DMSO, respectively. A standard working stock solution containing 1000 ng/mL each of BMS-068645 and BMS-068645-acid was prepared by appropriate dilution of the 1.0 mg/mL stock solutions with methanol. A 40 μL portion of the 1000 ng/mL stock solution was diluted to 4.0 mL with control human EDTA plasma containing 20 mM DFP to yield a combined stock solution of 10.0 ng/mL for each analyte. This plasma pool was diluted with plasma containing 20 mM DFP to obtain the final concentrations in plasma standards of 10.0, 7.0, 2.5, 1.0, 0.50, 0.25, 0.10, 0.05 and 0.02 ng/mL for BMS-068645 and 10.0, 7.0, 2.5, 1.0, 0.50, 0.25, 0.10 and 0.05 ng/mL for BMS-068645-acid. Standard curves were prepared fresh daily. Similar dilutions were used to prepare four levels of QC samples in human plasma at 0.06/0.15, 4.0/4.0, 8.0/8.0 and 50.0/50.0 (BMS-068645/BMS-068645-acid) ng/mL, which were aliquoted and stored frozen at −30 °C. An additional QC sample, containing only BMS-068645, was prepared to monitor the conversion of the BMS-068645 to BMS-068645-acid during storage and analysis. All QCs were prepared with human plasma containing 20 mM DFP to avoid the conversion of BMS-068645 to BMS-068645-acid.

2.5. Sample processing procedure for human plasma

The isotope analogues, D_5-BMS-068645 and D_5-BMS-068645-acid, at 1.0 and 5.0 ng/mL in methanol/water (50/50, v/v), were used as the internal standards. After the addition of 100 μL of the IS working solution and 0.5 mL of 0.1N HCl solution, 4 mL of methyl-*tert*-butyl ether were added to 0.5 mL of each calibration standard, QC sample and clinical sample. The concentration in plasma of each IS was 0.2 and 1.0 ng/mL. The samples were shaken for 20 min, and then centrifuged to separate the liquid phases. The organic layer from each sample was transferred to a clean tube and evaporated to dryness. The dried extracts were re-dissolved in 100 μL of the reconstitution solution containing 0.1% formic acid in acetonitrile/water (40/60, v/v) and transferred to injection vials. A 10 μL aliquot of the reconstitution solution was injected into the LC/ESI/MS/MS system.

2.6. Validation of the human plasma method

Calibration curve standards in plasma containing 20 mM DFP were prepared and analyzed in duplicate for three validation runs. Six replicates of each QC sample were assayed along with the standard curve. The stability QC samples, which contain BMS-068645 only, were analyzed in triplicate in all the validation runs and sample analysis runs. The specificity of the method was determined by extracting six lots of blank control human plasma containing 20 mM DFP both with and without the addition of BMS-068645 and BMS-068645-acid at the lower limit of quantitation level (0.02 and 0.05 ng/mL, respectively) to detect any potential interference problems. The stability of BMS-068645 and its acid metabolite in plasma with 20 mM DFP at room temperature and −30 °C, and after three freeze-thaw cycles were evaluated with low and high QCs in triplicates.

3. Results and discussion

3.1. Human blood collection procedure evaluation for stabilizing BMS-068645

BMS-068645, which contains a methyl ester functional group on its side chain, was rapidly metabolized to an acid (Fig. 1) in human and animal blood and plasma by endogenous esterases. In the previous study [5], BMS-068645 at 50 nM was incubated for various times in human, canine, rat, and mouse blood and plasma. BMS-068645 was rapidly metabolized in rodent blood with a $t_{1/2}$ of less than 30 s; which is consistent with reports that rodents have higher plasma esterase activity compared to higher species [9,14]. Longer blood stability was observed in dog, a $t_{1/2}$ of 41 min, and human, a $t_{1/2}$ of 54 min. To select an esterase inhibitor to stabilize BMS-068645, blood spiked with different esterase inhibitors was tested. BMS-068645 was incubated in blood with or without 10 mM esterase inhibitors and human urine at 25.0 ng/mL for 4 (blood) and 96 h (urine). Additional 25.0 ng/mL samples were prepared in methanol and served as controls. To insure complete enzyme inhibition, each of the esterase inhibitors were added to the blood sample prior to the analyte. In the absence of esterase inhibitor, only 41% of BMS-068645 remained after incubating in human blood for 4 h at room temperature (Table 1). Among the tested esterase inhibitors, 10 mM DFP and paraoxon appeared to stabilize BMS-068645 in blood with more than 95% BMS-068645 left after a 4-h incubation. In PMSF and eserine spiked blood, there were only approximately 85% and 46% BMS-068645 remained, respectively. The compound was determined to be stable in both methanol and urine, which indicates stability in the absence of esterase activity.

