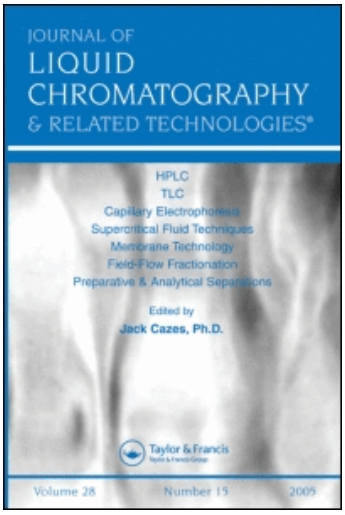

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Nuggehally R. Srinivas[a]; Eugene A. Papp[a]; Vinod R. Shah[a]; Wen Chyi Shyu[a]
[a] Department of Metabolism and Pharmacokinetics Bristol, Myers Squibb Pharmaceutical Research Institute, Princeton, NJ

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A SIMPLE HPLC ASSAY, WITH ULTRAVIOLET DETECTION, FOR DETERMINATION OF A MONOBACTAM ANTIBIOTIC

Nuggehally R. Srinivas,* Eugene A. Papp,
Vinod R. Shah, Wen Chyi Shyu

Department of Metabolism and Pharmacokinetics
Bristol-Myers Squibb Pharmaceutical
Research Institute
P.O. Box 4000
Princeton, NJ 08543-4000

ABSTRACT

BMS-180680 is a monobactam antibiotic currently under development for the treatment of gram negative bacteria including Pseudomonas aeruginosa. Simple, rapid, sensitive, precise, and reproducible assay methods have been developed for the quantification of BMS-180680 in dog and rat plasma using aztreonam as the internal standard. The assay methods involve a single-step protein precipitation by addition of acetonitrile, followed immediately by centrifugation, after which the supernatant is evaporated to dryness (65°C) under a gentle stream of nitrogen. The residue is re-constituted in the mobile phase, transferred to a micro-WISP vial and injected on to a Zorbax R_x C-18 HPLC column preceded by a guard column. Mobile phase comprised 17% acetonitrile and 83% of 40 mM dibasic ammonium phosphate and 6 mM tetrabutylammonium hydrogen sulfate. A small amount of tetrahydrofuran (20 mL/liter of mobile phase) was added and the apparent pH of the mobile phase was adjusted to 4.1 with 85% phosphoric acid. The column eluate was monitored by an ultraviolet

detector set at 252 nm. The nominal retention times were 8.0 and 9.0 min for aztreonam and BMS-180680, respectively. The lower limit of quantitation levels were 0.1 and 0.2 μg/mL in dog and rat plasma, respectively. The inter-assay and intra-assay precision values of the quality control (QC) samples were less than 6.4% relative standard deviation in both dog and rat plasma and the predicted concentrations of the QC samples deviated less than 10% of the corresponding nominal values. BMS-180680 was stable in dog and rat plasma at -20°C for at least 64 and 55 days, respectively and for at least three freeze/thaw cycles in either dog or rat plasma. Both BMS-180680 and aztreonam were stable in the autosampler for at least 59 hours at the room temperature. The assays were employed to measure BMS-180680 concentrations in heparinized dog and rat plasma samples obtained from a pharmacokinetic study.

INTRODUCTION

Monobactam antibiotics such as aztreonam[1,2] and carumonam[3,4] have been demonstrated to show pharmacological activity against several gram negative organisms including Pseudomonas aeruginosa. BMS-180680, ((2R-[2α,3α{Z}])-3-[[[{-(2-amino-4-thiazolyl)-2-[(2-methyl-4-oxo-1-sulfo-3-azetidinyl)- amino]-2-oxo-ethylidene} amino]oxy]methyl]-6,7,dihydroxy-2-quinoxaline carboxylic acid), a monobactam antibiotic structurally similar to both aztreonam and carumonam, is currently being developed for the treatment of infections caused by gram negative organisms. In order to support toxicokinetic and pharmacokinetic studies, a simple high performance liquid chromatographic (HPLC) assay was developed and validated for the quantitation of BMS-180680 in dog and rat plasma using aztreonam as the internal standard.

