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LC–MS/MS method using unbonded silica column and aqueous/methanol mobile phase for the simultaneous quantification of a drug candidate and co-administered metformin in rat plasma

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ABSTRACT

BMS-754807 and metformin were co-administered in drug discovery studies which required the quantitation of both compounds in plasma. Since the two compounds are chemically and structurally dissimilar, developing a single bioanalytical method presented a number of chromatographic challenges including the achievement of appropriate retention times and peak shapes on a single analytical column. To address this chromatographic challenge, we investigated different LC columns under different gradient elution schemes using aqueous/organic mobile phases. Using unbonded silica column and aqueous/methanol mobile phase, we were able to obtain robust and well-resolving chromatographic conditions to support the development and implementation of a single LC–MS/MS bioanalytical method. The use of sub-2 micron particle sizes and a high flow rate, which are attainable with UPLC systems, enhanced the method. The method performance evaluation showed that the method easily met the normally used acceptance criteria for bioanalytical methods, namely a deviation of ±15% from the nominal concentration except at lower limit of quantitation (LLOQ), where ±20% is accepted. The reported LLOQ of 7.8 ng/ml, for both BMS-754807 and metformin, was adequate to support the pharmacokinetic studies.

1. Introduction

The use of tandem mass spectrometry in conjunction with liquid chromatography (LC–MS/MS) has become indispensable for the quantification of drugs and metabolites in biological matrices in support of drug discovery and development [1–3]. Analysis of biological samples for the simultaneous determination of analytes with disparate physico-chemical properties usually presents challenges in method development, which include the achievement of good peak shape, chromatographic separation, and adequate retention to avoid analyte elution in the void volume.

BMS-754807 is an insulin-like growth factor (IGF-1R) kinase inhibitor that is currently in clinical development at Bristol-Myers Squibb [4]. Elevated glucose and insulin levels were observed during pre-clinical animal safety studies at doses that exceeded the efficacious exposure [4]. Metformin was co-administered along with BMS-754807 to test whether these elevated levels could be lowered. This presented the need for the simultaneous analysis of the two compounds.

Metformin and BMS-754807 (Fig. 1) are excellent examples of compounds that are structurally and chemically dissimilar. Polar compounds, such as metformin, are difficult to retain using traditional reversed-phase chromatography, but could be easily retained using hydrophilic interaction liquid chromatography (HILIC) [5]. On the other hand; BMS-754807 is relatively non-polar and thus could be easily retained using reversed-phase chromatography. Thus for simultaneous determination, the attainment of reasonable retention for both compounds on a single analytical column proved to be challenging.

HILIC is a variation of normal-phase chromatography which relies on a polar stationary phase and mobile phases containing high percentages of water-miscible organic solvents [6,7]. The introduction of HILIC has enabled analytical chemists to explore alternatives to traditionally used reversed-phase chromatography when applying bioanalytical strategies for the analysis of polar compounds. Jane first explored the use of underivatized silica columns for the separation of highly polar drugs in the forensic science field back in 1975 [7] but the term HILIC was not introduced until 1990 by Alpert [6]. HILIC has been slow to progress until recently when there has been an increased interest in the analytical and bioanalytical areas as indicated by several excellent articles [8–20]. With HILIC, the retention of a polar compound increases as the organic percentage in the mobile phase increases.

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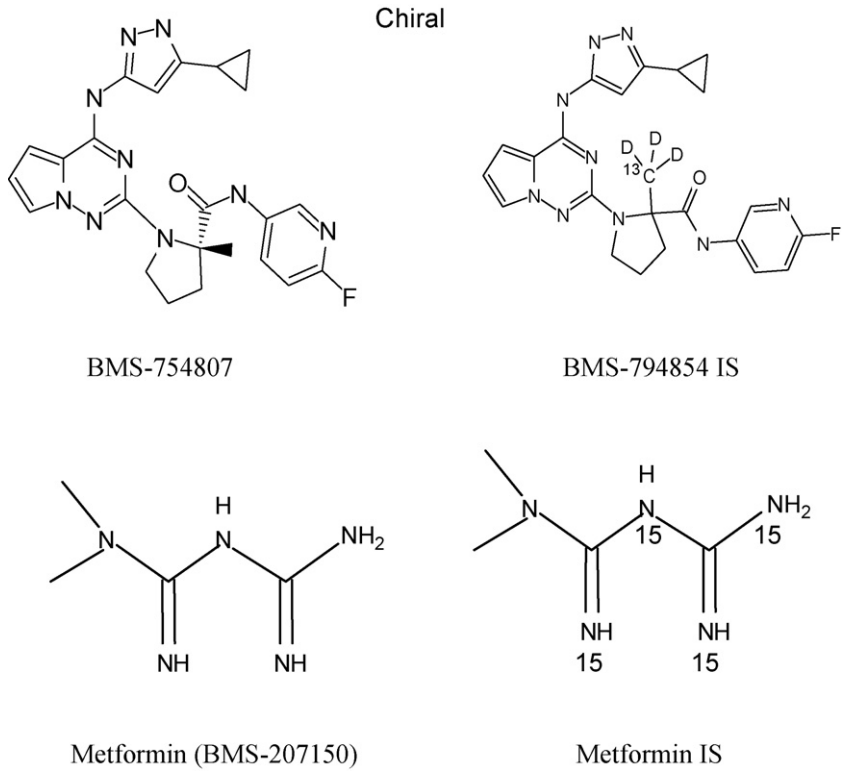