Table 1
BMS-068645 stability in methanol, human urine, human blood with or without 10 mM esterase inhibitors after incubation of 4 h or 96 h for human urine at 25.0 ng/mL

BMS-068645 in different matrix	BMS-068645 remaining (%)
Pure standard in methanol	100
Human blood control	41.0
Human blood with 10 mM DFP	97.2
Human blood with 10 mM Paraoxon	94.9
Human blood with 10 mM PMSF	85.3
Human blood with 10 mM Eserine	46.2
Human urine (4 h)	100
Human urine (96 h)	98.3

In addition, the conversion of BMS-068645 to its acid metabolite was determined to be dependent on the concentration of esterase inhibitor. In the previously described experiments, each esterase inhibitor was added to the whole blood prior to BMS-068645 to insure complete enzyme inhibition. However, this is not feasible during sample collection at the clinical sites where whole blood, including the parent drug and its metabolite, will be added to the K_2EDTA vacutainer tubes containing the esterase inhibitor. Therefore, it is necessary to ensure that the tubes contain sufficient amount of inhibitor to stop the conversion of the parent drug to its acid metabolite as soon as the blood was collected into the tubes.

Approximately 10% of BMS-068645 converted to its acid metabolite when the final concentration of DFP or paraoxon in blood were 10 mM and were added to the vacutainer tubes before blood collection. However, when the concentration of DFP in blood reached 20 mM, there was less than 3% conversion observed during the blood collection procedure. At the same time, paraoxon at the same concentration resulted in more conversion when compared to DFP. The vacutainer tubes containing either K_3EDTA or K_2EDTA showed no difference in the stability test. Unlike K_3EDTA in vacutainer tube, K_2EDTA is in solid form and therefore, contains no water to affect the stability of DFP spiked in the tube. Longer storage stability was expected for K_2EDTA tubes. Based on all the initial evaluation, 21 μL of DPF in 6 mL K_2EDTA vacutainer tube was chosen as the sample collection tubes. The DFP concentration in blood collected will be around 20 mM.

3.2. Stability of BMS-068645 and BMS-068645-acid in human blood containing 20 mM DFP

The stability of BMS-068645 and BMS-068645-acid in human blood containing 20 mM DFP was evaluated at two different concentrations. BMS-068645 at 4.0 and 0.03 μg/mL or BMS-068645-acid at 4.0 and 0.075 μg/mL were spiked individually into the vacutainer tubes after blood collection with 20 mM DFP tubes. Each concentration level was prepared in triplicate. The blood was centrifuged to obtain plasma immediately after collection (time = 0 h) and after 1 h of being stored on ice. The plasma samples were processed with the analysis procedure described Section 2. The deviations between the measured concentration at 0 and 1 h samples were less than 3.2% and 10.3% for low and high QCs, respectively. The 4.0 μg/mL BMS-068645 stock solution was spiked into the DFP tubes that had been stored at −30 °C for 1 day and 3 months. The concentration deviations for the 3-month samples compared to the day 1 were less than 0.1%. The observed conversion of BMS-068645 to its acid metabolite was less than 0.9% in both the day 1 and the 3-month samples. This made it possible to prepare the

sample collection tubes 3 months in advance and made it easier to support the clinical studies. Due to the toxicity of DFP, it is recommended that the blood be collected from an indwelling catheter.

