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Determination of BMS-186318 in dog, rat and monkey plasma by liquid chromatography–ionspray mass spectrometry ☆

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Abstract

BMS-186318 is a member of the recently discovered "aminodiol" class of HIV protease inhibitors. A simple but sensitive method was developed for the determination of BMS-186318 in dog plasma and then applied to monkey and rat plasma. The compound was extracted from dog plasma with methyl *tert*-butyl ether at basic pH. The dried extract was reconstituted in mobile phase and injected into a 150 × 2.1 mm i.d. Zorbax Rx-C_{18} HPLC column. A portion of the effluent was directed into the LC–ionspray MS system, where the $[M + H]^+$ ion of the secondary amine compound was monitored. The HPLC conditions were chosen in order to achieve a short run time and large sample throughput, with both analyte and internal standard eluting within 1.5 min. The liquid–liquid extraction procedure provided very clean extracts so that sufficient signal-to-noise ratio was obtained with single-stage mass spectrometry instead of the more costly tandem mass spectrometry. The required lower limit of quantitation of 2.5 ng ml^{-1} was easily achieved. The method has also been validated for BMS-186318 in monkey plasma without modification. The method has been modified for rat plasma. Owing to irreproducibility observed when applying the liquid–liquid extraction method to rat plasma, a solid-phase extraction method was developed. The addition of phenylmethylsulfonyl fluoride was necessary to stabilize BMS-186318 in rat blood and plasma.

Keywords: BMS-186318; Liquid chromatography–mass spectrometry; Ionspray; Plasma

1. Introduction

BMS-186318 (Fig. 1) is a member of the recently discovered "aminodiol" class of HIV protease inhibitors. In support of non-clinical studies, a simple but sensitive method was developed for the determination of BMS-186318 in dog, rat and monkey plasma. The method is based on LC–ionspray (pneumatically assisted electrospray) single-stage MS. With a simple liquid–liquid extraction, a single-ion monitoring (SIM) method with a short run time was devel-

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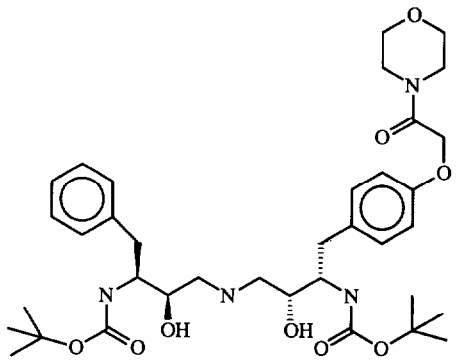
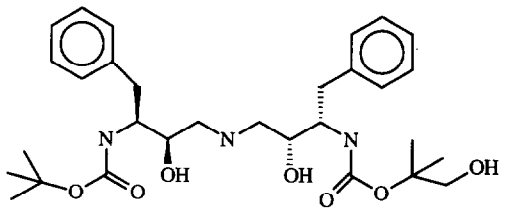


BMS-186318 (MW 686.85)



BMS-185313 (MW 559.71)

Fig. 1. Structures of BMS-186318 and BMS-185313.

oped, which achieved the sensitivity required. The use of the electrospray-based LC–MS technique for the sensitive determination of drug substances and other compounds of interest in a variety of matrices is becoming very popular [1–4]. The mechanisms and practical operational parameters of electrospray mass spectrometry have been discussed in recent papers [5–7]. While tandem mass spectrometry (MS–MS) with electrospray may have the benefit of added selectivity and sensitivity, the less costly single-stage mass spectrometry with electrospray is, in many instances, capable of providing sensitive methods with simple sample preparations. The capability exists because, with electrospray, it is often feasible simply to change the energy applied to the pseudo-molecular ion (or any other parent ion) and bring about fragmentation of the parent ion, providing the analyst with a broad choice of ions to choose from for selected-ion monitoring [8]. The method described in this paper illustrates the utility of liquid chromatography–electrospray single-stage mass spectrometry with a simple sample preparation, short chromatographic run time and good sensitivity.