Fig. 1. Chemical structures of BMS-754807, BMS-754807 IS, metformin and metformin IS.

While the column we used for the finally adopted bioanalytical method was packed with unbonded (bare) silica (designated as a HILIC column by the manufacturer), the retention mechanism may not be purely HILIC under the elution conditions used for the method, described under Section 2.5. Using UPLC technology with sub-2 μm polar stationary phases, a robust and rapid LC–MS/MS-based bioanalytical method for the simultaneous quantitation of metformin and BMS-754807 in plasma was developed. This method was superior to other LC–MS/MS-based methods developed earlier to measure the two compounds in support of discovery.

2. Experimental

2.1. Chemicals and reagents

BMS-754807, the stable isotope labeled analog of BMS-754807 used as the internal standard (IS) for BMS-754807, metformin, and the stable isotope labeled analog of metformin used as the IS for metformin, were all obtained from Bristol-Myers Squibb (Fig. 1). The chemical structures for BMS-754807, BMS-754807 IS, metformin and metformin IS are shown in Fig. 1. Ammonium formate and ammonium bicarbonate were obtained from Sigma–Aldrich (St. Louis, MO, USA). HPLC grade acetonitrile, methanol and formic acid were obtained from Burdick & Johnson (Muskegon, MI, USA).

2.2. Materials and equipment

Leap 4X Flux UPLC quaternary pump was used with a CTC Analytics HTC PAL autosampler (LEAP Technologies, Carrboro, NC) equipped with a cooling stack that maintained samples at 10 °C. The mass spectrometer was the TSQ Quantum Ultra with a HESI source (Thermo Fisher Scientific, San Jose, CA, USA) controlled by Xcalibur version 2.0. The column used in the final bioanalytical method was the Acquity UPLC BEH HILIC column, 2 mm × 50 mm, 1.7 μm (Waters, Franklin, MA, USA). The stationary phase in this column is unbonded (bare) silica. 96-Well filter plates for plasma protein precipitation were obtained from Millipore (Burlington, MA, USA).

2.3. Preparation of standard and QC samples

A high concentration stock solution of BMS-754807 and metformin in EDTA rat plasma were used to prepare rat plasma calibrations standards via serial dilution using blank rat plasma. The calibration set consisted of 7.8, 15.6, 31.25, 125, 500, 2000 and 8000 ng/ml standards for BMS-754807 and metformin. A separate high concentration stock solution of BMS-754807 and metformin in EDTA rat plasma was used to prepare rat plasma quality control (QC) samples via serial dilution with rat blank plasma. The QC set consisted of 40, 400, 4000 and 6400 ng/ml QCs for BMS-754807 and metformin. BMS-754807 IS and metformin IS were prepared as a combined solution in acetonitrile, 100 and 1000 ng/ml, respectively.

2.4. Sample preparation

Rat plasma sample extraction was accomplished by protein precipitation using a PTFE (0.45 μm) filter plate and acetonitrile containing the internal standards. This was performed by placing an aliquot of 50 μL of the internal standard solution into the PTFE filter plate attached to a 96-well plate. A 25 μL aliquot of each standard or QC was added to the IS solution. The plate was covered, vortexed for 2 min, transferred to a refrigerated centrifuge (4 °C), and centrifuged for 5 min at 3000 rpm to separate the plasma proteins from the supernatant. A 2-μL portion of the sample supernatant was injected into the LC–MS/MS system for analysis.

