Journal of Chromatography B, 877 (2009) 471–476

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Remediation of undesirable secondary interactions encountered in hydrophilic interaction chromatography during development of a quantitative LC–MS/MS assay for a dipeptidyl peptidase IV (DPP-IV) inhibitor in monkey serum

Eugene P. Kadar[a,*], Chad E. Wujcik[b,1]

[a] Pharmacokinetics, Dynamics & Metabolism, Pfizer Global Research and Development, Pfizer, Inc., Groton, CT 06340, United States
[b] Monsanto, 800 North Lindbergh Boulevard, Mail Code V1B, St. Louis, MO 63167, United States

ARTICLE INFO

Article history:
Received 7 August 2008
Accepted 26 November 2008
Available online 9 December 2008

Keywords:
Dipeptidyl peptidase IV (DPP-IV) inhibitor
Bioanalysis
Hydrophilic interaction chromatography
HILIC
LC–MS/MS

ABSTRACT

PF-00734200 (3,3-Difluoropyrrolidin-1-yl)-((2S,4S)-4-(4-(pyrimidin-2-yl) piperazin-1-yl)pyrrolidin-2-yl)methanone) is an inhibitor of dipeptidyl peptidase IV (DPP-IV) for the treatment of diabetic complications and other disorders. A sensitive and selective LC–MS/MS assay capable of quantifying PF-00734200 in monkey serum was required to support regulated safety studies. Due to the polar nature of this compound and for ease of sample processing, hydrophilic interaction chromatography (HILIC) was identified as an ideal assay technique. During the initial phase of method development significant peak tailing was observed. The effects of polar organic modifier percentage, buffer concentration, column particle size, and flow rate were assessed to determine the final optimal conditions. PF-00734200 demonstrated a strong dependence on buffer concentration with respect to height equivalent to a theoretical plate (HETP), capacity factor (k'), and tailing factor (T). Improvements in chromatography were observed with increasing buffer concentration due to reduction of electrostatic secondary interactions with ionized silanols. A plot of $\log k'$ versus percentage organic modifier at an elevated buffer concentration, produced a linear fit with a correlation coefficient of 0.996, indicating that the primary chromatographic retention mechanism was partitioning. A LC–MS/MS assay was successfully developed and validated for GLP bioanalysis of PF-00734200 in monkey serum utilizing the optimized HILIC conditions. Additionally, carryover was effectively minimized through fortification of ethylene glycol to the sample extract.

1. Introduction

Hydrophilic interaction chromatography (HILIC) is a pseudo normal phase technique that can retain and separate polar analytes using polar stationary phases coupled with more traditional reversed-phased mobile phase systems. The most commonly used HILIC stationary phase is silica [1], but aminopropyl, cyanopropyl, amide, diol, poly(succinimide), cyclodextrin, sulfonated S-DVB, and sulfoalkylbetaine have also been successfully employed [1,2]. HILIC mobile phases are typically comprised of aqueous and semi-polar organic solvents (*i.e.*, acetonitrile and methanol) [1–6]. The aqueous component is initially maintained at a relatively low level to promote the formation of a water layer on the surface of the stationary phase particles [3]. This hydrated interface provides the polar medium for analytes to interact. Analytes that are more polar have a greater affinity for the hydrated layer, spend more time interacting, and are therefore better retained. Hydrophobic compounds remain solubilized in the more organic mobile phase and are rapidly flushed through the system.

Alpert suggested that the mechanism of separation is based on the partitioning of a given analyte between the fixed hydrated layer and the more hydrophobic organic mobile phase [3]. Our recently published work demonstrates a relationship between k' and the distribution coefficient of a compound, D [5], giving further evidence to the presence of a partitioning mechanism.

HILIC coupled with tandem mass spectrometry has been utilized in numerous quantitative bioanalytical assays involving a variety of matrices [6–14]. The ability to directly inject extracts from standard protein precipitation without the need for altering the final sample extract organic composition can result in significant time savings and less overall variability [6]. Since the initial aqueous composition of typical HILIC mobile phases ranges between 5 and 25%, direct injection of the extracts can allow for the focusing of analytes and the maintenance of peak symmetry. An added benefit of

* Corresponding author at: Pharmacokinetics, Dynamics & Metabolism, Pfizer Global Research and Development, Groton/New London Laboratories, Pfizer, Inc., Mail Stop: MS 8118D-2041, Eastern Point Road, Groton, CT 06340, United States. Fax: +1 860 715 7205.
E-mail addresses: eugene.p.kadar@pfizer.com (E.P. Kadar), chad.e.wujcik@monsanto.com (C.E. Wujcik).
[1] Fax: +314 694 8774.

1570-0232/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2008.11.048

Table 1
Physicochemical properties of test compounds.

