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Short communication

Application of the dried spot sampling technique for rat cerebrospinal fluid sample collection and analysis

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ABSTRACT

Dried blood spotting (DBS) sample collection is gaining favor in the pharmaceutical industry due to benefits that include reduced animal usage and easier sample shipment and storage when compared to traditional plasma collection/analysis. The applicability of the DBS card to alternate, limited-volume, matrices has not been as fully characterized as their use with whole blood. In this paper we explored the application of the DBS sample collection technique to rat cerebrospinal fluid (CSF). A reverse phase HPLC–MS/MS method was developed and characterized for the quantitative bioanalysis of the α7 neutonal nicotinic acetylcholine receptor agonist PHA-00543613 in CSF using the dried spot sampling technique. The characterized assay and dried spot sampling technique was employed to analyze serially collected *in vivo* rat CSF samples after a single 4 mg/kg dose of PHA-00543613 in CSF-cannulated rats. The DBS strategy enabled the collection of more timepoints and produced comparable exposure results to those obtained by the collection and analysis of liquid CSF samples but notably with eight less animals.

1. Introduction

Most Americans have been introduced to Dried Blood Spot (DBS) sample collection within the first days of their birth. A simple heel prick followed by collection of bloodspots onto absorbent paper has been the standard sample collection route for the infant testing of phenylketonuria since its introduction by Guthrie and Sui [1]. Indeed, the United States Accounting Office reported that blood was sampled from 4 million infants in 2003 using this technique [2]. So while DBS collection and analysis is not a new concept, it has been primarily a tool for qualitative infant screening.

Recently however, there has been a great interest from the pharmaceutical industry that DBS is an option for PK/TK sample collection in lieu of the industry standard of larger blood draws followed by the harvesting of plasma. As the DBS strategy is currently being practiced, 15–50 μL of whole blood is spotted onto a card which is then air dried under ambient conditions. A 3–5 mm punch is taken from the sample spot, the analytes are removed by liquid extraction, and the extracts are analyzed by conventional bioanalytical techniques. The benefits of the approach for sampling plasma and urine have been discussed in detail [3–8]. Study costs in toxicological species can be significantly reduced because the small volumes sampled eliminates the need for satellite TK groups. Savings are potentially realized on animal, animal husbandry, sample drawing/processing and active pharmaceutical ingredient costs while the quality of the data is improved because exposure data is collected from the toxicological animal group. Similarly the opportunity for significant bioanalytical cost savings are expected in human clinical trials because samples would not have to be shipped on dry ice or stored in freezers. The potential benefits of DBS collection for regulated studies and clinical trials are readily apparent.

Although similar avenues of cost savings can be realized for non-regulated discovery studies, these studies typically involve much smaller animal and sample numbers so that the cost savings are offset by the increased assay development time and the current lack of automation to process DBS samples. In the discovery phase it is the technical aspects of the DBS strategy, the liquid handling of very small volumes of sample that may yield benefits greater than the cost savings. Especially when intrinsically low volume body fluids such as synovial and cerebral spinal fluid (CSF) are sampled and analyzed to establish that drug is being delivered to the target tissue [9].

The direct sampling of CSF from animal models as a surrogate route to measure central nervous system (CNS) availability is often preferred and more accurate than *in vitro* penetration models or microdialysis sampling of brain interstitial fluid [10]. While serial sampling is desirable to determine systematic uptake and elimination, the small volume of CSF in the common CNS model species of rat and mouse along with the low re-charge rate of CSF makes

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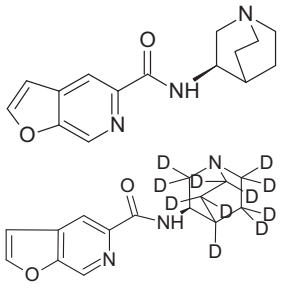

Fig. 1. Chemical structures of PHA-00543613 (top) and its d_{11}-deuterated internal standard PF-03079417 (bottom).

serial sampling very challenging and often impractical for early discovery programs. After collection, the bioanalytical assay is further complicated because small sample volumes are not amenable to automation and tend to be lost on the sides of the collection vials and during transfers. In this paper we use the DBS strategy for serial, low volume (15-μL) sample collection and handling of CSF on paper cards to establish the pharmacokinetic (PK) time-course of a pharmaceutical in rat. Data from the CSF dried spot collection is compared to data obtained via traditional liquid CSF collection as a demonstration of the advantages of expanding the DBS strategy to other biological matricies.