2.5. LC–MS/MS conditions

The UPLC conditions used for the final method based on using a HILIC UPLC column are summarized in Table 1. The mobile phase

Table 1
Chromatographic conditions used in the final bioanalytical method for the simultaneous quantitation of BMS-754807 and metformin.

Chromatography		
Analytical column	Acquity UPLC BEH HILIC, 2 mm × 50 mm, 1.7 μm	
Mobile phase	(A) 5 mM ammonium bicarbonate in 100% water (B) 5 mM ammonium bicarbonate in 80% methanol/20% water	
Injection	2 μL	
Flow rate	1000 μL/min	
Gradient		
Time (min)	% A	% B
0.00	95	5
0.60	60	40
1.20	60	40
1.60	95	5
2.00	95	5

consisted of 5 mM ammonium bicarbonate in water as eluent A and 5 mM ammonium bicarbonate in 80:20 methanol–water as eluent B. The gradient elution used started with 5% B which was programmed to increase % B to 40.0% in 0.6 min. The mobile phase was maintained at 40.0% B until 1.2 min after which % B was decreased back to starting point of 5%, where it was maintained until 2.0 min before the start of the next run. Thus, the gradient elution was conducted from low-percentage organic mobile phase to medium-percentage organic mobile phase. Under this elution condition, the chromatographic separation mechanism may not be purely HILIC; it probably involves ion exchange with the silanol groups of bare silica.

The mass spectrometer, TSQ Quantum Ultra equipped with a HESI source, was used in the positive ionization mode. Ultra high purity nitrogen was used as the sheath, ion sweep, and auxiliary gas with flow rates of 35, 2 and 15 (arbitrary units), respectively. The electrospray voltage was set at 3500 V. The vaporized temperature was 350 °C and the heated capillary temperature was 222 °C. The following selected reaction monitoring (SRM) transitions were employed: m/z 462.2 → m/z 322.5 for BMS-754807; m/z 466.2 → m/z 326.5 for BMS-754807 IS; m/z 130.0 → m/z 71.4 for metformin; and m/z 134.0 → m/z 71.3 for metformin IS. The scan width was 0.30 Da and scan time was 0.05 s per SRM. The collision energy was set to 27, 26, 25, and 30 eV for BMS-754807, BMS-754807 IS, metformin, and metformin IS, respectively. The Thermo Finnigan Xcalibur 2.0 was used for acquiring and processing the LC–MS/MS data.

2.6. Method performance evaluation

The performance of the bioanalytical method was evaluated by conducting accuracy and precision runs using rat plasma calibration standards and QCs. Each QC level was analyzed in five replicates, with a standard curve set analyzed at the beginning and end of a run. Peak area ratios (analyte/internal standard) were used for the regression of the calibration curves and quantitation the QC samples.

3. Results and discussion

3.1. Method development

The chromatographic conditions adopted in the final bioanalytical method used for the simultaneous quantitation of BMS-754807 and metformin in rat plasma, summarized in Table 1, achieved a balance between reasonable retention times and peak shapes of the two analytes. Typical chromatograms obtained with the final method are shown in Fig. 2 at the lower limit of quantitation (LLOQ) concentrations and in Fig. 3 at higher concentrations. The retention times of BMS-754807 and metformin are approximately 0.62 and 0.89 min, respectively. Although the retention time of BMS-754807 was relatively short, the effective retention factor (k^*) obtained was 3.4, which was adequate for these studies. The k^* value was computed using the equation $k^* = (20t_GF)/Vm(\Delta\%\ \mathrm{B})$, where t_G is the gradient time (min), F is the flow rate (ml/min), Vm is the column dead volume and Δ% B is the difference between the initial and final % B values [21]. Vm is estimated using the equation $Vm = 0.5Ld^2$, where L is the column length (cm) and d is the column diameter (cm) [22]. It should be noted that adequate k^* value was obtained for BMS-754807 in spite of the apparently short retention time, which was approximately six times the column dead time (t_0) of 0.10 min. The t_0 value was obtained using the equation $t_0 = Vm/F$ [22].