Compound identification no.	Purpose	Molecular weight (Da)	Basic pK_a[a]	log $D_{pH\ 3.0}$[b]	log P[b]
PF-00734200	Analyte	366	9.37, 5.65, 4.25	−8.68	−0.02
PF-03771846	Internal Standard	374	9.37, 5.65, 4.25	−8.68	−0.02
Torcetrapib	t_0 Marker	600	−1.87, −3.14	8.16	8.16

[a] Calculated using ACD/pK_a DB software, ACD/Labs Release: 9.00, Product Version 9.04, Advanced Chemistry Development, Inc., Copyright © 1997–2005.
[b] Calculated using ACD/log D software, ACD/Labs Release 9.00, Product Version 9.03, Advanced Chemistry Development, Inc., Copyright © 1997–2005.

high organic mobile phase concentration is the enhancement of signal and overall sensitivity gained with electrospray ionization by reducing surface tension and enhancing desolvation.

PF-00734200 is an active pharmaceutical ingredient under development at Pfizer in the cardiovascular and metabolic disease therapeutic area. Stable labeled analog, [$^2H_8$]-3,3-Difluoropyrrolidin-1-yl)-((2S,4S)-4-(4-(pyrimidin-2-yl)piperazin-1-yl) pyrrolidin-2-yl)methanone (PF-03771846), and a hydrophobic compound, 4-[(3,5-Bis-triflouromethylbenzyl)-methoxycarbonyl-amino]-2-ethyl-6-trifluoromethyl-3,4-dihydro-2-H-quinoline-1-carboxylic acid ethyl ester (Torcetrapib), were identified for use as an internal standard and void volume marker, respectively. The physicochemical properties of these three compounds are tabulated in Table 1 [15,16]. The log $D_{pH\ 3.0}$ value indicates that PF-00734200 is a relatively polar compound, making it an appropriate choice for hydrophilic liquid chromatography, especially at low pH.

Initial method development indicated considerable peak tailing. This paper describes the optimization of HILIC conditions taking into consideration the physicochemical parameters of the compound, as well as the chromatographic parameters: number of theoretical plates (N), height equivalent to a theoretical plate (HETP), capacity factor (k'), and peak tailing factor (T).

2. Experimental

2.1. Materials

(3,3-Difluoropyrrolidin-1-yl)-((2S,4S)-4-(4-(pyrimidin-2-yl) piperazin-1-yl)pyrrolidin-2-yl)methanone (PF-00734200), [$^2H_8$]-3,3-Difluoropyrrolidin-1-yl)-((2S,4S)-4-(4-(pyrimidin-2-yl)piperazin-1-yl)pyrrolidin-2-yl)methanone (PF-03771846), 4-[(3,5-Bis-triflouromethylbenzyl)-methoxycarbonyl-amino]-2-ethyl-6-trifluoromethyl-3,4-dihydro-2-H-quinoline-1-carboxylic acid ethyl ester (Torcetrapib) were produced by Pfizer, Inc. (NY, USA). The chemical structures of these compounds are illustrated in Fig. 1. HPLC grade water, methanol, and acetonitrile were obtained from J.T. Baker (NJ, USA). Reagent grade ammonium formate was obtained from Sigma–Aldrich (MO, USA). Reagent grade formic acid was obtained from Acros Organics (NJ, USA). Reagent grade ethylene glycol was obtained from J.T. Baker (NJ, USA). HPLC grade acetone and isopropyl alcohol were obtained from Sigma–Aldrich (MO, USA). Cynomolgous monkey control serum was obtained in-house and from Biochemed (SC, USA).

2.2. LC–MS/MS

Experiments were performed using a Shimadzu SCL-10A controller, Shimadzu LC-10AD pumps, and a CTC Analytics (LEAP) HTLS PAL autosampler. The autosampler was equipped with a SGE syringe (P/N 005330) that was used to load 10 μL onto a 3 μL sample loop. Analytical columns evaluated for this application were Atlantis™ HILIC silica column, 2.1 mm × 50 mm, 5 μm, P/N 186002012 (Waters Corporation, MA, USA) and Atlantis™ HILIC Silica column, 2.1 mm × 50 mm, 3 μm, P/N 186002011 (Waters Corporation, MA, USA). A 200 mM ammonium formate stock solution was prepared and adjusted to pH 3.0 using concentrated formic acid. Serial dilutions of this stock solution with HPLC grade water produced additional 50 and 10 mM stock concentrations. HPLC grade water, HPLC grade acetonitrile, and the appropriate 200, 50, or 10 mM ammonium formate solutions were delivered using pump channels A, B, and C, respectively.