3.3. Stability of BMS-068645 and BMS-068645-acid in human plasma containing 20 mM DFP

The stability of BMS-068645 and BMS-068645-acid in human EDTA plasma containing 20 mM DFP during sample storage, processing and analysis was evaluated in triplicate using QC samples at 0.060/0.15 and 8.0/8.0 ng/mL. The QC samples were stored at room temperature for 24 h, 3 months at −20 °C and brought through three freeze thaw cycles prior to analysis. The deviations of the mean predicted concentrations of the test QC samples from the nominal concentrations were used as an indicator of the stability of BMS-068645 and BMS-068645-acid in human plasma. Both analytes were stable for at least 24 h at room temperature, 3 months at −20 °C and after three freeze thaw cycles with maximum mean deviation of −9.5%. In processed samples stored at 4 °C for 48 h and re-injected into the mass spectrometer, the maximum mean deviation for the low and high QCs was less than 4.5% compared to the nominal.

3.4. Chromatography and mass spectrometry

During method development, it was observed that DFP treated plasma gradually changed the property of the proteins in the plasma. The plasma color became greenish and small amount of protein has been precipitated. When plasma samples containing DFP were prepared and loaded onto C18 SPE cartridges, the SPE wells were clogged easily. In addition, the plasma containing DFP took much longer to pass through the SPE bedding than plasma samples without DFP. Due to the matrix effect, the sensitivity from the C18 SPE extraction method was insufficient and the necessary LLOQ was not obtained. At the same time, sensitivity decreased during the run. When strong cation exchange SPE cartridges were used, even though cleaner samples were obtained, the conversion from BMS-068645-acid to parent was observed. Liquid–liquid extraction offered cleaner samples and sufficient sensitivity to achieve the LLOQ of 0.020 ng/mL for BMS-068645 and 0.050 ng/mL for BMS-068645 acid. Although some background response was observed in the blank plasma (Figs. 2 and 3) the response of the LLOQ samples were at least 10-fold greater than blank plasma samples. The DFP treated plasma samples were acidified with buffer and then extracted with organic solvents. Two pH modifiers (0.1N HCl and ammonium acetate at pH of 6) were examined for their impact on recovery and 0.1N HCl was chosen for its higher recovery. Methyl *tert*-butyl ether was the extraction solvent chosen because it provided sufficient recovery and cleaner samples compared with other solvents (e.g. methyl *tert*-butyl ether with ethyl acetate (50/50) and ethyl acetate).

Under the LC/ESI/MS/MS conditions used, BMS-068645 and BMS-068645-acid and their internal standards showed

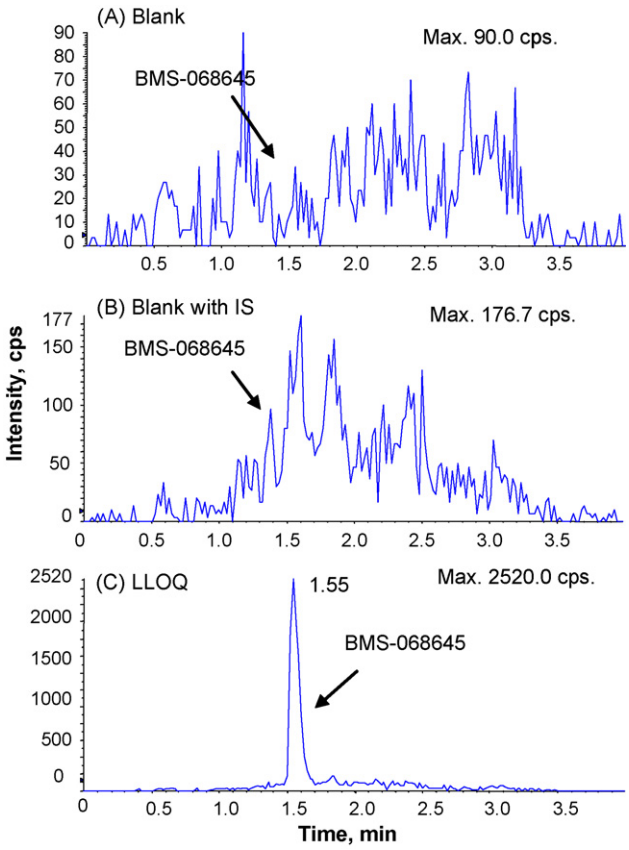

Fig. 2. Selected reaction monitoring chromatograms for BMS-068645 obtained from: (A) blank human plasma; (B) human plasma containing only the internal standard at 0.20 ng/mL; (C) human plasma containing BMS-068645 at lower limit of quantitation (0.020 ng/mL) and its internal standard at 0.20 ng/mL.