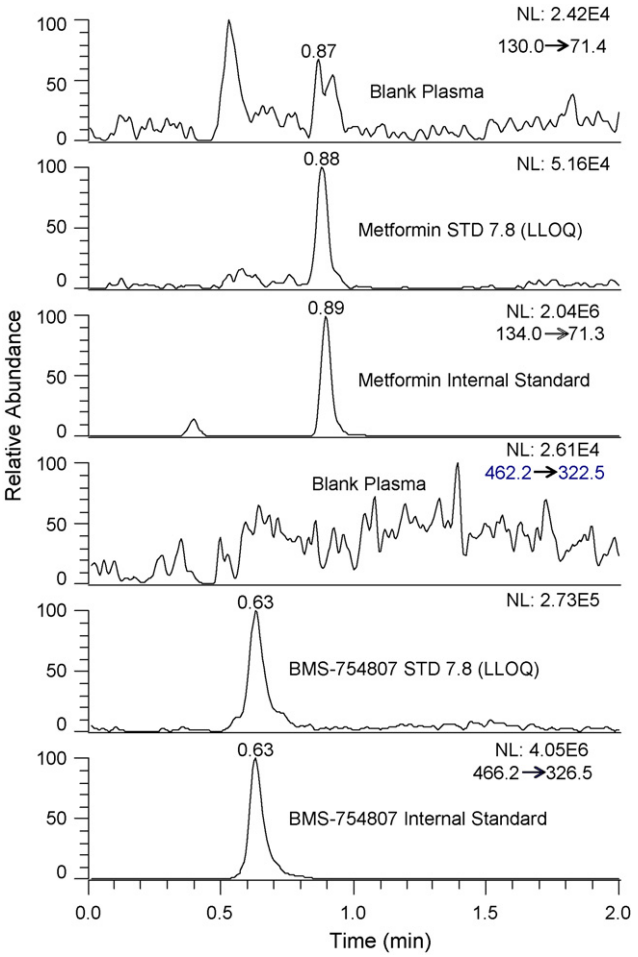

Fig. 2. Selected reaction monitoring chromatograms obtained using the final method from rat blank plasma and plasma containing metformin and BMS-754807 at LLOQ levels and their respective internal standards: blank plasma with no metformin, metformin at LLOQ level and metformin internal standard at 1000 ng/ml (the top three traces from top to bottom, respectively); blank plasma with no BMS-754807, BMS-754807 at LLOQ level and BMS-754807 internal standard at 100 ng/ml (the bottom three traces from top to bottom, respectively).

During the course of method development, various reversed-phase and HILIC columns with different mobile phase gradients were evaluated. The chromatographic conditions evaluated, designated as methods A–E, are summarized in Table 2. As shown in Fig. 4 for method A, desirable retention and excellent peak shape were

Table 2
Various chromatographic conditions, designated as methods A-E, evaluated prior to the adoption of the final bioanalytical method summarized in Table 1.

Mobile phases evaluated			
Mobile phase 1	(A) 5 mM ammonium bicarbonate in 100% water (B) 5 mM ammonium bicarbonate in 80% acetonitrile/20% water		
Mobile phase 2	(A) 0.1% formic acid, 10 mM ammonium formate in 100% water (B) 0.1% formic acid, 10 mM ammonium formate in 90% acetonitrile/10% water		
Columns and gradient schemes evaluated			
Method	Column	Mobile phase	Gradient scheme and flow rate
A	Acquity UPLC BEH C18, 2 mm × 50 mm, 1.7 μm	1	From low to high % acetonitrile; 800 μL/min
B	Acquity UPLC BEH C18, 2 mm × 50 mm, 1.7 μm	1	From high to low % acetonitrile; 800 μL/min
C	Thermo Hypersil Gold PFP, 2 mm × 50 mm, 3.0 μm	2	From high to low % acetonitrile; 300 μL/min
D	Acquity UPLC BEH HILIC, 2 mm × 50 mm, 1.7 μm	1	From high to low % acetonitrile; 800 μL/min
E	Acquity UPLC BEH HILIC, 2 mm × 50 mm, 1.7 μm	1	From low to high % acetonitrile; 800 μL/min

achieved for BMS-754807 using a C18 reversed-phase column with a traditional reversed gradient that started with a low percentage of the organic eluent. On the other hand, metformin eluted closer to the void volume region (retention time: 0.19 min; t_0: 0.13 min) under these conditions and the peak was broad (Fig. 4). As shown in Fig. 5, retention of both BMS-754807 and metformin was achieved with a PFP column using a gradient elution that started with a high % of the organic eluent (method C). However, the metformin peak shape was not symmetrical and was relatively broad. It should be noted that PFP columns have been reported to exhibit U-shaped retention as a function of mobile phase organic content and that this behavior is more dramatic to that seen using C18 columns [23]. Thus, depending on the analyte, PFP could exhibit HILIC characteristics in a high organic mobile phase. Initial evaluation of a bare silica (underivatized silica) column did not yield adequate results, with BMS-754807 eluting near the void volume region and the well-retained metformin exhibiting a poorly defined broad peak (Fig. 6, method D). It took further optimization of the gradient elution to achieve the conditions adopted in the final method using the bare silica column, as summarized in Table 1. It is interesting to note that metformin exhibited a very broad asymmetrical peak on the same column when a gradient scheme starting with a low % of the organic