An Applied Biosystems API 4000 tandem quadrupole mass spectrometer equipped with a TurboIonspray™ source operated in positive-ion electrospray mode was utilized to detect the selected reaction monitoring (SRM) precursor > product ion transitions of 367 > 122, 375 > 127, and 618 > 300 for PF-00734200, PF-03771846, and Torcetrapib, respectively. The SRM transition for each compound was determined during the mass spectrometer tuning process by teeing in the infusion solutions under generic mobile phase flow conditions. Molecular ions $[M+H]^+$ were chosen as the precursor ions for PF-00734200 and PF-03771846, while the ammonium adduct of Torcetrapib $[M+NH_4]^+$ was employed to obtain adequate sensitivity. The following optimal mass spectrometer source parameters were utilized: ion spray voltage: 1500, source temperature: 650 °C, CUR 25, GS1 50, and GS2 60. Collision energy (CE) values were set at 47, 55, and 39 for PF-00734200,

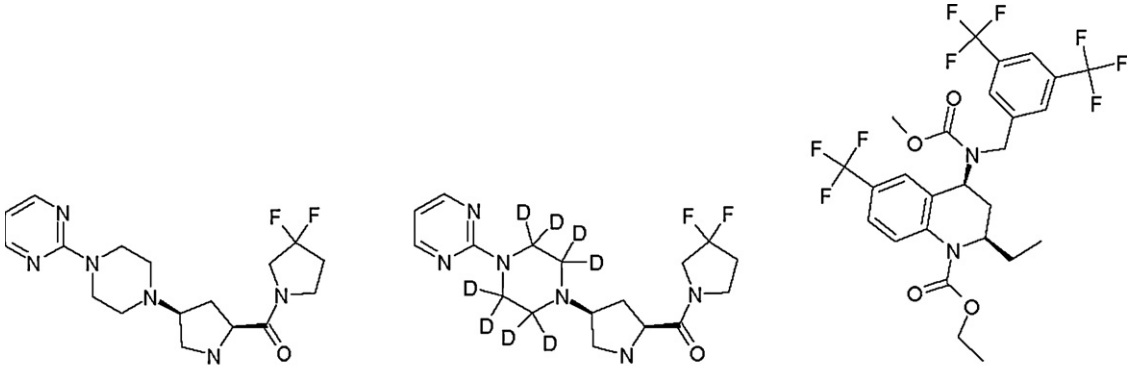

Fig. 1. Chemical structures of PF-00734200, PF-03771846, and Torcetrapib.

PF-03771846, and Torcetrapib, respectively. Data acquisition and chromatographic assessments were performed with Analyst, version 1.4 software.

2.3. Selection of appropriate column void volume marker

Hydrophobic compounds have a high affinity for the organic mobile phase in a HILIC chromatographic system and therefore are eluted in the void volume. A series of available compounds was evaluated for hydrophobicity utilizing ACD/LogD software [16]. Torcetrapib was chosen as a void volume marker based on its relatively high log *P* and log *D* (pH 3.0) value of 8.16.

2.4. Effect of organic mobile phase concentration on capacity factor

Chromatography was performed using the 5 μm particle size HILIC silica column at a flow rate of 1.0 mL/min. The polar organic modifier concentration, acetonitrile, was varied over the range of 75–95%. A constant ammonium formate concentration of 10 mM was maintained by utilizing pump channels A, B, and C. Pump A delivered the make-up volume of water, Pump B delivered the appropriate percentage of acetonitrile, and pump C delivered the 200 mM ammonium formate, pH 3.0, buffer at a constant 5%. A solution containing PF-00734200 (200 ng/mL) and Torcetrapib (1000 ng/mL) in acetonitrile was injected in triplicate at each of the acetonitrile mobile phase concentrations of 75, 80, 85, 90, and 95%.

2.5. Impact of buffer concentration on HETP, capacity factor, and peak symmetry

Chromatography was performed using the 5 μm particle size HILIC silica column at a flow rate of 1.0 mL/min. Pump A delivered the required make-up volume of water, Pump B provided acetonitrile at a constant percent of 90, and Pump C delivered the appropriate molarity ammonium formate, pH 3, stock buffer (*i.e.,* 10, 50 or 200 mM) to produce a net mobile phase concentration of 1, 5, 10, 15 and 20 mM. The PF-00734200 (200 ng/mL) and Torcetrapib (1000 ng/mL) mixture was injected in triplicate at each respective ammonium formate concentration after adequate system equilibration was achieved.

2.6. Influence of column particle size and flow rate on HETP

The flow rate was varied over the range of 0.1–1.00 mL/min and 0.1–1.75 mL/min for the 3 and 5 μm particle size HILIC silica columns, respectively. A minimum flow rate of 0.100 mL/min was chosen for both columns in order to produce a reasonable run time. The high end flow rates for each column were limited by back pressure. A net 10 mM ammonium formate concentration and an acetonitrile concentration of 90% were maintained for both columns. Pumps A and B provided water and acetonitrile at 5 and 90%, respectively. Pump C delivered the 200 mM ammonium formate, pH 3.0, stock buffer at 5% to produce a constant 10 mM delivery. The standard mixture of PF-00734200 (200 ng/mL) and Torcetrapib (1000 ng/mL) was injected in triplicate for each flow rate evaluated.