EXPERIMENTAL

Materials

Analytical supplies of BMS-180680 and aztreonam, the internal standard (I.S.), were obtained from Bristol-Myers Squibb Co., New Brunswick, USA. Monobasic potassium phosphate, dibasic ammonium phosphate, 85% phosphoric acid, and concentrated hydrochloric acid (Fischer Scientific, Fairlawn, NJ, USA); acetonitrile and methanol (J.T. Baker Inc., Phillipsburg, NJ, USA); tetrahydrofuran (Burdick and Johnson Div., Muskegon, MI, USA); and tetrabutylammonium hydrogen sulfate (Aldrich Chemical Co., Milwaukee, WI, USA) were purchased

from the commercial suppliers. Control heparinized rat and dog plasma was obtained from Cocalico Biologicals Inc., Reamstown, PA, USA. Distilled water was deionized and filtered through a Millipore (Milford, MA, USA) Milli-Q system. All other solvents and chemicals used were of analytical grade and were used without further purification.

Equipment

The HPLC system comprised: a Waters Model 590 solvent delivery pump and a Waters 710B WISP autosampler (Water, Division of Millipore, Milford, MA, USA), and a model 785A Kratos programmable ultraviolet (UV) absorbance detector (Applied Biosystem, Inc., Ramsey, NJ, USA) set at 252 nm. The UV detector's output was interfaced through an analog-to-digital converter to a Model HP-3357 laboratory automation system (Hewlett Packard, Cupertino, CA, USA). Other equipment included a Model 720A digital pH meter (Orion, Cambridge, MA, USA), a Model IEC HN-SII centrifuge with a fixed-head 6/84 rotator (Damon/IEC Division, International Equipment, Needham Heights, MA, USA) and a turbo-vap LV evaporator (Zymark Co., Hopkinton, MA, USA).

Standards and Quality Control Samples

Stock concentrations of 400 μg/mL of BMS-180680 and 100 μg/mL of the I.S. were prepared in a buffer solution (pH 6) containing 100 mM potassium phosphate and 10 mM ammonium phosphate. The stock solution of BMS-180680 was used to prepare the standards in dog plasma (range, 0.1 to 40 μg/mL) and rat plasma (range, 0.2 to 40 μg/mL).

Three quality control (QC) samples were prepared separately in the dog (1.30, 11.3, and 30.8 μg/mL) and rat plasma (1.60, 16.2, and 30.3 μg/mL). These QC samples served to: (i) validate the assays for accuracy and precision, (ii) assess the plasma stability of BMS-180680 upon storage at -20°C and (iii) evaluate the freeze-thaw stability of BMS-180680.

Preparation of Samples

Aliquots (100 μL for the rat assay or 150 μL for the dog assay) of plasma samples (standards, QCs and blank samples) were transferred into disposable borosilicate culture tubes. Fifty μL (rat assay) or 30 μL (dog assay) of the I.S. solution (1 part of 100 μg/mL solution of aztreonam mixed with 1 part of 0.1 N

hydrochloric acid solution) was added and vortexed for a few seconds. Plasma proteins were precipitated by the addition of a 200 μL (rat assay) or 300 μL (dog assay) of acetonitrile. The samples were mixed thoroughly, followed by centrifugation at 2600 rpm for 10 minutes. Following centrifugation, the entire supernatant was transferred into another labelled tube and evaporated to dryness using a turbo-evaporator (65°C) under a gentle stream of nitrogen (7-10 psi). The residue was re-constituted in the mobile phase (130 μL) within 15-30 minutes. The entire mixture was transferred to a WISP vial with an insert. Approximately 75 μL of the mixture was injected on to the HPLC column.

Mobile Phase

The mobile phase comprised 17% acetonitrile and 83% of Milli-Q water containing 40 mM dibasic ammonium phosphate and 6 mM tetrabutylammonium hydrogen sulfate. In each liter of the mobile phase, a 20 mL volume of tetrahydrofuran was added. The apparent pH of the mobile phase was adjusted to 4.1-4.2 with 85% phosphoric acid.