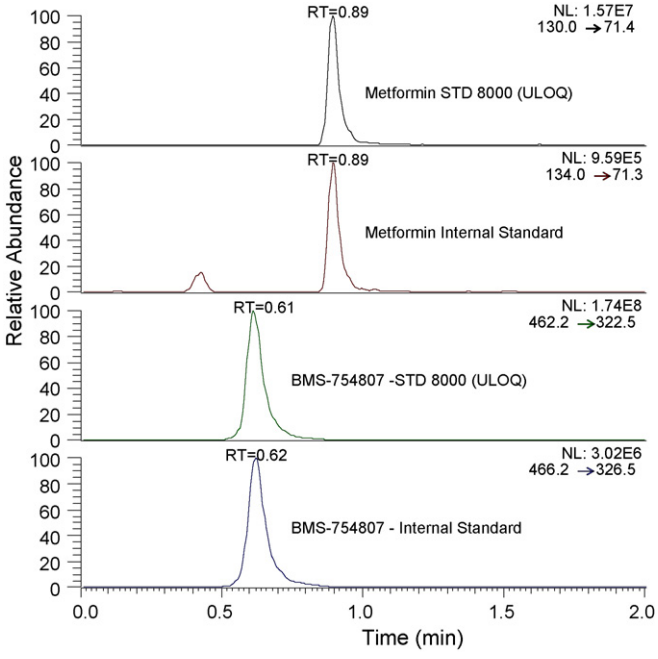

Fig. 3. Selected reaction monitoring chromatograms obtained using the final method from rat plasma containing metformin and BMS-754807, each at 8000 ng/ml concentration, and their respective internal standards: metformin at 8000 ng/ml and metformin internal standard at 1000 ng/ml (the top two traces from top to bottom, respectively); BMS-754807 at 8000 ng/ml and BMS-754807 internal standard at 100 ng/ml (the bottom two traces from top to bottom, respectively).

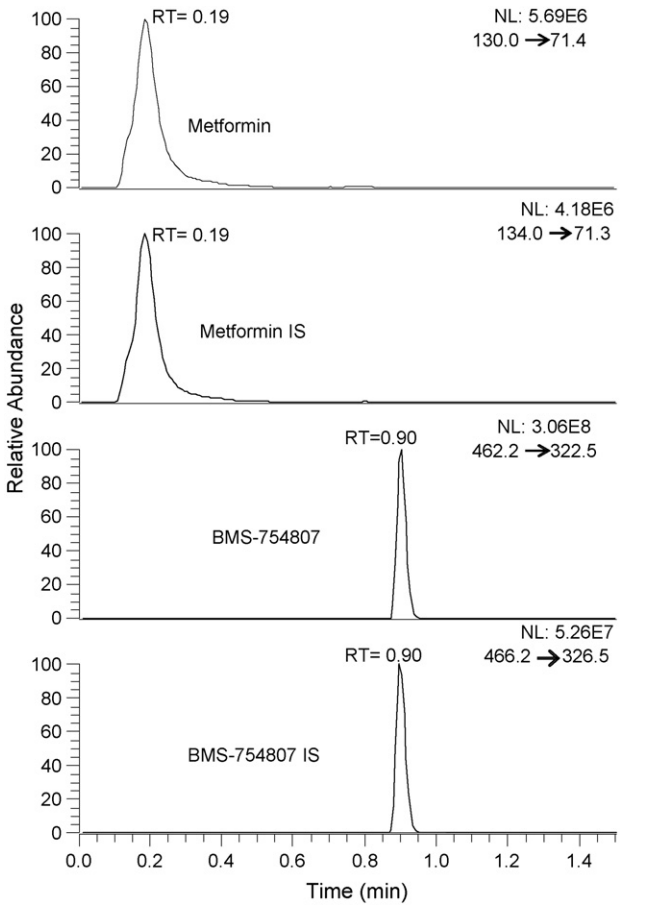

Fig. 4. Selected reaction monitoring chromatograms obtained using method A (Table 2) from rat plasma containing metformin and BMS-754807, each at a high concentration, and their respective internal standards: metformin at 1000 ng/ml and metformin internal standard at 1000 ng/ml (the top two traces from top to bottom, respectively); BMS-754807 at 1000 ng/ml and BMS-754807 internal standard at 100 ng/ml (the bottom two traces from top to bottom, respectively). Gradient elution for method A: start with 15% B, which provides a low % of the organic eluent B; hold there until time = 0.01 min; then ramp up for 0.69 min to obtain 95% B; hold there until time = 1.0 min; ramp down to the starting % B; hold there until time = 1.5 min and then stop.