2. Experimental

2.1. Reagents and chemicals

BMS-186318 and BMS-185313 (the internal standard) are characterized products of Bristol-Myers Squibb Pharmaceutical Research Institute. HPLC-grade acetonitrile (B & J brand), ethanol, 190 proof (Mallinckrodt brand), methyl *tert*-butyl ether (B & J brand) and glacial acetic acid (Mallinckrodt brand) were purchased from Baxter Scientific Products (McGaw Park, IL, USA). House deionized water further purified with a Milli-Q water-purifying system (Millipore, Bedford, MA, USA) was used. Ammonium acetate (ACS grade) was obtained from Sigma (St. Louis, MO, USA). Phenylmethylsulfonyl fluoride (PMSF) was purchased from Aldrich (Milwaukee, WI, USA).

Drug-free dog, monkey and rat plasma and blood were obtained from the Department of Metabolism and Pharmacokinetics or purchased from Pel Freez Biologicals (Rogers, AK, USA). When needed, the appropriate drug-free biological matrix was fortified with PMSF prior to spiking with BMS-186318. A 25 mM stock solution of PMSF was prepared by dissolving 4.36 mg of PMSF in 0.5 ml of ethanol and diluting the solution with 0.5 ml of water. Unless specified otherwise, 50 μl of the PMSF stock solution was used per ml of blood and 100 μl per ml of plasma.

2.2. Equipment

The LC–MS analysis was performed using a Sciex (Thornhill, Ontario, Canada) API I mass spectrometer equipped with an articulated ionspray interface and a Macintosh data system. The API I was coupled to a Hewlett-Packard (Palo Alto, CA, USA) 1090L HPLC system equipped with a ternary pumping unit and a variable automatic sampler. The HPLC column, Zorbax Rx-C_{18}, 5 μm, 150 × 2.1 mm i.d., was obtained from MAC MOD Analytical (Chadds Ford, PA, USA).

A microbore guard column, unpacked, with a 0.5 mm collared frit was purchased from Keystone Scientific (Bellefonte, PA, USA). A Turbovap LV evaporator from Zymark (Hopkinton, MA, USA) was used. Solid-phase extraction columns (C_{18}) were purchased from Varian (Harbor City, CA, USA).

2.3. Chromatographic and mass spectrometric conditions

The mobile phase used was a combination of solvents A and B (in the ratio 4:6, v/v), where A was obtained by mixing 25 parts of methanol, 75 parts of water and ammonium acetate (final concentration: 10 mM), with the final pH adjusted to 5.5 with glacial acetic acid, and B was 100% acetonitrile. The flow rate through the HPLC column was 0.4 ml min^{-1} and the effluent was split so that only one-seventh of the effluent was directed to the mass spectrometer. The injection volume was 10 µl. House nitrogen was used as the nebulizing gas and ultra-high-purity nitrogen as the curtain gas. The sprayer voltage was set at +3500 V and the orifice was set at +45 V. The multiplier voltage was optimized daily.

2.4. Standard and quality control preparations

Two separate stock standard solutions of BMS-186318 were prepared by dissolving separately weighed amounts of BMS-186318 in 190 proof ethanol. One stock solution was used for the preparation of the calibration curve set; the second stock solution was used for the preparation of quality control (QC) samples. The calibration set consisted of ten concentrations, each in duplicate, prepared by spiking the specified amounts of BMS-186318 into a specified volume of drug-free plasma (0.5 ml of plasma for dog, 0.2 ml for monkey, 0.1 ml with PMSF for rat). The concentration ranges were 2.5–500 ng ml^{-1} for dog, 5–1500 ng ml^{-1} for monkey plasma and 10–2500 ng ml^{-1} for rat plasma. Four levels of QCs, prepared in drug-free plasma and stored at −20 °C until analysis, were used for each set of QC samples used for method validation. Three QC levels were in the first quartile, near the mid-point and fourth quartile of the curve. The fourth QC, known as the dilution QC, had a concentration several-fold higher than the highest concentration of the calibration set.

2.5. Extraction and reconstitution

A liquid–liquid extraction was performed for the dog and monkey plasma samples. To a 0.5 ml portion of each dog plasma standard, QC or study plasma sample, internal standard (452 ng ml^{-1}) was added and then 0.5 ml of 0.1 N sodium hydroxide solution was added and mixed. For monkey samples, where 0.2 ml of plasma was used for extraction, 0.5 ml of 0.04 N sodium hydroxide was added and the internal standard was at a concentration of 1035 ng ml^{-1}. Methyl *tert*-butyl ether (3.0 ml) was added and the sample was shaken for 10 min. The aqueous and ether layers were separated by centrifugation and the organic layer was transferred into a clean tube. The ether was removed by evaporation in a Turbovap at 40 °C under nitrogen. The dried extract was reconstituted by dissolving it in 60–75 µl of reconstitution solution (4 parts of solvent A and 6 parts of acetonitrile) and transferred into a conical vial for injection. A 5–10 µL aliquot of this solution was injected into the HPLC system.