2. Experimental

2.1. Chemicals, reagents, and materials

Methanol, acetonitrile and water were of HPLC grade and were purchased from Sigma–Aldrich (St. Louis, MO, USA). Ammonium formate was reagent grade and purchased from Sigma–Aldrich (St. Louis, MO, USA). PHA-00543613, N[(3R-1-Azabicyclo[2.2.2]oct-3-yl]furo[2,3]pyridine-5-carboxamide), and its deuterated internal standard were synthesized by Pfizer Global Research & Development and their structures are presented in Fig. 1. FTA® Elute DMPK-A, DMPK-B, and DMPK-C blood spot cards, Harris punch, cutting mat and pre-packaged Whatman Desiccant 1000 were purchased from GE Healthcare (Piscataway, NJ, USA).

Artificial CSF was prepared internally by Pfizer Global Research & Development and consisted of a buffer of four salts in water: sodium chloride (147 mM), calcium chloride (1.3 mM), potassium chloride (2.7 mM), and magnesium chloride (1 mM). All buffer salts were reagent grade and purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Preparation of standard stock and working solutions

Primary stock solutions of the analyte PHA-00543613 (in duplicate) and internal standard PF-03079417 were prepared in acetonitrile/dimethyl sulfoxide (1:1, v/v, 1 mg/mL). Analyte working solutions of PHA-00543613 in acetonitrile/dimethyl sulfoxide (1:1, v/v) were prepared from the primary stocks at a concentration of 50,000 ng/mL. An internal standard working solution (20 ng/mL) was prepared from the primary stock using methanol.

2.3. Preparation of calibration standards and quality control (QC) samples on DBS cards

Calibration standards were prepared fresh on the day of analysis by diluting the standard working solution with blank artificial CSF to concentrations of 1.20, 2.40, 4.80, 9.70, 19.5, 39.0, 78.0, 156, 312, 625, and 1250 ng/mL.

QC samples for all analyses were prepared from a separate stock solution than that used for the calibration standards. The working stock solution was diluted in artificial CSF to achieve the concentrations of 1.22, 3.91 (low QC), 125, and 1000 (high QC) ng/mL. Separate low and high QC pools were prepared to evaluate the analyte storage stability.

Additional QC samples were prepared in pooled rat CSF ($n = 8$ animals) at all three QC concentrations and at 10,000 ng/mL (Dilution QC), to assess the ability to quantitate actual rat CSF against standards prepared in artificial CSF.

The standards and QC samples were prepared on DBS cards by aliquotting 15 μL of the appropriate calibration standard or QC sample onto FTA® Elute (DMPK-B) cards and allowing the card to dry at room temperature for at least 2 h prior to analysis. The dried QC samples were stored at room temperature with desiccant in a sealed plastic bag until analysis.

2.4. Sample preparation

Analyte was extracted from the dried CSF spots by punching a 3 mm diameter disk from the center of the dried sample and placing it into a clean polypropylene tube. 100-μL of working internal standard was added to the punch and the tube was vortexed for approximately 3 min. 80-μL of the supernatant was transferred to a 96 well injection block, diluted with an equal amount of water, and injected onto the HPLC–MS/MS system.

Dilution QC samples were prepared by combining 10-μL of extracted 10,000-ng/mL QC with 190-μL of extracted artificial CSF blank with internal standard to accomplish a 20-fold dilution.