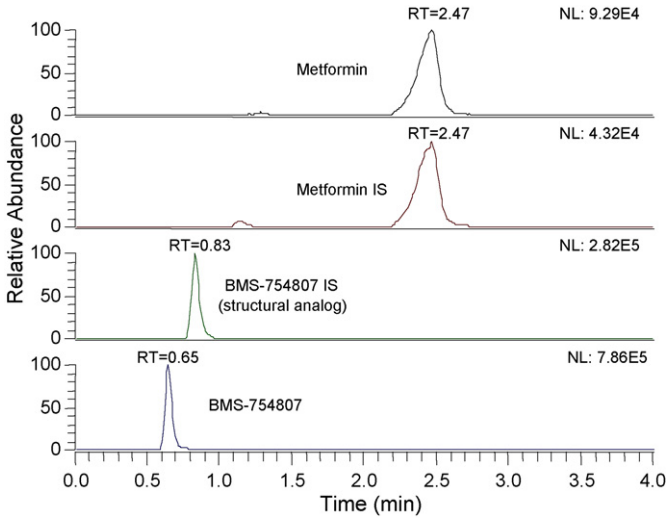

Fig. 5. Selected reaction monitoring chromatograms obtained using method C (Table 2) from rat plasma containing metformin and BMS-754807, each at a high concentration, and their respective internal standards: metformin at 250 ng/ml and metformin internal standard at 1000 ng/ml (the top two traces from top to bottom, respectively); BMS-754807 at 250 ng/ml and BMS-754807 internal standard at 100 ng/ml (the bottom two traces from top to bottom, respectively). Gradient elution for method C: start with 90% B, which provides a high % of the organic eluent B; hold there until time = 0.01 min; then ramp down for 1.0 min to obtain 50% B; hold there until time = 3.0 min; ramp up to the starting % B; hold there until time = 4.0 min and then stop.

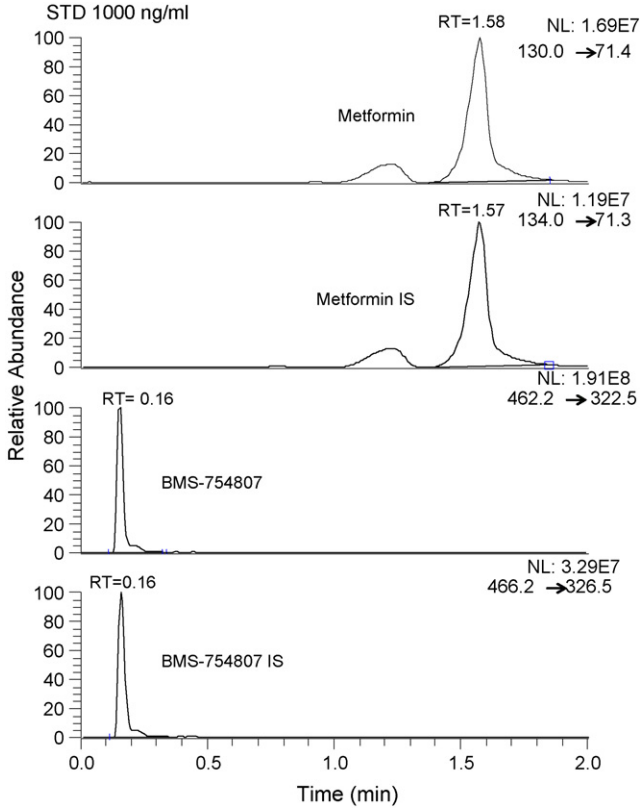

Fig. 6. Selected reaction monitoring chromatograms obtained using method D (Table 2) from rat plasma containing metformin and BMS-754807, each at a high concentration, and their respective internal standards: metformin at 1000 ng/ml and metformin internal standard at 1000 ng/ml (the top two traces from top to bottom, respectively); BMS-754807 at 1000 ng/ml and BMS-754807 internal standard at 100 ng/ml (the bottom two traces from top to bottom, respectively). Gradient elution for method D: start with 85% B, which provides a high % of the organic eluent B; hold there until time = 0.01 min; then ramp down for 0.25 min to obtain 5% B; hold there until time = 1.7 min; ramp up to the starting % B; hold there until time = 2.0 min and then stop.