A solid-phase extraction, with a 100 mg C_{18} solid-phase extraction (SPE) column, was performed for the rat samples. The SPE column was conditioned with 1.0 ml of methanol and then with 1.0 ml of water. A 0.1 ml plasma sample, fortified with internal standard (2070 ng ml^{-1}),

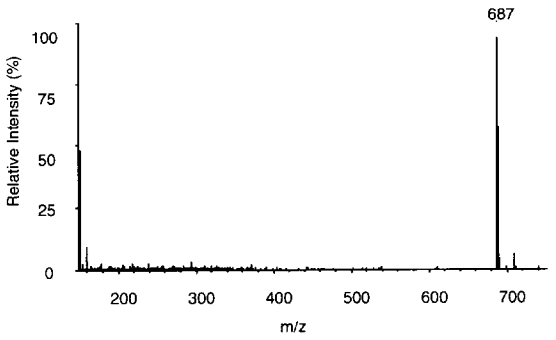


Fig. 2. Positive-ion electrospray mass spectrum of BMS-186318. $[M + H]^+$: m/z 687.

was diluted by adding 1.0 ml of water. The diluted sample was applied to the column and rinsed with 1.0 ml of water, followed by 1.0 ml of 10% methanol in water. Elution was carried out with 1.0 ml of methanol containing 0.1% triethylamine. The eluate was dried in a Turbovap at 40 °C under nitrogen and the extract was dissolved in 75 µl of reconstitution solution. A 10 µl aliquot of this solution was injected into the HPLC system.

3. Results and discussion

From the outset of the method development for BMS-186318 in biological matrices, the focus was to achieve a simple extraction procedure and a short chromatographic time, without sacrificing ruggedness of the method. The method developed and presented in this paper meets those objectives.

The positive-ion electrospray mass spectrum of BMS-186318, at an orifice voltage of +45 V, is shown in Fig. 2. The base peak at *m/z* 687 is due to $[M + H]^+$. There was no significant difference among the sectra acquired at the orifice voltages of +45, +65 and +85 V, either in the total ion abundance or in the degree of fragmentation. On the other hand, the tandem mass spectrum of BMS-186318 (not shown) exhibited a prominent daughter ion at *m/z* 487. This ion was due to the loss of the two *tert*-butyl carbonate groups from the parent ion of *m/z* 687. The positive-ion electrospray mass spectrum of BMS-185313, shown in Fig. 3, had $[M + H]^+$ at *m/z* 560, as expected from the spectrum of BMS-186318. The tandem mass spectrum of BMS-185313 (not shown) also showed a prominent daughter ion of *m/z* 344 due to loss of the *tert*-butyl carbonate and hydroxy-*tert*-butyl carbonate groups. Among a large number of analogues of BMS-186318 investigated as potential internal standards, BMS-185313 was found to be the most acceptable since, in the HPLC system used, it nearly co-eluted with BMS-186318 with no mass spectral interference arising at *m/z* 687, the analytical ion used for BMS-186318. This permitted the development of a method with a very short retention time (less than 1.5 min) for both the analyte and internal standard.

The liquid–liquid extraction with methyl *tert*-butyl ether was adopted after a brief investigation of extractions with a number of solvents. While ethyl acetate and methyl *tert*-butyl ether gave comparable recoveries (>80%), toluene and n-butyl chloride gave lower recoveries, 66% and 47%, respectively. The ether was finally chosen because of ease of removal by evaporation. Extractions based on protein precipitation with acetonitrile or ethanol were deemed less desirable because the evaporation time of ethanol–water or acetonitrile–water supernate is significantly longer than that of water-immiscible solvents such as methyl *tert*-butyl ether. Moreover, reconstitution of the dried extract obtained from a protein precipitation procedure invariably entails centrifugation after reconstitution to remove the insoluble materials and requires more instrument maintenance due to blockage of the orifice, clogging of the sprayer or splitter and cleaning of the interface plate. Because the pK_a of BMS-186318 is about 8.0, the plasma samples were made alkaline before extraction. Because of the potential instability of the carbamate groups in highly alkaline media, the pH of the plasma samples before extraction was raised to only 11.