2.5. HPLC–MS/MS analysis

The HPLC–MS/MS system consisted of an HTS PAL Leap autosampler (Carrboro, NC, USA) and Shimadzu LC-20 ADVP pumps (Columbia, MD, USA). Chromatographic separation was achieved with a Thermo Hypersil Gold AQ C18 Silica column (2.1 mm × 50 mm, 3 μm) and a gradient of 5 mM ammonium formate in 0.1% formic acid (A) and acetonitrile (B) at a flow rate of 0.45 mL/min. An initial mobile phase composition of 5% B was held for 0.3 min followed by a linear gradient over 1.9 min to a final mobile phase composition of 90% B which was then held for 0.5 min. Sample injections were 5 μL.

Detection of the analytes was achieved with an Applied Biosystems Sciex API4000 triple-quadrupole mass spectrometer (Foster City, CA, USA) using positive Turbo IonSpray™ electrospray ionization (ESI) followed by multiple reaction monitoring (MRM). The ionization source temperature was 600 °C with ion source gas 1 and 2 settings of 60 (nitrogen). The curtain gas and collision gas settings were 10 and 7, respectively (nitrogen). The MRM transition for PHA-00543613 was m/z 272.1 → m/z 110.2 and the MRM transition for the deuterated internal standard was m/z 283.1 → m/z 121.2. The HPLC–MS/MS system was controlled and data acquired and processed using Analyst software version 1.4.2 (Applied Biosystems/MDS Sciex, Canada).

2.6. Assay characterization

2.6.1. Specificity

Specificity was defined as no signal in blank matrix greater than or equal to 20% of the signal achieved at the lowest limit of quantitation (LLOQ) at the retention time of the analyte. Assay specificity for the analyte from endogenous matrix components was evaluated in actual blank rat CSF and two lots of artificial CSF. Similarly, specificity for the internal standard was established as no signal greater than or equal to 5% of the signal of the internal standard. Specificity

from cross-interference between the analyte and internal standard was also established.

2.6.2. Linearity of calibration

Calibration curves were generated by plotting the peak area ratios (analyte/internal standard) versus the theoretical concentration. The calibration curves were run in singlet and the linearity of the calibration curve was evaluated by linear regression analysis using a $1/(\text{concentration})^2$ weighting.

2.6.3. Accuracy and precision

The intra-day assay accuracy and precision were established through the performance of six replicates of the QC samples in artificial CSF at the four concentrations described. The inter-day assay accuracy and precision were established through the performance of three consecutive intra-day runs. Additionally intra-day assay performance was established for QC samples prepared in pooled rat CSF. The assay accuracies were evaluated by the deviation of the mean concentration measurement of the replicates versus the theoretical concentration value expressed as a percentage (%DEV). The assay precisions were evaluated from the relative standard deviation (RSD) of the concentration measurements and expressed as the percent coefficient of variation (%CV) from the mean concentration of the replicates. Intra- and inter-day accuracies and precisions at all QC concentrations of less than or equal to ±20% were deemed to be acceptable.

2.6.4. Lower limit of quantitation

The lowest limit of quantitation of the assay was established to be the lowest concentration from which the performance of six replicates of the QC pool met the intra- and inter-day accuracies and precisions requirements of ±20%.

2.6.5. Assessment of extraction efficiency

Analyte extraction efficiency from the DBS card was evaluated for several extraction procedures and conditions. Extraction efficiency (recovery) was assessed in triplicate from 15-μL spots of artificial CSF fluid at high (1000 ng/mL) and low (3.91 ng/mL) QC concentrations. Percent recovery was determined by cutting out and extracting the entire sample spot. Recovery standards were prepared by similarly spotting, cutting, and extracting blank artificial CSF from a card and fortifying the resulting extract to a concentration corresponding to quantitative recovery of the analyte.