Chromatography

The processed dog and rat plasma samples were analyzed using a 250 mm X 4.6 mm I.D. Zorbax R_x-C18 column with a particle size diameter of 5 μM (Mac-Mod Analytical Inc., Chadds Ford, PA, USA), immediately preceded by a 30 mm X 4.6 mm I.D. (ODS 5 μM particle diameter) pre-packed Brownlee™ guard column (Chrom Tech. Inc., Apple Valley, MN, USA). The mobile phase was delivered at a rate of 1.5 mL/min and the column eluate was monitored by an UV detector set at 252 nm.

Data Processing

The analog output of the UV detector was collected and processed by the HP-3357 laboratory automation system according to previously described procedures.[5] Briefly, the standard data (peak height ratio versus concentration) generated from each analytical run were weighted by the reciprocal of the corresponding concentrations and fit to a linear regression equation. The procedure described by Prescott was used to perform the outlier rejection of the standards.[6] The concentrations of unknown samples were determined by inverse prediction from the appropriate regression line.

Validation Procedures

The validation of the assay methods included documentation of assay linearity, accuracy, precision, lower limit of quantitation (LLQ), and specificity. The stability of BMS-180680 in the auto sampler, after repeated freeze/thaw cycles, and upon storage (-20°C) was also evaluated. All the validation procedures were performed separately for the dog and rat plasma matrices.

LLQ and specificity were evaluated concurrently using the plasma matrix from ten individual animals. For LLQ assessment, the ten individual samples were processed, both as blank samples and as samples spiked at LLQ, defined as 0.1 μg/mL for the dog and 0.2 μg/mL for the rat plasma matrix. Paired t tests were performed ($\alpha = 0.05$) to assess the statistical significance between peak heights for blank and those for spiked LLQ samples. For specificity evaluation, the individual paired chromatograms of blank and spiked samples were inspected to assess the degree of interference by endogenous constituents.

The accuracy and precision of the assays were assessed at concentrations in the upper, middle and lower quartile of the standard curve range, assaying at least six replicate samples for each concentration, on three different days. Accuracy was defined as the deviation of the mean observed concentration from the nominal and expressed as a percentage of the nominal concentration. Intra-assay precision estimate was expressed as the percentage relative standard deviation (RSD) of the predicted concentrations of the replicate samples from each group. The inter-assay precision estimate was calculated using the following equation: $100[(TrMS-EMS)/N]^{0.5}/GM$, where TrMS, EMS, and GM refer to treatment and error mean squares and grand mean, respectively.

The stability of BMS-180680 during storage at -20°C and after freeze-thaw cycles was evaluated using the plasma QC samples used to predict the accuracy and precision of the assay. The autosampler stability of BMS-180680 at the room temperature was evaluated by periodically injecting aliquots of the processed QC samples (dog: 2.5, 10 and 40 μg/mL; rat: 10, 20 and 40 μg/mL) over a 59 hr period and determining peak height as a function of time.

Pharmacokinetic Application

The assay methods were applied to analyze the unchanged BMS-180680 concentrations in plasma samples obtained from pharmacokinetic studies in dogs and rats.