2.7. Chromatographic performance calculations

Chromatographic performance was evaluated by calculating the number of theoretical plates (*N*), height equivalent to a theoretical plate, capacity factor (*k′*), and peak tailing factor (*T*), by the following equations [4]:

$$N = 5.54\left(\frac{t_r}{W_{1/2}}\right)^2 \quad (1)$$

where t_r is the analyte retention time and $W_{1/2}$ is the peak width at half-height.

$$\text{HETP} = \frac{L}{N} \quad (2)$$

where *L* is the column length in millimeters and *N* is the number of theoretical plates.

$$k' = \frac{t_R - t_0}{t_0} \quad (3)$$

where t_R is the retention time of the analyte, and t_0 is the retention time of the void volume marker.

$$T = \frac{A + B}{2A} \quad (4)$$

where *A* is the width from the leading edge of the analyte peak to the peak maximum at 5% of peak height and *B* is the width from the peak maximum to the tailing edge of the analyte peak at 5% of peak height.

2.8. Validation

A LC–MS/MS bioanalytical method validation for PF-00734200 in monkey serum was conducted in accordance with the U.S. Food and Drug Administration, Guidance for Industry, Bioanalytical Method Validation [17] and Pfizer standard operating procedures. The guard and analytical columns consisted of a Waters Corporation Atlantis™ HILIC Silica guard column, 5 μm, 2.1 mm × 10 mm, P/N 186002006 (Waters Corporation, MA, USA) coupled to the 2.1 mm × 50 mm, 5 μm particle size Atlantis™ HILIC silica column described previously. The larger 5 μm particle size was utilized to accommodate higher flow rates and throughput. The mobile phase composition and chromatographic program are listed in Table 2.

Pump C delivered the 200 mM ammonium formate, pH 3.0, stock buffer at a constant level to produce a final mobile phase ammonium formate concentration of 20 mM. A wash step utilizing 50% water in the mobile phase was employed to wash polar material from the column following elution of PF-00734200, simulating the column regeneration conditions. A two minute re-equilibration period was employed to allow the column to equilibrate back to initial conditions following the wash step and ensure the formation of a static hydrated layer prior to the next injection. Needle wash solutions one and two consisted of acetonitrile:methanol:water:formic acid (40:40:20:1) and acetonitrile:isopropyl alcohol:acetone (6:3:1), respectively.

Ninety-six well polypropylene blocks utilized for validation batch runs were prepared as follows: twenty microliters of sample or control matrix were added to appropriate wells of a 96-well polypropylene block. Three hundred microliters of working internal standard solution were added to control blank, calibration standard, and quality control samples. Additionally, 300 microliters of acetonitrile were added to double blank wells. The block was capped, vortexed and centrifuged at approximately 1450 × *g* for 10 min. Forty microliters of supernatant was transferred to a new 96-well polypropylene block. Three hundred and sixty microliters of acetonitrile:ethylene glycol (9:1) solution were added. The block was capped, vortexed, and analyzed.

Watson Laboratory Information Management Software (LIMS) (ThermoElectron, MA), version 7.2 was used to perform regression, quantitation, and statistical calculations**.** Three separate validation batch runs were performed with calibration standards covering a range of 10–10,000 ng/mL and replicate quality control samples at four different concentration levels (10, 30, 400, and 7500 ng/mL). Selectivity, bench top, long term and freeze/thaw stability assessments were also performed.

Table 2
Tabulation of mobile phase gradient program.

Time (min)	Flow rate (mL/min)	%Mobile phase A (water)	%Mobile phase B (acetonitrile)	%Mobile phase C (200 mM Amm. formate, pH 3.0)
0.01	1.000	5	85	10
2.00		5	85	
2.01		40	50	
3.00		40	50	
3.01		5	85	
5.00		5	85	

3. Results and discussion

3.1. Effect of organic mobile phase concentration on capacity factor

The k' of PF-00734200 increased as a function of elevated acetonitrile levels in the mobile phase as expected. A linear relationship between k' and the percentage of acetonitrile in the mobile phase (correlation coefficient of 0.996) was observed when the y-axis was converted to the log scale as illustrated in Fig. 2. This linear relationship is indicative of a partitioning mechanism [1]. A net 10 mM ammonium formate concentration was utilized in this experiment to minimize secondary electrostatic interactions between PF-00734200 and ionized silanol groups, allowing partitioning to act as the primary retention mechanism.