2.6.6. Matrix effects

Despite the use of a stable-labeled internal standard in this assay which should compensate for ionization variability, matrix effects from the artificial CSF and DBS cards were evaluated using two approaches. First analyte peak areas obtained from the above recovery standards were compared to peak areas obtained from neat solutions to determine if matrix effects changed the apparent extraction recovery. Second, ion suppression was evaluated using the strategy reported by King et al. [11]. Stable response for the analyte was achieved by post-LC column infusion of 250 ng/mL of PHA-00543613 at a flow rate of 10 μL/min. Blank artificial and rat CSF sample extracts were injected onto the LC system and suppression or enhancement of the analyte signal was monitored over the chromatographic gradient (Fig. 2).

2.6.7. Storage stability assessment of PHA-00543613 on DBS card

The storage stability of PHA-00543613 on DBS cards was assessed at the high and low QC concentrations. The spotted DBS cards were stored in sealed plastic bags containing desiccant and stored at ambient temperature for up to 30 days. Storage stability samples were quantitated from freshly prepared standards and their stability assessed against the concentrations determined on their day of preparation (Day 0).

2.7. Application of the CSF spot technology to an in vivo study

The *in vivo* portion of the study was conducted in accordance with the animal care and *in vivo* procedures of the Pfizer Animal Care and Use Committee.

2.7.1. Dried CSF collection

Adult, male Sprague–Dawley rats (275–300 g) were fitted with intracisternal and jugular vein cannulas (Charles River Laboratories International, Inc., Wilmington, MA 01887). PHA-00543613 was dissolved in normal saline and subcutaneously dosed to rats at 1 mL/kg (4 mg/kg). Serial CSF samples were collected at 10 min, 20 min, 30 min, 1 h, 2 h, and 4 h post-dose by opening of the intracisternal cannula for $n = 2$ rats. A single CSF spot of approximately 15 μL was collected onto the DBS card at each timepoint. The sample was then dried under ambient conditions for approximately 2-h, placed in a sealed bag with desiccant and stored at room temperature until concentration analysis.

2.7.2. Liquid CSF collection

Adult, male Sprague–Dawley rats (275–300 g) were fitted with jugular vein cannulas (Charles River Laboratories International, Inc., Wilmington, MA 01887). PHA-00543613 was dissolved in normal saline and subcutaneously dosed to rats at 1 mL/kg (4 mg/kg). A CSF collection device was fashioned by breaking a 23G needle in half, and connecting it to a 1 mL syringe by a two-foot long section of PE50 tubing. Rats were euthanized by CO_2, blood was collected by cardiac puncture, and CSF was collected into the tubing from the cisterna magna and transferred to a polypropylene tube. CSF was terminally collected from $n = 2$ rats at each of the timepoints of 15 min, 30 min, 1, 2, and 4 h post dose for a total of 10 animals.

3. Results and discussions

3.1. Method development

3.1.1. Card selection

Currently a variety of a DBS cards are available commercially. For this study, three cards, DMPK-A, DMPK-B, and DMPK-C were evaluated. The DMPK-A and-B cards are chemically treated with the manufacturer's proprietary compounds while the DMPK-C card is untreated. All cards were spotted with artificial CSF and allowed to dry for 2 h, but upon drying the CSF spot could not be discerned from the unspotted background on the A and C cards. Therefore, for this practical reason, it was decided to develop the extraction procedure with the DMPK-B card, where the spot was visible after drying. Since the conclusion of these experiments other authors have reported the use of dye-indicating cards to alleviate this issue [9].

3.1.2. Extraction solvent and procedure

Methanol has been shown in the literature to be a viable extraction solvent for DBS [3] and for this evaluation was the initial extraction solvent choice. Vortexing the dried spot with 100-μL of methanol for 2 min produced the desired 1 ng/mL lower limit of detection with good precision but the measured recovery was only 40%. Increasing the vortexing time to 15 min did not change recovery (40%), however, extracting the spot with two aliquots of methanol and 5-min of vortexing improved the recovery slightly, up to 47%. Pre-soaks with water, pH 10.5 sodium bicarbonate or pH 11.5 sodium phosphate buffers followed by methanol extraction yielded no better recovery (29–35%) and extraction with acetonitrile also demonstrated no recovery improvement (31%). In order