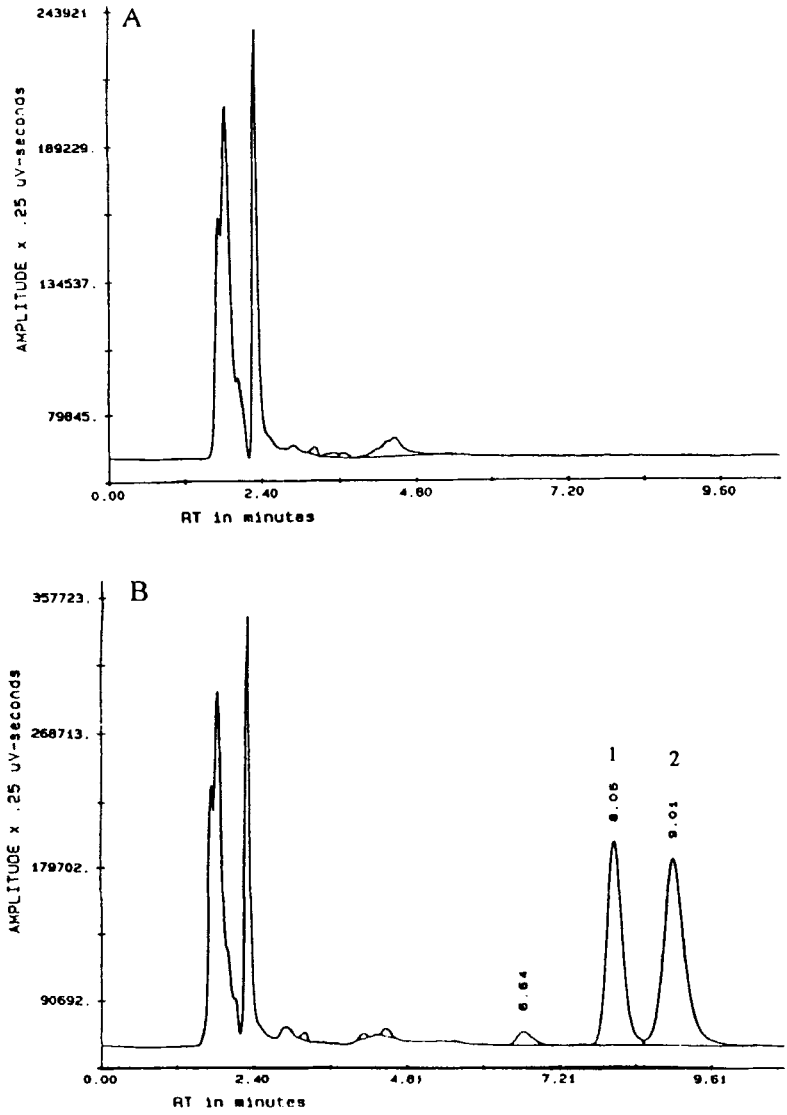

Figure 1. Representative chromatograms of a blank rat plasma [A] and a spiked standard containing aztreonam and BMS-180680 (20 μg/mL) [B] (aztreonam (1) and BMS-180680 (2) peaks correspond to retention times of 8 and 9 minutes, respectively).

Table 1

Accuracy and Precision Data for the Analysis of BMS-180680 in Dog and Rat Plasma

(a) Intra-assay Accuracy and Precision

Species	Nominal Concentration (μg/mL)	Mean Predicted Conc'n (μg/mL)	N	Precision (% RSD)	Accuracy (%Deviation from nominal)
Dog	30.8	31.3	9	6.4	1.7
	11.3	11.2	9	3.9	-0.8
	1.30	1.18	9	6.1	-9.1
Rat	30.3	33.3	9	3.5	9.9
	16.2	17.8	9	1.6	9.6
	1.60	1.57	9	3.8	-2.0

(b) Intra-assay Accuracy and Precision

Species	Nominal Concentration (μg/mL)	Mean Predicted Conc'n (μg/mL)	N	Precision (% RSD)	Accuracy (%Deviation from nominal)
Dog	30.8	33.7	24	4.5	-13.8
	11.3	11.7	24	3.7	3.3
	1.30	1.12	25	6.4	9.3
Rat	30.3	31.7	27	5.0	4.5
	16.2	17.2	27	5.5	6.0
	1.60	1.61	27	7.8	0.6

RESULTS AND DISCUSSION

Since BMS-180680 is zwitter ionic in nature (presence of both acidic and basic functionalities in the molecule), a controlled pH environment of the mobile phase is essential to maintain satisfactory and consistent peak shape without peak splitting or peak tailing. In preliminary experiments, it was assessed that a mobile phase pH of about 4.1-4.2 was best suited for the separation of BMS-180680. At this pH value, the I.S. peak was also sharp and well separated from the BMS-180680 peak.

Table 2

Stability of BMS-180680 inDog and Rat Plasma QC Samples on Storage at -20°C

(a) Dog Plasma

Time (Days)	Mean Predicted Concentrations (± DEV) at Various Concentrations		
	30.8 (μg/mL)	**11.3 (μg/mL)**	**1.30 (μg/mL)**
9	31.3 (1.7)	11.2 (-0.8)	1.18 (-9.1)
44	26.5 (-14.1)	11.6 (2.5)	1.32 (2.6)
64	25.9 (-15.9)	9.38 (-16.8)	1.23 (-4.5)