3.2. Impact of buffer concentration on capacity factor, HETP, and peak tailing

Fig. 3 illustrates the influence of buffer concentration on HETP for the 5 μm particle size column. A decrease in HETP occurred as the buffer concentration was elevated from 1 to 20 mM ammonium formate. The most significant reduction in HETP was observed between buffer concentrations of 1–5 mM ammonium formate. Less impact on HETP occurred between 10 and 20 mM ammonium formate. Overall, a 20 mM ammonium formate concentration produced the lowest HETP, which corresponds to the most efficient chromatographic peak and yields an adequate k' of 3. The Center for Drug Evaluation and Research (CDER) recommends a minimum k' value >2 [23], which ensures adequate separation of the analyte from un-retained matrix components.

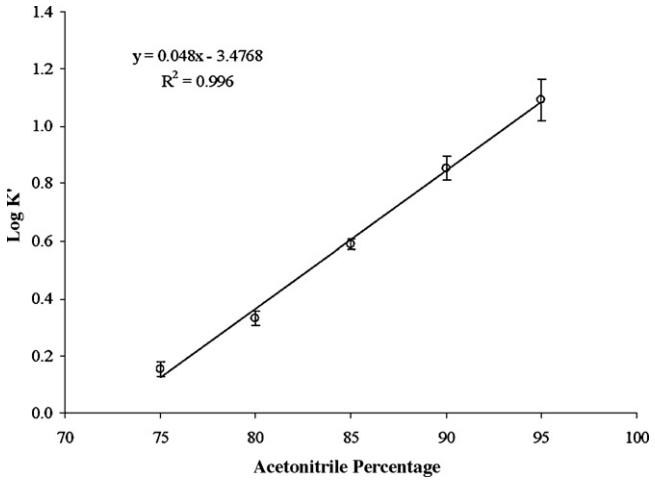

Fig. 2. Influence of acetonitrile mobile phase concentration on capacity factor. Error bars represent ± one standard deviation (n = 3). Chromatography was performed using a 5 μm particle size HILIC silica column at a flow rate of 1.0 mL/min. The polar organic modifier concentration, acetonitrile, was varied over the range of 75–95%. A constant ammonium formate concentration of 10 mM was utilized in this experiment to minimize secondary electrostatic interactions between PF-00734200 and ionized silanol groups, allowing partitioning to act as the primary retention mechanism, which is demonstrated by the presence of a linear relationship between log k' and acetonitrile percentage.

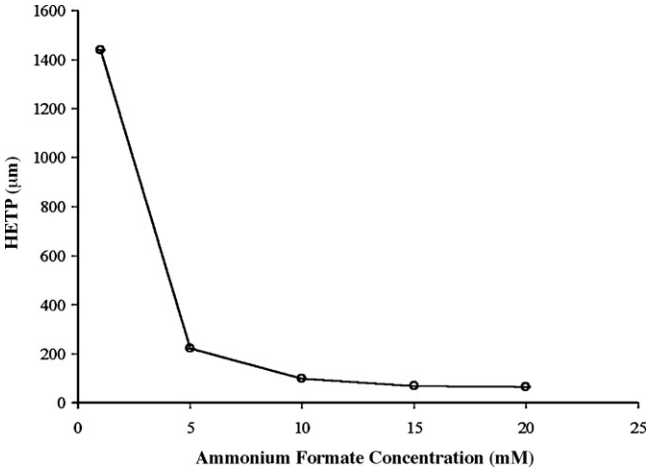

Fig. 3. Influence of buffer concentration on HETP. Chromatography was performed using a 5 μm particle size HILIC silica column at a flow rate of 1.0 mL/min. The mobile phase acetonitrile concentration was 90% and the ammonium formate concentration was varied from 1 to 20 mM. Error bars represent ± one standard deviation (n = 3). The standard deviations did not exceed 0.125 μm and therefore the error bars cannot be seen on this plot due to the ordinate scale. A 20 mM ammonium formate concentration produced the lowest HETP, which corresponds to the most efficient chromatographic peak and yields an adequate k' of 3.

Fig. 4 demonstrates the influence of buffer concentration on tailing factor (T). The tailing factor was substantial at an ammonium formate concentration of 1 mM. Peak symmetry continued to