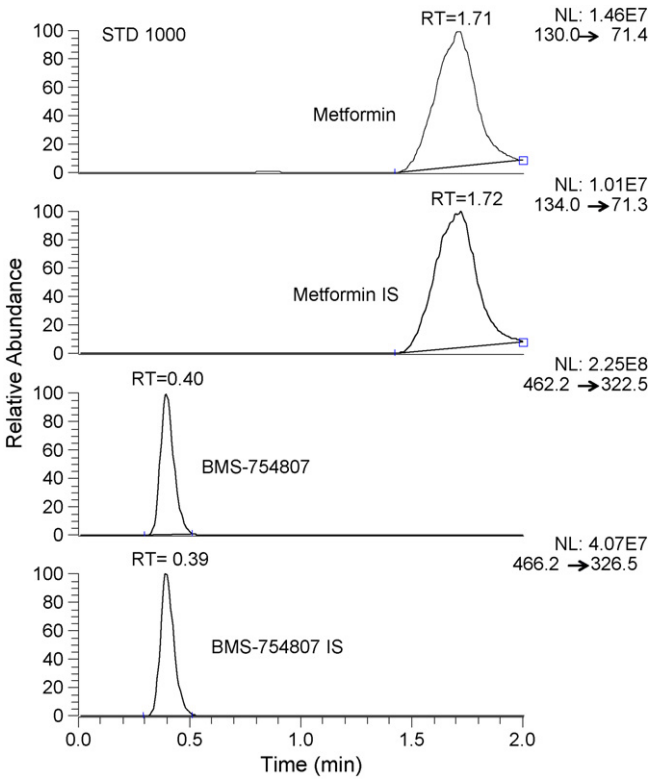

Fig. 7. Selected reaction monitoring chromatograms obtained using method E (Table 2) from rat plasma containing metformin and BMS-754807, each at a high concentration, and their respective internal standards: metformin at 1000 ng/ml and metformin internal standard at 1000 ng/ml (the top two traces from top to bottom, respectively); BMS-754807 at 1000 ng/ml and BMS-754807 internal standard at 100 ng/ml (the bottom two traces from top to bottom, respectively). Gradient elution for method E: start with 15% B, which provides a low % of the organic eluent B; hold there until time = 0.01 min; then ramp up for 0.69 min to obtain 95% B; hold there until time = 2.0 min; ramp down to the starting % B; hold there until time = 2.5 min and then stop.

eluent was used; although BMS-754807 exhibited a symmetrical peak with acceptable retention (Fig. 7, method E). The information obtained from Figs. 6 and 7 provided guidance in choosing the appropriate gradient program for the finally adopted bioanalytical method, which resulted in improved peak shape for metformin and enhanced retention for BMS-754807 (Figs. 2 and 3). Typically, in HILIC, gradient elution chromatography is conducted using an initial mobile phase composition that has a very high percentage of the organic component. For the simultaneous analysis of two analytes, the gradient scheme adopted was found to be optimal.

3.2. Method performance evaluation

The performance of the final method used for the quantitation of BMS-754807 and metformin in rat plasma is summarized in Tables 3–6, where the results of the standard curves and QC samples obtained on three different days are presented. The results for the standards (Table 3 for BMS-754807 and Table 5 for metformin) easily met the normally used acceptance criteria for bioanalytical methods, namely a deviation of ±15% from the nominal concentration except at LLOQ where ±20% is accepted. The results for the QC samples (Table 4 for BMS-754807 and Table 6 for metformin) also met the normally used acceptance criteria for bioanalytical methods, namely a deviation of ±15% from the nominal concentration and % RSD of 15%. The LLOQ of 7.8 ng/ml, for both BMS-754807 and metformin, was adequate to support the pharmacokinetic studies.

Table 3
Summary of the performance of the BMS-754807 calibration standards.

BMS-754807	Set 1		Set 2		Set 3	
Nominal conc. (ng/ml)	Found conc. (ng/ml)	% Accuracy	Found conc. (ng/ml)	% Accuracy	Found conc. (ng/ml)	% Accuracy
7.8	7.1	91	7.3	93	7.4	95
	8.4	108	NF	NF	7.3	94
15.6	14.4	92	16.8	108	15.1	97
	16.5	106	16.3	104	18.6	119
31.25	31.1	99	29.9	96	33.2	106
	34.8	111	32.6	104	32.8	105
125	124.0	99	130.6	104	124.1	99
	128.7	103	132.8	106	131.3	105
500	483.3	97	497.2	99	514.4	103
	529.4	106	491.3	98	526.4	105
2000	2015.0	101	2004.5	100	1859.7	93
	2049.7	102	1927.6	96	1825.8	91
8000	7204.7	90	7380.9	92	7403.9	93
	7596.4	95	7786.6	97	7570.8	95

Linear regression with $1/x^2$ weighting. NF: not found.