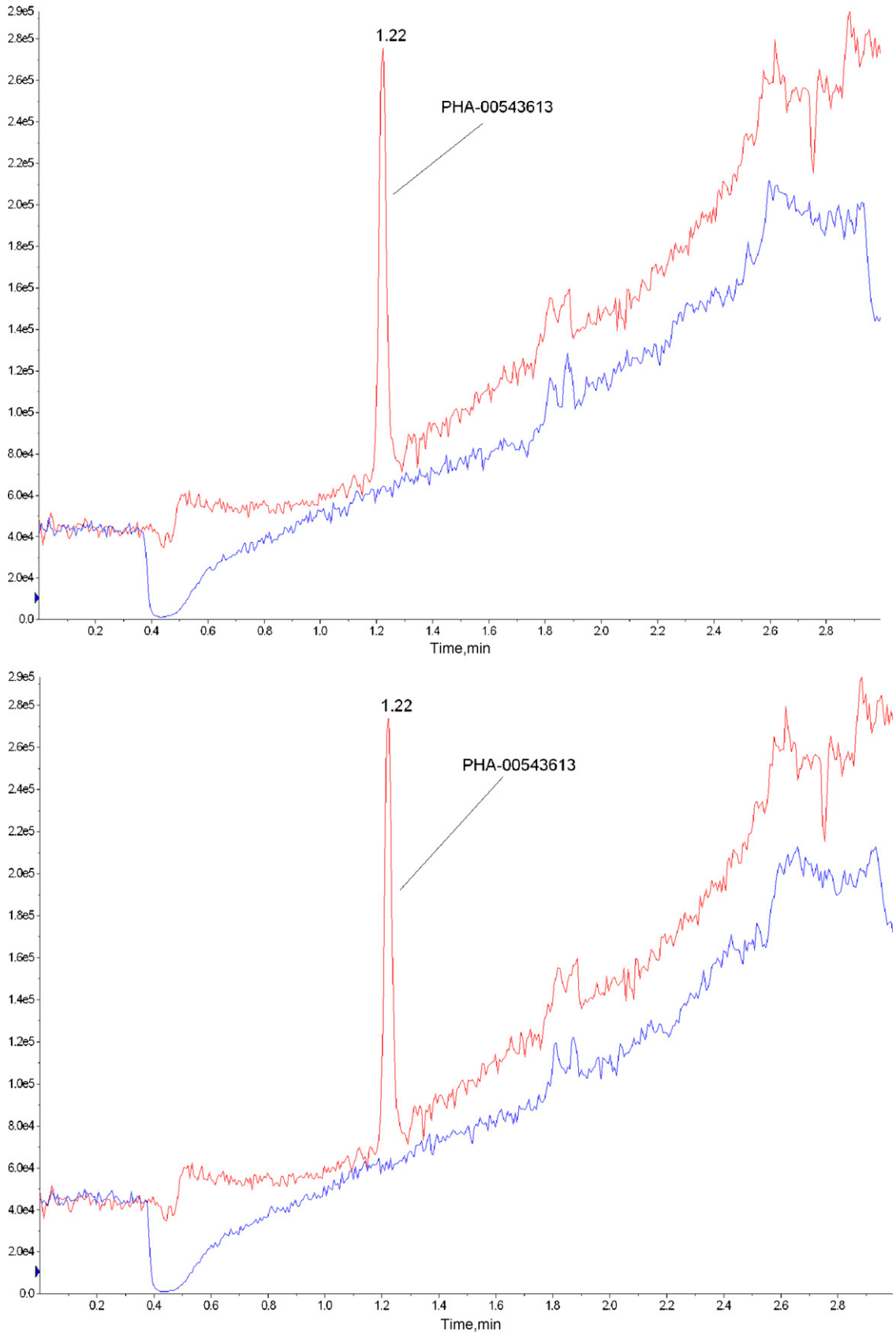

Fig. 2. Supressograms [11] generated by infusing PHA-00543613 and injecting extracted blanks from artificial csf (top), and rat csf (bottom). The chromatogram from the 25 ng/mL PHA-00543613 standard is overlayed for reference. Note: The rise in baseline is due to the effect of the chromatographic gradient on ionization.