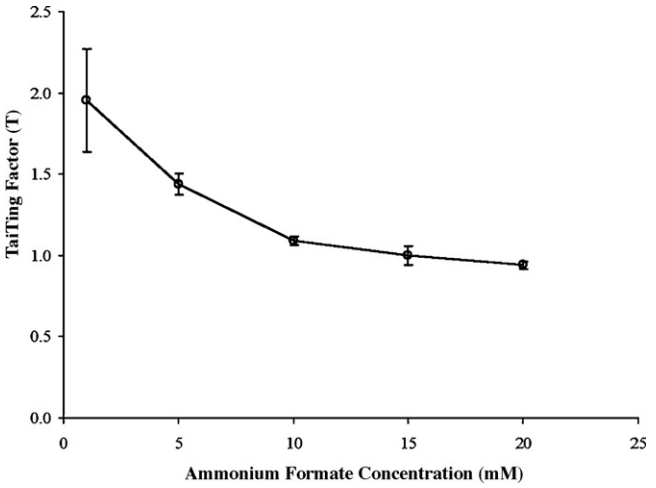

Fig. 4. Influence of buffer concentration on tailing factor (T). Error bars represent ± one standard deviation (n = 3). Chromatography was performed using the 5 μm particle size HILIC silica column at a flow rate of 1.0 mL/min. Pump A delivered the required make-up volume of water, Pump B provided acetonitrile at a constant percent of 90, and Pump C delivered the appropriate molarity ammonium formate, pH 3, stock buffer (*i.e.*, 10, 50 or 200 mM) to produce a net mobile phase concentration of 1, 5, 10, 15 and 20 mM. An ammonium formate concentration of 20 mM produced the most symmetrical peak.

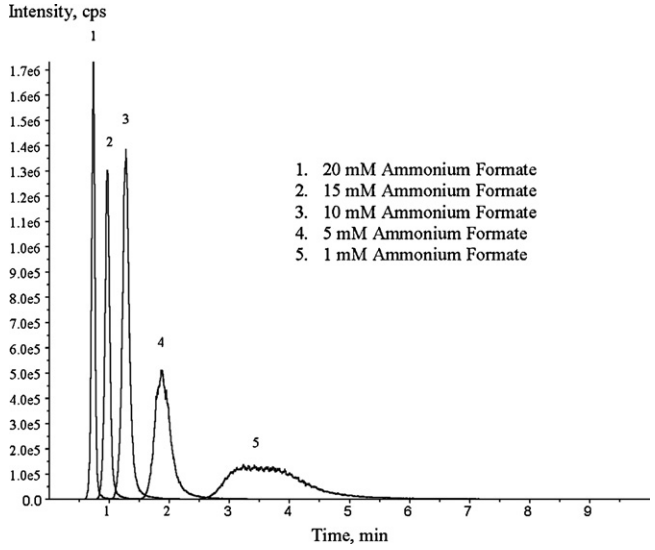

Fig. 5. Influence of buffer concentration on overall peak shape. Chromatography was performed using a 5 μm particle size HILIC silica column at a flow rate of 1.0 mL/min. The mobile phase acetonitrile concentration was 90% and the ammonium formate concentration was varied from 1 to 20 mM. Acetonitrile concentration was kept constant at 10% (v/v). Peak symmetry continued to improve as the buffer concentration approached 20 mM with the most significant changes occurring from 1 to 10 mM.

improve as the buffer concentration approached 20 mM with the most significant changes occurring from 1 to 10 mM. Fig. 5 illustrates the influence of buffer concentration on the overall peak shape of PF-00734200.

Elevated ammonium formate concentrations were found to have a positive impact on HETP, T, and overall peak shape. These benefits are believed to be attributed to the alleviation of secondary electrostatic interactions between the protonated analyte (PF-00734200) and ionized silanols [22].

3.3. Influence of column particle size and flow rate on HETP

Van Deemter plots for the 5 μm (A) and 3 μm (B) particle size HILIC silica columns are shown in Fig. 6. Analysis of the 5 μm plot, Fig. 6(A), indicated that the most efficient flow rate was the lowest value tested (*i.e.*, 0.1 mL/min). HETP continued to increase to 1.5 mL/min where it then appeared to reach a steady state. However, the number of theoretical plates at 1.0 mL/min ($N = 633$) provides adequate retention and separation potential for PF-00734200 from endogenous matrix components (*i.e.*, salts, phospholipids, etc.) at a reasonable back pressure of 600 psi.

The most efficient flow rate for the 3 μm particle size HILIC silica column was identified to be 0.2 mL/min. The higher optimum flow rate for the smaller particle size column was expected due to the dependence of the van Deemter equation on particle size [24]. The HETP values continued to increase slightly to 0.6 mL/min where they then significantly increased to a value approximately 4 times the optimum value. The peak efficiency of the 3 μm particle size column was only about 30% greater than the 5 μm particle size column under this condition. For further development of the bioanalytical assay, analysis throughput was weighed against the benefit of a modest increase in efficiency. The heightened back pressure that occurs during the wash phase (*i.e.*, 50% aqueous) for the 3 μm column would not allow for flow rates much above 600–650 μL/min. To increase throughput for the PF-00734200 method validation the 5 μm column at a flow rate of 1.0 mL/min was used.