Table 4
Summary of the performance of the BMS-754807 quality control samples.

BMS-754807	QC 40	QC 400	QC 4000	QC 6400
Mean – Set 1 (ng/ml)	40.5	412.7	3835.1	6114.9
SD – Set 1	1.4	26.9	138.9	288.7
% RSD – Set 1	3.5	6.5	3.6	4.7
% Accuracy – Set 1	101	103	96	96
Mean – Set 2 (ng/ml)	43.0	398.9	3885.0	5927.7
SD – Set 2	3.0	21.7	98.8	503.0
% RSD – Set 2	7.1	5.4	2.5	8.5
% Accuracy – Set 2	107	100	97	93
Mean – Set 3 (ng/ml)	42.9	400.0	3972.3	6013.7
SD – Set 3	1.7	14.6	117.1	199.4
% RSD – Set 3	4.1	3.6	2.9	3.3
% Accuracy – Set 3	107	100	99	94
Mean – overall (ng/ml)	42.1	403.9	3897.4	6018.8
SD – overall	2.4	21.1	125.2	337.2
% RSD – overall	5.6	5.2	3.2	5.6
% Accuracy – overall	105	101	97	94

SD: standard deviation. RSD: relative standard deviation.

Table 6
Summary of the performance of the metformin quality control samples.

Metformin	QC 40	QC 400	QC 4000	QC 6400
Mean – Set 1 (ng/ml)	37.4	395.1	3868.7	6281.3
SD – Set 1	1.1	21.7	133.2	327.7
% RSD – Set 1	2.9	5.5	3.4	5.2
% Accuracy – Set 1	94	99	97	98
Mean – Set 2 (ng/ml)	43.3	406.5	3803.6	6023.5
SD – Set 2	2.6	12.9	136.0	508.2
% RSD – Set 2	6.1	3.2	3.6	8.4
% Accuracy – Set 2	108	102	95	94
Mean – Set 3 (ng/ml)	39.7	405.8	3917.6	6522.1
SD – Set 3	3.1	36.6	125.1	126.4
% RSD – Set 3	7.7	9.0	3.2	1.9
% Accuracy – Set 3	99	101	98	102
Mean – overall (ng/ml)	40.1	402.5	3863.3	6275.6
SD – overall	3.4	24.4	131.0	391.7
% RSD – overall	8.4	6.1	3.4	6.2
% Accuracy – overall	100	101	97	98

SD: standard deviation. RSD: relative standard deviation.

Table 5
Summary of the performance of the metformin calibration standards.

Metformin	Set 1		Set 2		Set 3	
Nominal conc. (ng/ml)	Found conc. (ng/ml)	% Accuracy	Found conc. (ng/ml)	% Accuracy	Found conc. (ng/ml)	% Accuracy
7.8	7.5	96	7.4	95	8.6	110
	8.0	102	7.4	95	6.8	87
15.6	14.4	93	15.2	97	15.1	97
	15.7	100	17.5	112	19.3	124
31.25	31.4	100	35.0	112	32.7	105
	37.1	119	34.1	109	35.1	112
125	132.2	106	125.5	100	117.0	94
	128.8	103	136.4	109	131.9	106
500	498.8	100	520.7	104	498.9	100
	492.5	98	470.2	94	516.0	103
2000	1950.3	98	1829.5	91	1933.5	97
	1971.7	99	2003.1	100	1958.9	98
8000	7416.4	93	7399.4	92	7492.8	94
	7505.6	94	7050.2	88	7883.6	99

Linear regression with $1/x^2$ weighting.

4. Conclusion

The use of bare (unbonded) silica column enabled the retention of both BMS-754807 and metformin for the development of a LC–MS/MS-based bioanalytical method for the simultaneous quantitation of the two compounds in rat plasma. The use of the 1.7 μm version of the bare silica column (2 mm × 50 mm) and a high flow rate of 1.0 ml/min resulted in rapid chromatography for fast turnaround samples analyzed in support of discovery programs where metformin was co-administered with test compounds.

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