Table 1
Intra- and inter-assay performance in artificial CSF.

QC concentration (ng/mL)	Intrarun Day 1 ($n = 6$)		Intrarun Day 2 ($n = 6$)		Intrarun Day 3 ($n = 6$)		Inter-run ($n = 18$)	
	Accuracy (%)	% CV	Accuracy (%)	% CV	Accuracy (%)	% CV	Accuracy (%)	% CV
LLOQC1.22	103.4	9.1	99.3	8.8	95.6	14.4	99.4	10.8
LQC3.91	111.3	8.0	105.2	10.5	96.0	12.8	104.1	11.6
MQC 125	97.9	10.8	94.3	6.5	94.6	5.4	95.6	7.7
HQC 1000	93.8	11.3	92.5	10.4	93.6	7.6	93.3	9.3

to assess that the observed low recovery was not due to ionization matrix effects, the response of the recovery standards were compared to neat solutions at the low and high QC concentrations as suggested by Li et al. [3]. Here the recovery was 94.6% and 90.9% for the low and high QC, respectively, indicating that the poor recovery is real and that PHA-00543613 may irreversibly bind to the DBS card. It is known that CSF is almost devoid of proteins [12] and that hydrophobic compounds in low protein matricies are known to irreversibly bind to surfaces [13,14]. Interestingly methanol extraction on the untreated DMPK-C card produced 60% recovery indicating that the card pretreatment likely impacts irreversible binding leading to the conjecture that there may be preferred card pre-treatments or compositions to alleviate non-specific binding of hydrophobic compounds. Despite the higher recovery from the DMPK-C card, the assay was developed using the lower recovery DMPK-B card for the reason of spot visibility cited earlier. Results of the extraction efficiency experiment can be found in Table 3. Although the extraction efficiency was <50%, there was sufficient analyte extracted off of the card to provide quality data to support subsequent studies.

3.1.3. Sample spot size

Experiments in whole blood have demonstrated that the volume of blood spotted onto the card does not impact the accuracy of the analysis as long as the spot does not wick beyond the sample area of the card and the extraction sample is uniformly punched near the center of the spot [4]. This behavior was verified for dried CSF spots over the range of 15–25 μL by comparison of triplicate analysis of the low and high artificial CSF QC samples. No relationship between the determined analyte concentration and the volume spotted was observed over this range. The percent CV's observed for all spots at the low and high QC concentrations were 6.1% and 2.1%, respectively. Results of the sample spot size experiment can be found in Table 4.

3.2. Sensitivity, linearity, and selectivity

The lowest limit of quantitation (LLOQ) for the assay was determined to be 1.22 ng/mL in artificial CSF and the assay was linear over the concentration range of 1.22 and 1250 ng/mL. The mean correlation coefficient (r^2) over the three batch characterization runs was $r^2 \geq 0.9956$.

The analytical method was shown to be specific and selective. No interfering peak co-eluting with the analyte was observed in artificial CSF (Fig. 3).