protonated ions $[M+H]^+$ at *m/z* 487, 473, 492 and 478, respectively. The fragmentation of the protonated molecule using collision-induced dissociation resulted in dominant product ions, representing the loss of the sugar moiety, for BMS-068645 and its internal standard at 314 amu and BMS-068645-acid and its internal standard at 300 amu (Figs. 4 and 5). Typical SRM mass chromatograms of blank human plasma, blank human plasma with IS and human plasma spiked with 0.020 ng/mL of BMS-068645, 0.050 ng/mL of BMS-068645-acid, 0.20 ng/mL of D_5-BMS-068645 and 1.0 ng/mL of D_5-BMS-068645-acid are shown in Figs. 2 and 3 for the analyte channels. No significant interfering peaks from the plasma were found at the retention time and in the ion channel of either of the analytes or the IS when six different lots of control human EDTA plasma blanks were analyzed.

3.5. Accuracy and precision of calibration curve standards in human plasma

BMS-068645 and its acid metabolite plasma concentrations were determined by inverse-prediction following a 1/*x* weighted quadratic regression analysis of the standard curve data. A summary of the regression parameters (quadratic slope, slope, intercept and *R*-squared) for the calibration curves in plasma for

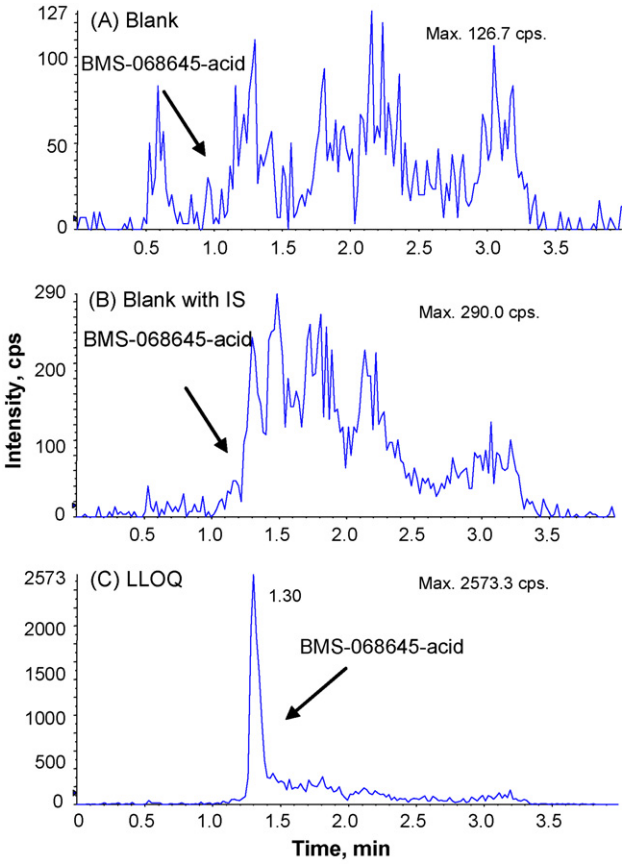

Fig. 3. Selected reaction monitoring chromatograms for BMS-068645-acid obtained from: (A) blank human plasma; (B) human plasma containing only the internal standard at 1.0 ng/mL; (C) human plasma containing BMS-068645-acid at lower limit of quantitation (0.050 ng/mL) and its internal standard at 1.0 ng/mL.