3.4. Validation

A bioanalytical LC–MS/MS method for quantitation of PF-00734200 in monkey serum was successfully validated, meeting

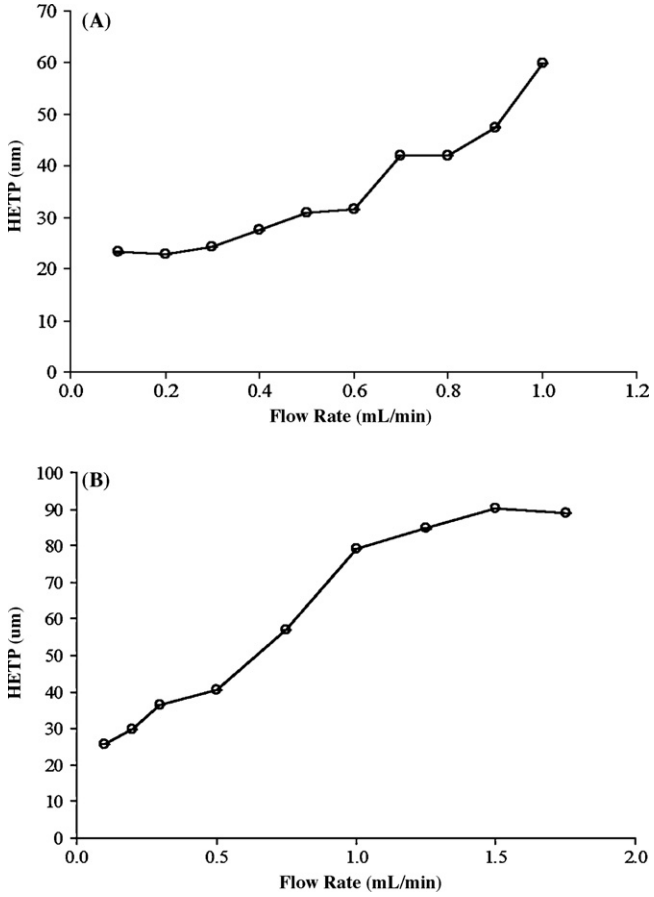

Fig. 6. van Deemter plots for 3 μm particle size column (A) and 5 μm particle size column (B). The flow rate was varied over the range of 0.1–1.00 mL/min and 0.1–1.75 mL/min for the 3 and 5 μm particle size HILIC silica columns, respectively. A net 10 mM ammonium formate concentration and an acetonitrile concentration of 90% were maintained for both columns in isocratic mode. Error bars represent ± one standard deviation ($n = 3$). The standard deviations did not exceed 0.020 μm and therefore the error bars cannot be seen on this plot due to the ordinate scale.

all guideline recommendations [17] and Pfizer SOP requirements. A quadratic least squared fit with a $1/x^2$ weighting factor was utilized for the calibration curve to accommodate covering a concentration range spanning three orders of magnitude. Correlation coefficient values >0.95 were observed for all of the validation batch runs. Acceptable carry-over, ≤20% area response relative to the LLOQ, was achieved throughout the course of the validation. Table 3 provides a tabulation of the inter-assay precision and accuracy from the three validation batch runs. Selectivity assessments utilizing two separate lot of monkey serum matrix showed no interferences with the analyte or IS. Bench top and long-term stability were established for 169 h (ambient) and 7 days (−20 °C), respectively. Three freeze/thaw cycles were achieved.

3.5. Injection carryover

The use of acetonitrile:ethylene glycol (9:1) in the final extract diluent alleviated an unacceptable level of analyte carry-over. Carryover was evaluated as the area response of the analyte in a double blank immediately following the injection of a high standard compared to the area response of the analyte in the lowest calibration standard (LLOQ). An acceptable carry-over level is ≤20% given these conditions [17]. We hypothesize that ethylene glycol may be capable of minimizing carryover by reducing the interactions between the analyte and surface of the glass injection syringe. The proposed mechanism by which this occurs may be a

Table 3
Inter-assay precision and accuracy from validation batch runs.

Validation QC levels	LLOQ 10.0 ng/mL	QCL 30.0 ng/mL	QCM 400 ng/mL	QCH 7500 ng/mL
Mean concentration found (ng/mL)	9.63	29.2	403	7510
Inter-run SD	0.577	1.78	10.4	262
Inter-run %CV	6.0	6.1	2.6	3.5
Inter-run %bias	−3.7	−2.7	0.8	0.1
n	18	18	18	18

combination syringe surface coating and/or co-solvent properties. Ethylene glycol, like other glycol compounds, could coat the surface of the silica based injection syringe. This may subsequently reduce interactions between silanols and our protonated amine. The polymer form of ethylene glycol (*e.g.*, PEG) has been applied to silicon/glass surfaces of microfluidic systems to reduce non-specific analyte interactions [18]. PEG is used in capillary electrophoresis to coat fused silica capillaries to minimize undesired analyte interactions with silica [19,20]. Additionally, ethylene glycol is commonly used as a co-solvent in many pharmaceutical formulations [21]. Interactions between ethylene glycol and our analyte of interest, PF-00734200, may reduce the net overall affinity between the protonated amine groups and silanols (protonated or free). This may be affected through ion-dipole interactions between the glycol alcohols and analyte amines and/or the net modification of the overall solution polarity, allowing for better overall solubilization of the analyte in its given state. Further investigations will need to be performed in order to fully understand how ethylene glycol minimizes carry-over.