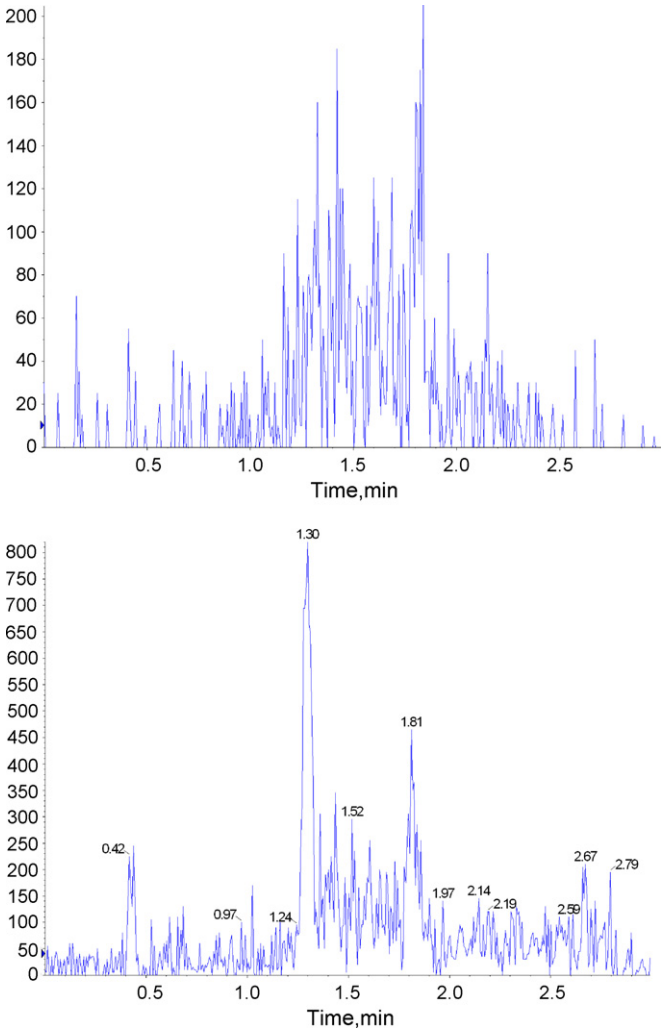

Fig. 3. Representative HPLC–MS/MS chromatograms of artificial csf matrix blank (top) and 1.22 ng/mL PHA-00543613 in artificial csf (bottom).

3.3. Accuracy and precision

Evaluation of the QC pools over three analytical runs demonstrate that the assay produces acceptable intra- and inter-day accuracy (%RE) and precision (%CV) which in fact would meet the FDA guidance for bioanalytical method validation [14] (Table 1).

Table 2
Intra-assay performance in rat CSF.

QC concentration (ng/mL)	Intrarun Day 1 ($n = 6$)	
	Accuracy (%)	%CV
LQC3.91	96.5	9.6
MQC 125	94.5	10.1
HQC 1000	102.5	10.3
Dilution QC 10,000	108.3	10.5

Table 3
Extraction recovery and matrix effect for PHA-00543613 in artificial CSF spots.

Nominal concentration (ng/mL)	Recovery CSF spot vs. extracted blank standards (%) ($n = 6$)	Recovery extracted blank standards vs. spiked liquid standards (%) ($n = 6$)
3.91	40.2	94.6
1000	39.5	90.9

Table 4
Sample spot size comparison.

Nominal concentration (ng/mL)	Mean response	Ave	%cv
3.91			
15 μLspot	0.079	0.085	6.1
20 μLspot	0.086		
25 μLspot	0.089		
1000			
15 μLspot	18.3	18.5	2.1
20 μLspot	18.3		
25 μLspot	19.0		

The performance of the QC's prepared in pooled rat csf, establish that analyte in rat csf can accurately and precisely be quantitated from standards prepared in artificial csf. The observed accuracies of the rat csf pools ranged from 94.5 to 102.5% and their precision ranged from 9.6 to 10.3% (Table 2).

The ability to accurately dilute and quantitate rat csf with artificial csf was established by the performance of the dilution QC samples where the observed accuracy and precision was 108.3% and 10.5%, respectively (Table 2).

3.4. Matrix effects

No suppression or enhancement of signal was observed at the retention time of the analyte from rat or artificial CSF extracts (Fig. 2).

3.5. Storage stability of PHA-00543613 on DBS card

The storage stability of PHA-00543613 in dried artificial CSF spots on the FTA® Elute (DMPK-B) card at days 15 and 30 were assessed against the determined day 0 values using freshly prepared standard curves. Results are presented in Table 5 and indicate that PHA-00543613 in dried artificial CSF is stable for at least 30 days when stored at room temperature with desiccant.