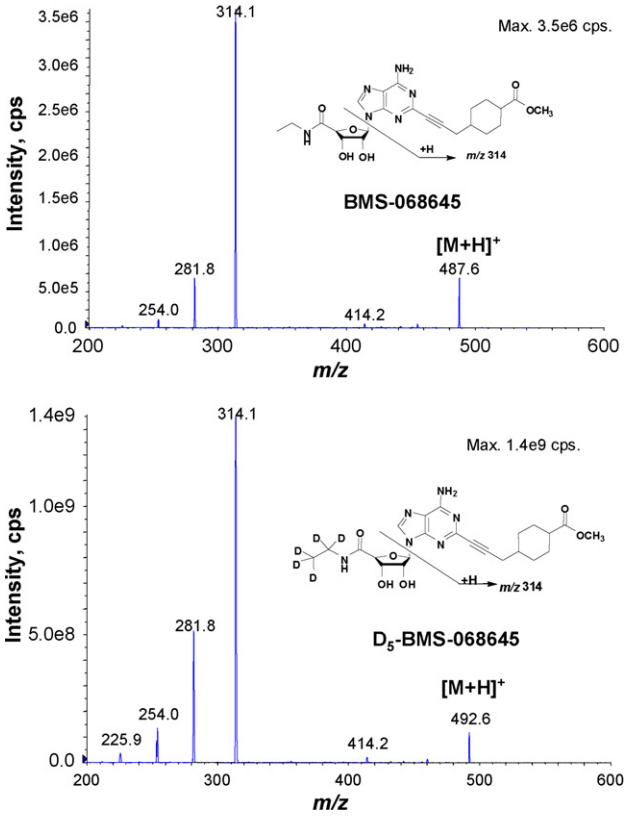

Fig. 4. Electrospray positive ion MS/MS product ion spectra of $[M+H]^+$ for BMS-068645 (top) and its internal standard (bottom).

BMS-068645 and BMS-068645-acid is provided in Table 2. For all the runs, the deviations of the back-calculated concentrations from their nominal values were within the range from −13.4% to 13.5% for all the calibration standards for both BMS-068645 and BMS-068645-acid except for two outliers. The mean concentration for six different lots of control human plasma spiked with BMS-068645 and BMS-068645-acid at 0.020 and 0.050 ng/mL (LLOQ levels) were 0.021 and 0.045 ng/mL with a mean deviation of 5.0% and −10.0%, respectively.

3.6. Accuracy and precision of quality control samples in human plasma

The accuracy and precision of the method was assessed by analyzing QC samples at concentrations within the lower, the second, and the upper quartile of the standard curve. To evaluate the dilution linearity and matrix effect, a fourth QC sample, with a concentration higher than the upper limit of the standard curve range, was also analyzed. This QC sample was diluted 1:20 with control human plasma containing 20 mM DFP and then the diluted sample was processed and analyzed. Six replicate samples at each concentration were analyzed in three separate runs. The accuracy was determined by calculating the deviations of the predicted concentrations from their nominal values. The intra-

Table 2
Standard curve regression analysis results for BMS-068645 and BMS-068645-acid in human EDTA plasma containing 20 mM DFP

Run number	BMS-068645				BMS-068645-acid			
	Quadratic slope ($\times10^{-2}$)	Slope	Intercept ($\times10^{-2}$)	*R*-squared	Quadratic slope ($\times10^{-3}$)	Slope	Intercept ($\times10^{-3}$)	*R*-squared
1	1.2443	6.17031	−1.2020	0.9990	8.3648	0.995699	5.4323	0.9988
2	1.9754	6.12231	0.8628	0.9996	8.8004	1.034217	−2.0116	0.9986
3	3.2032	8.31879	−5.4299	0.9990	10.968	1.142456	−3.8069	0.9985
4	6.3928	8.70695	−2.1363	0.9992	6.5374	1.150615	−1.4793	0.9990
Mean	3.2039	7.32959	−1.9763		8.6678	1.0807	−0.0466	
S.D.	2.2744	1.37563	2.6213		1.8203	0.0776	0.4056	

Model: Area ratio = $(\text{conc})^2(\text{quad slope}) + (\text{conc})(\text{slope}) + \text{intercept}$.

Table 3
Accuracy and precision for BMS-068645 and BMS-068645-acid in human EDTA plasma with 20 mM DFP, $n = 18$

	BMS-068645				BMS-068645-acid			
Nominal conc (ng/mL)	0.0600	4.00	8.00	50.0[a]	0.150	4.00	8.00	50.0[a]
Mean observed conc (ng/mL)	0.0580	4.03	8.20	49.2	0.142	4.00	7.71	48.1
Deviation (%)	−3.3	0.9	2.5	−1.5	−5.4	−0.1	−3.6	−3.7
Inter-run precision (%)	8.7	3.4	0.7	2.7	5.3	5.8	4.8	1.2
Intra-run precision (%)	3.1	2.3	2.9	4.0	4.0	3.2	2.9	3.0

[a] Dilution factor = 20.