(b) Rat Plasma

Time (Days)	Mean Predicted Concentrations (± DEV) at Various Concentrations		
	30.3 (μg/mL)	**16.2 (μg/mL)**	**1.60 (μg/mL)**
1	29.8 (-1.6)	16.0 (-1.5)	1.48 (-2.0)
22	34.6 (14.2)	18.5 (14.2)	1.71 (7.0)
33	30.7 (1.5)	17.9 (10.5)	1.65 (3.1)
55	29.1 (-4.1)	16.3 (0.5)	1.49 (-6.7)

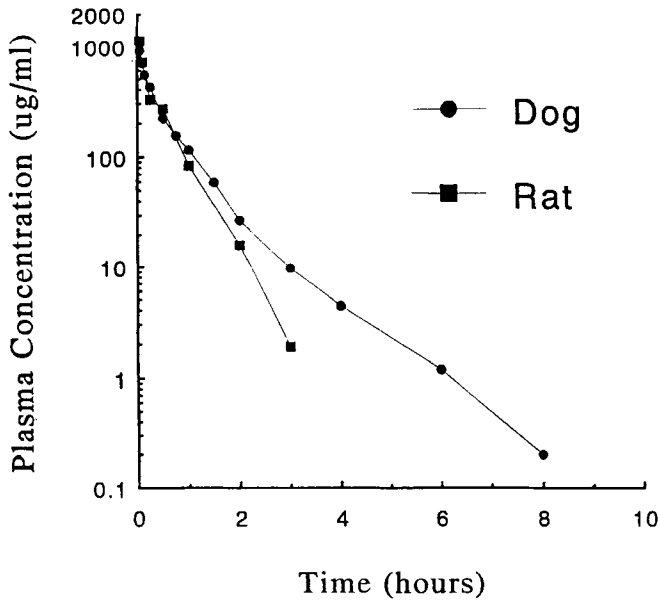

Figure 2. Plasma concentration-time profiles of BMS-180680 in a dog (closed circles) and in a rat (closed squares) receiving intravenous doses of 100 and 200 mg/kg of BMS-180680, respectively.

Representative chromatograms of a blank and a spiked standard in the rat plasma matrix are shown in Figure 1. Inspection of the chromatograms obtained from rat plasma indicated no interference at the peaks of interest by endogenous materials. Similar inferences were drawn from the chromatograms obtained from dog plasma.

The nominal retention times for the peaks of I.S. and BMS-180680 were 8 and 9.0 min, respectively. The standard curves demonstrated linearity ($R^2 > 0.995$) over the concentration range of 0.1 to 40 μg/mL in the dog plasma and 0.2 to 40 μg/mL in rat plasma.

LLQ was established as the lowest standard curve concentration, 0.10 μg/mL for the dog and 0.20 μg/mL for the rat plasma assay. The mean value for LLQ in the dog plasma assay had a precision of 14.5% RSD and an accuracy of 1.2% deviation. The mean value for LLQ in the rat plasma assay had a precision of 8.9% RSD and an accuracy of 5% deviation.

The intra-assay (within-day) and inter-assay (between-day) accuracy and precision data for the dog and rat plasma assays are shown in Table 1. The inter- and intra-assay (between days) precision estimates (RSD) for the dog plasma assay were less than 7% and for the rat plasma assay were less than 8%. The inter- and intra-assay accuracy estimates were within ± 14% for either of the two assays.

The autosampler stability of BMS-180680 in the processed dog or rat plasma samples was demonstrated over a period of 59 hr. The freeze/thaw stability study indicated that BMS-180680 in dog or rat plasma was stable (within ± 15% of nominal values) through three freeze/thaw cycles. Finally, BMS-180680 was found to be stable in the dog and rat plasma at -20°C for at least 2 months (Table 2).

The developed and validated assays in dog and rat plasma were utilized in the analysis of plasma samples from pharmacokinetic studies. A representative plasma concentration-time profile of intact BMS-180680 following a single intravenous dose in dogs (100 mg/kg) and rats (200 mg/kg) are shown in Figure 2.

CONCLUSIONS

The assay methods developed for the quantification of BMS-180680 in the dog and rat plasma are simple, specific, sensitive, accurate, precise, and reproducible. The advantages of this assay includes the rapidity of the sample analysis due to a minimal sample preparation and the use of small sample volumes.

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