3.6. Application of the CSF spot technology to in vivo rat studies

PHA-00543613, is an agonist of the α7 neutonal nicotinic acetylcholine receptor (α7nA ChR) and has been investigated as a potential treatment of cognitive deficits in schizophrenia [15]. The chemical series from which it is derived is characterized by rapid brain penetration so that CSF concentration profiles of the series analogs in rodent models would provide a direct avenue for the optimization and understanding of pharmacokinetics and dynamics. Rat CSF was collected and a drug concentration time course of PHA-0053613 in CSF was conducted during the development of the compound under a routine study protocol. In this protocol, 10 rats received a single dose of PHA-0053613 and a single collection of liquid CSF was collected from each rat at staggered timepoints and analyzed. The determined concentrations were compiled to give an *average* time-course plot and exposure with an $n = 2$ for each timepoint [16]. A new protocol was generated to repeat this study as a proof of concept of the DBS strategy for CSF collection. In the new protocol, two CSF-cannulated rats received a single dose of PHA-0053613 and serial collections of approximately 15-μL of CSF was collected on the DBS card, dried, and analyzed using the above assay. Here the determined concentrations give the *individual* time-course plot and exposure for $n = 2$ rats (Fig. 4). Not only did the dried spot protocol enable the collection of additional timepoints, it did so with the reduction of eight rats and 80% less drug needed to obtain the data. Measured mean exposures as determined by the area under the curve (AUC) for the dried spot and liquid collections were 596 ng × mL/h and 760 ng × mL/h, respectively. Comparison of the two exposures shows a relative percent difference of 24%. It is assumed that some of the percent difference can be attributed to inter-animal variability and to the fact that the studies were conducted approximately three years apart.

Table 5
Stability of PHA-00543613 in artificial CSF spots.

Nominal concentration (ng/mL)	Mean	S.D.	%cv	%Bias
3.91				
Day O	3.99	0.31		
Day 15	4.41	0.53	12.03	10.52
Day 30	3.36	0.36	10.62	15.95[a]
1000				
DayO	963	180		
Day 15	901	120	13.3	6.51
Day 30	947	133	14.1	1.7

[a] Value falls outside of typical GLP criteria for acceptance.

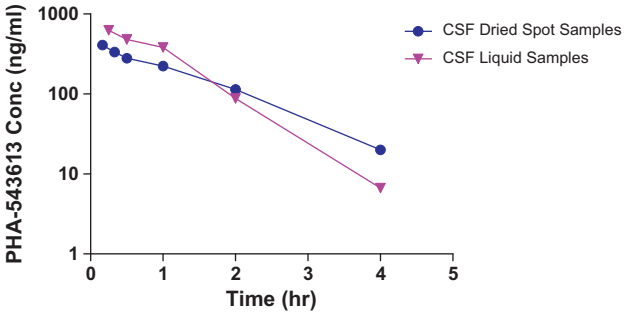

Fig. 4. PHA-00543613 in rat CSF time course determined from *in vivo* studies collecting dried CSF spot and liquid CSF samples.

4. Conclusion

Dried spot sample collection provides an avenue for small sample volume collection and manipulation. These traits are particularly valuable when a pharmaceutical series is in the discovery phase and the analysis of intrinsically low volume fluids such as rat cerebral spinal fluid (CSF) are needed to optimize a chemical series. Here the dried spot technology has been successfully used for the collection and analysis of rat CSF fluid for PHA-00543613; a α7 neutonal nicotinic acetylcholine receptor agonist. The developed assay demonstrated good intra- and inter-day accuracy, precision, and selectivity and the analyte proved stable on the DBS sampling card for at least 30 days. Despite low recovery from the card a lowest limit of detection of 1.22 ng/mL was achieved. Serial dried spot sample collection and analysis of rat CSF following a single dose of PHA-0053613 generated more timepoints and comparable measured PK exposures to those obtained from traditional staggered CSF liquid collection and analysis of samples. In addition to producing individual PK the dried spot sample protocol required eight less rats to produce equivalent data making the ethical reduction in animal usage another motivation to explore and employ dried sample collection technologies for limited volume matrices.

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