4. Conclusion

Several HILIC parameters were investigated during the course of this work to produce a robust LC–MS/MS assay for the GLP bioanalysis of PF-00734200 in monkey serum. The evaluation of the effect of k' on the percent organic composition provides further evidence of a partitioning mechanism. The k' of PF-00734200 was reduced by increasing the polar organic modifier and buffer concentrations. HETP was directly influenced by increasing flow rate. A minimal improvement in HETP values was observed using the 3 μm over the 5 μm particle size columns at the same flow rates, while the 5 μm column afforded a considerably lower back pressure for higher throughput applications. HETP and T for PF-00734200 were reduced by increasing the buffer concentration up to 20 mM. This effect was believed to be a result of the disruption of the ion-exchange interactions between PF-00734200 and the ionized silanol groups. Utilizing the optimized HILIC conditions, a bioanalytical HILIC LC–MS/MS was successfully developed and validated for the GLP bioanalysis of PF-00734200 in monkey serum. Furthermore, injection carryover was effectively minimized by addition of ethylene glycol to the sample extract.

References

[1] P. Hemstrom, K. Irgum, J. Sep. Sci. 29 (2006) 1784.
[2] Y. Guo, S. Gaiki, J. Chromatogr. A 1074 (2005) 71.
[3] A.J. Alpert, J. Chromatogr. 499 (1990) 177.
[4] L.R. Synder, J.J. Kirkland, J.L. Glajch, Practical HPLC Method Development, 2nd Ed., John Wiley & Sons, Inc., New York, 1997, p. 280.
[5] E.P. Kadar, C.E. Wujcik, D.P. Wolford, O. Kavetskaia, J. Chromatogr. B 863 (2008) 1.
[6] W. Naidong, J. Chromatogr. B 796 (2003) 209.
[7] I. Paek, Y. Moon, H. Ji, H. Kim, H. Lee, Y. Lee, H. Lee, J. Chromatogr. B 809 (2004) 345.
[8] P. Uutela, R. Reinilä, P. Pipponen, R.A. Ketola, R. Kostianinen, Rapid Commun. Mass Spectrom. 19 (2005) 2950.
[9] R. Oertel, V. Neumeister, W. Kirch, J. Chromatogr. A 1058 (2004) 197.
[10] M.S.S. Curren, J.W. King, J. Chromatogr. A 954 (2002) 41.
[11] Y. Hsieh, R. Chen, Rapid Commun. Mass Spectrom. 19 (2005) 3031.
[12] Q. Song, W. Naidong, J. Chromatogr. B 830 (2006) 135.
[13] A.C. Li, H. Junga, W.S.Z. Shou, M.S. Bryant, X. Jiang, W. Naidong, Rapid Commun. Mass Spectrom. 18 (2004) 2343.
[14] S.D. Brown, C.A. White, M.G. Barlett, Rapid Commun. Mass Spectrom. 16 (19) (2002) 1871.
[15] ACD/pKa DB, ACD/Labs Release: 9.00, Product Version 9.04, Advanced Chemistry Development, Inc., Copyright © 1997–2005.
[16] ACD/LogD, ACD/Labs Release 9.00, Product Version 9.03, Advanced Chemistry Development, Inc., Copyright © 1997–2005.
[17] Guidance for Industry, Bioanalytical Method Validation, U.S. Food and Drug Administration, May 2001.
[18] K.C. Popat, T.A. Desai, Biosens. Bioelectron. 19 (2004) 1037.
[19] J. Horvath, V. Dolník, Electrophoresis 22 (2001) 644.
[20] I. Rodriguez, S.F.Y. Li, Anal. Chim. Acta 383 (1–2) (1999) 1.
[21] N. Seedher, S. Bhatia, AAPS PharmSci Tech. 2003 4 (2004) (issue 3) Article 33.
[22] J. Nawrocki, J. Chromatogr. A 779 (1997) 29.
[23] Center for Drug Evaluation and Research, U.S. Food and Drug Administration, Reviewer Guidance, Validation of Chromatographic Methods, FDA, Rockville, MD, November 1994.
[24] J.J. van Deemter, F.J. Zuiderweg, A. Klinkenberg, Chem. Eng. Sci. 5 (1956) 271.