When the extraction procedure developed for dog plasma was applied to monkey plasma, the method worked well; however, it did not work when applied to rat plasma, showing unacceptably high variability. Consequently, a solid-phase

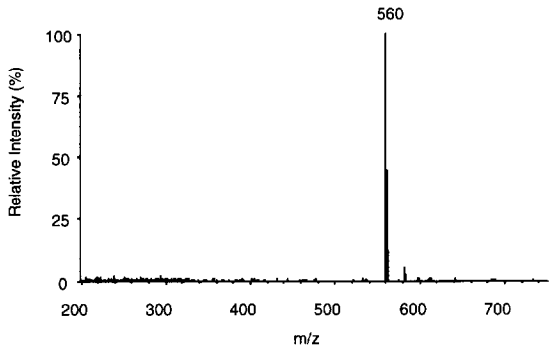


Fig. 3. Positive-ion electrospray mass spectrum of BMS-185313. $[M + H]^+$: *m/z* 560.

extraction (SPE) procedure was developed. For quantitative elution of BMS-186318 from the SPE C_{18} column, it was necessary to add triethylamine to the methanol used for elution.

Whereas BMS-186318 was found to be stable in dog or monkey plasma at room temperature or 4 °C, the compound was found to be unstable in rat plasma under these conditions. Less than 10% of the initial concentration of BMS-186318 remained intact in rat plasma after 3 h at room temperature and less than 40% remained after 3 h at 4 °C. Stability of BMS-186318 in rat plasma was achieved by adding PMSF to the plasma at a concentration of 0.40 mg ml^{-1}. In the presence of PMSF, BMS-186318 in rat plasma was stable for at least 6 h at 4 °C or room temperature. Thus, all the standards and QCs were prepared in drug-free rat plasma fortified with PMSF. Long-term storage stability data at −20 °C showed that BMS-186318 is stable in dog plasma for at least 11 months, in monkey plasma for at least 5 months and in rat plasma (with PMSF) for at least 6 months. BMS-186318 was found to be stable in monkey blood and in rat blood with PMSF for at least 1 h at 4 °C, the temperature at which the blood was kept during the process of plasma formation.

It was of interest to compare a method based on MS–MS versus one based on single-stage MS, as it is generally difficult to predict a priori which of the two approaches would give higher sensitivity. To compare sensitivities, the same extracts from spiked plasma samples were analyzed on a Sciex API III (a triple-quadrupole instrument) in the single-stage mode and in MS–MS mode. For the single-stage MS method the ion at m/z 687 was monitored and for the MS–MS method the daughter ion (m/z 487) was monitored. It was found that the signal-to-noise ratio values of the two methods were comparable, although the signal obtained from the MS–MS method was about 80 times lower. Hence the sensitivities by the two methods were comparable. This result suggested that for this analyte, a relatively simple extraction procedure and a single-stage MS instrument were sufficient, and it was not necessary to resort to the more costly MS–MS instrument.

The single-stage LC–MS method exhibited excellent linearity in dog, monkey and rat plasma. The results of a linear regression analysis (weighted to $1/X$, where X is the concentration in plasma in ng ml^{-1}) of a typical calibration curve in dog plasma are shown in Table 1. The accuracy of each standard point and the precision of the duplicate points were very good. Table 2 presents

Table 1
Linear regression analysis results for a typical calibration curve for dog plasma[a]

Theoretical concentration (ng ml^{-1})	Area ratio	Experimental concentration (ng ml^{-1})	Deviation (%)
2.500	0.00155, 0.00160	2.49, 2.56	−0.4, 3.4
5.000	0.00297, 0.00296	4.89, 4.86	−2.1, −2.8
10.00	0.00536, 0.00642	8.91, 10.69	−10.9, 6.9
20.00	0.01212, 0.01186	20.28, 19.84	1.4, −0.8
50.00	0.02990, 0.03195	50.19, 53.64	0.4, 7.2
100.0	0.05859, 0.06102	98.47, 102.6	−1.5, 2.6
150.0	0.09168, 0.08695	154.1, 146.2	2.8, −2.5
250.0	0.1499, 0.1475	252.1, 248.1	0.8, −0.8
300.0	0.1683, 0.1757	283.1, 295.5	−5.6, −1.5
500.0	0.3011, 0.3032	506.5, 510.1	1.2, 2.0

[a] The pairs of values given under the headings area ratio, experimental concentration and deviation are for the duplicate standards for each level of the calibration curve set. The percentage deviation was obtained by subtracting the experimental (back-calculated) value from the theoretical (nominal) value and then normalizing to the theoretical value. Intercept, 0.000 032 68; slope, 0.000 598 7; r^2, 0.999.