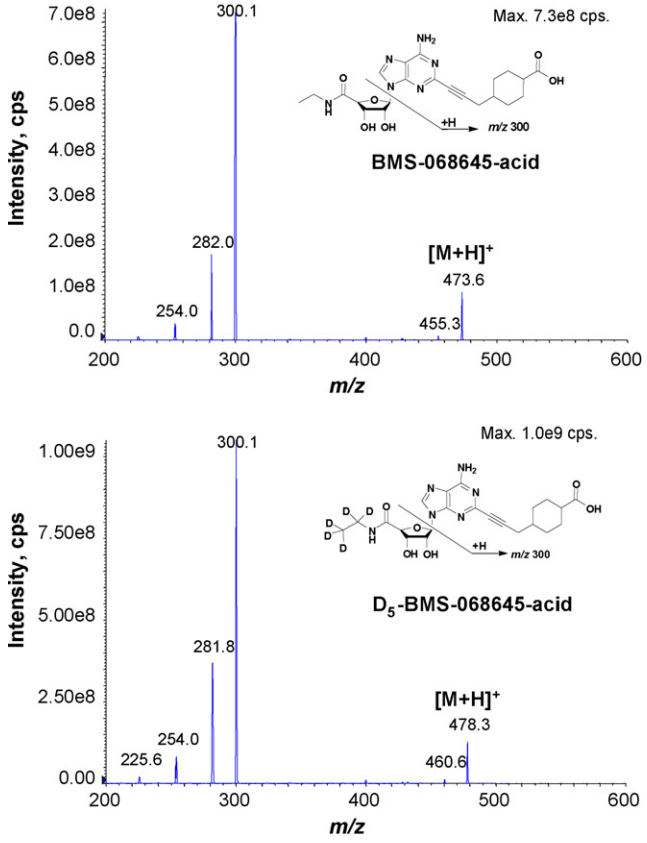

Fig. 5. Electrospray positive ion MS/MS product ion spectra of $[M+H]^+$ for BMS-068645-acid (top) and its internal standard (bottom).

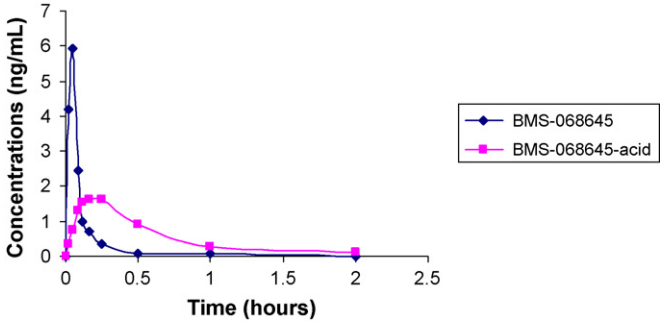

Fig. 6. The human plasma concentration vs. time profile in a typical subject following the administration of a single intravenous dose of 2.0 μg/kg of BMS-068645.

and inter-assay precision was determined by calculating the %R.S.D. values using a one-way ANOVA. The intra-assay precision was within 4.0% R.S.D. and the inter-assay precision was within 8.7% R.S.D. for both BMS-068645 and BMS-068645-acid, data is shown in Table 3. The assay accuracy was within ±5.4% of the nominal values for all the QC samples analyzed for both analytes.

4. Assay applications

The LC/ESI/MS/MS method was applied to evaluate the pharmacokinetics of BMS-068645 in a human single ascending dose study in which the dose started from 0.1 μg/kg intravenous dose of the drug. Serial blood samples were collected for up to 2 h to characterize the pharmacokinetics of BMS-068645. Fig. 6 shows the BMS-068645 and its acid metabolite plasma concentration versus time profile in a typical subject following the administration of a single intravenous dose of 2.0 μg/kg of BMS-068645 using the assay.

5. Conclusions

The validated LC/ESI/MS/MS method in the human plasma containing 20 mM DFP demonstrated high sensitivity, selectivity, accuracy, precision and ruggedness. The developed assays are suitable for the determination of the pharmacokinetics of BMS-068645 and BMS-068645-acid in humans. DFP effectively inhibited the esterase activity that would have converted the parent drug to the metabolite *ex vivo*. Also, the collection tubes containing DFP are convenient to prepare, store and use for the clinical studies.

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