Table 2
Summary of the results for QC samples analyzed on three different days in dog plasma

Parameter	Theoretical concentration (ng ml^{-1})			
	75.23	200.6	351.1	2508
Grand mean (ng ml^{-1})	75.19	202.37	347.00	2779.24
Deviation (%)	−0.05	0.88	−1.17	10.81
Inter-day precision (%)	0.00[b]	0.16	1.09	0.00[b]
Intra-day precision (%)	2.78	2.57	1.36	2.03

[a] The theoretical concentrations of the QCs are shown at the top. The dilution QC (2508 ng ml^{-1}), which is outside the calibration curve range of 2.5–500 ng ml^{-1}, was analyzed by using 0.05 ml of the sample and diluting with 0.45 ml of drug-free dog plasma.

[b] No significant additional variation was observed as a result of performing the assay on different days.

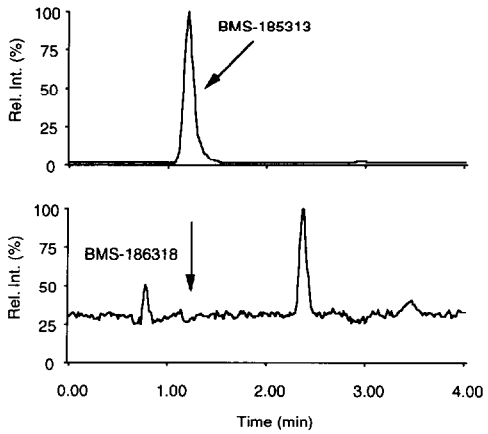


Fig. 4. Ion chromatograms of dog plasma spiked with internal standard (BMS-185313) only. The top trace is for the m/z 560 channel (internal standard) and the bottom trace for the m/z 687 channel (analyte).

a summary of the results for the dog plasma method validation on three different days. The deviation of the grand mean was less than 2.0% for the three levels of QC that are within the range of the curve. The inter- and intra-day precisions were better than 3.0% at all levels. The performance parameters of the monkey and rat methods were generally as good as those of the dog method.

Typical chromatograms for dog plasma analysis are shown in Figs. 4 and 5. A good-sized peak was obtained for BMS-186318 at a concentration in dog plasma of 2.5 ng ml^{-1}, which was the lowest point in the calibration curve.

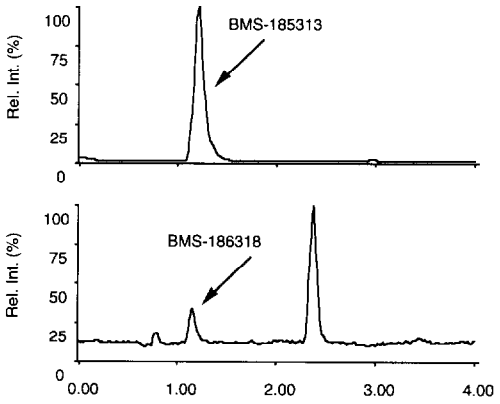


Fig. 5. Ion chromatograms of dog plasma spiked with the internal standard (BMS-185313) and 2.5 ng ml^{-1} of BMS-186318. The top trace is for the m/z 560 channel (internal standard) and the bottom trace for the m/z 687 channel (analyte).

4. Conclusions

A simple method, with excellent precision and accuracy and a short run-time, based on LC–positive-ion electrospray single-stage MS, has been developed for the determination of BMS-186318 in dog, monkey and rat plasma. Both the analyte and internal standard elute within 1.5 min. The method utilizes a one-step liquid–liquid extraction for dog or monkey plasma and solid-phase extraction for rat plasma. BMS-186318 is stable in dog or monkey plasma but is unstable in rat plasma without PMSF.

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