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Novel and rapid LC–MS/MS method for quantitative analysis of methylphenidate in dried blood spots

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Aim: Development and validation of a novel, sensitive, specific and rapid dried blood spots (DBS)-LC–MS/MS method for methylphenidate (MPH), an attention-deficit hyperactivity disorder drug. **Methodology & results:** Protein precipitation with acetonitrile was used to extract MPH from the DBS cards. Chromatographic separation was achieved on a Zorbax C18 column using an isocratic mobile phase composed of acetonitrile and 5 mM ammonium formate buffer (20:80, v/v) at a flow rate of 0.5 ml/min. MPH was quantified over a linear range of 200–25,000 pg/ml. **Conclusion:** The clinical DBS-LC–MS/MS method was successfully validated as per the US FDA's Bioanalytical Method Validation Guidance to support an ongoing pediatric pharmacokinetic study.

Graphical abstract:

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Figure 1. Chemicals structures of methylphenidate and methylphenidate-d₁₀ (internal standard).

Attention-deficit hyperactivity disorder (ADHD) is a neurobehavioral disorder that can be defined as ‘a persistent pattern of inattention and/or hyperactivity–impulsivity disorder that interferes with normal functioning or development’ [1,2]. ADHD begins in childhood, with an overall prevalence of 1.4–3.0%. Although largely diagnosed during childhood and adolescence, 30–50% of people diagnosed in childhood continue to have persistent symptoms into adulthood with 2–5% of adults having the condition [2,3,4,5]. The WHO estimated that ADHD affected about 39 million people as of 2013. Stimulants remain the mainstay of treatment for ADHD across the life cycle because of their well-documented safety and efficacy [2,3,4,5]. Methylphenidate (MPH; Figure 1), a CNS stimulant, was first approved by the US FDA in 1955 and medical use began in 1960. Over the last decade, MPH prescriptions increased tremendously due to increased acceptance of ADHD as a life-threatening disorder worldwide [6,7]. MPH in immediate release formulations exhibits a pharmacokinetic (PK) time of maximum concentration ($T_{\max}$) of about 2 h, a half-life ($t_{1/2}$) of 2–3 h and a pharmacodynamic (PD) duration of action of up to 4 h. In the USA, MPH is available in various brand name formulations such as Ritalin, Concerta, Aptensio XR, Metadate CD, Daytrana, etc. and a variety of generic formulations are also approved by the FDA. MPH is available in both immediate and extended release for use as oral solutions, tablets, capsules or suspension.

Although PK profiling of MPH has been characterized in healthy adult volunteers [8,9,10] and pediatric ADHD patients [11], PK/PD data generated from pediatric classroom clinical trial can provide additional information on the efficacy and safety of extended release MPH to aid the pediatric ADHD labeling. To support the investigation of the MPH exposure–response relationship in the pediatric patient population, it was very important to accurately measure the concentration of MPH in clinical samples by means of a rugged, sensitive and reliable bioanalytical method. Previous studies were developed for quantifying MPH in human plasma, urine and hair by different analytical techniques such as ultraviolet-based detection [12,13,14,15,16], CE–MS [17], GC–MS [7,18,19] and LC–MS/MS [20,21].

Although MPH has a partition ratio of approximately 1 in plasma and red blood cells [22], until now, no studies have been reported for the quantitative determination of MPH in dried blood spots (DBS). DBS technology was first introduced more than 50 years ago for screening of inborn errors of metabolism in neonates. However, it was seldom accepted as the matrix of choice for routine PK studies. DBS has several pragmatic advantages over traditional plasma/serum analyses in pediatric ADHD classroom studies due to the pediatric subject population, such as it is a minimally invasive (peripheral blood draw) procedure, quick and cheaper sample processing (can be automated) [23], quantitative analysis possible with 5–10 μ l blood (allowing multiple spots), long-term storage possible at ambient conditions (saves freezer space) [24], off-site sample collection and shipping can be done with ease even with limited resource settings, and compatible with MS, HPLC and immunoassays [25,26,27]. DBS can be used for quantitative analysis of small molecules, large molecules, elements as well as endogenous components (viral load, lipids, DNA, peptides) [28,29,30,31]. Although challenges in using DBS technology include – hematocrit effect (common with partial punch due to different blood viscosity) and protein alteration during drying of DBS cards that could affect sensitivity, these challenges can be overcome by using a full punch and utilizing adequately controlled storage conditions [32,33]. In addition, PK sample collection in pediatric ADHD classroom studies by means of intravenous blood draws would require more blood to be taken and thus reduce the number of samples that can be safely taken, and can interfere with the behavior of the students in the classroom, affecting clinical outcome measurements. On the other hand, DBS requires less volume so more samples can be taken and it is less

invasive. Thus, DBS can be a valuable technique to collect concurrent PK data in these types of studies. In the present study, for the first time, the authors report development and validation of a LC–MS/MS assay for MPH in DBS specimens based on the FDA *Guidance for Bioanalytical Method Validation* ([34,35]). The method validation criteria (in addition to hematocrit effect) from this guidance for small-molecule quantitation in non-DBS-related matrices using LC–MS/MS assay were still applicable to this study and were evaluated for quantifying MPH in DBS using 10- μ l whole blood. The validated method presented in this study will be used to quantify MPH in children 6–13-year old dosed with an optimized dose of two different extended release formulations of MPH along with placebo control arm over a period of 4 weeks.

Although DBS was used for measuring MPH using Orbitrap mass spectrometer in single-ion monitoring mode [36]. This detection, as stated by the authors, provided only qualitative measurement for MPH measurement and the method reported an LOD value with no LOQ. Thus, considering the rarity of validated bioanalytical assays using DBS in clinical settings, we thoroughly investigated probable variables in DBS validation assay [37,38,39] and that this assay can be successfully applied for PK, PK/PD or bioequivalence studies of MPH in both adults or adolescent patient populations.

Experimental section

Chemicals & reagents

MPH-hydrochloride (99.9%) was obtained from United States Pharmacopoeia (MD, USA) and the internal standard (IS) MPH-d10 (98.4%) was purchased from Toronto Research Chemicals (Toronto, ON, Canada). LC–MS grade methanol, acetonitrile and water were purchased from Merck Pharmaceuticals (NJ, USA). GR grade ammonium formate and formic acid were procured from Sigma-Aldrich (MO, USA). The DBS cards (PDMS 4) were purchased from Tomtec (CT, USA). Blank adult male human blood (stored at 4°C and used within 1 week of collection) for calibrators and quality controls (QCs) was procured from Bioreclamation IVT (MD, USA).

Preparation of stock solutions, calibration standards & QCs on DBS cards

Stock solutions of MPH and MPH-d10 (IS) were prepared in methanol at a conc. of 1 mg/ml. Two separate working stock solutions of MPH were prepared and used for the preparation of calibration curve (CC) standards and QCs. Working stock solutions of MPH (100-times higher than the final conc.) were prepared in 60% methanol. Similarly, working stock solution of MPH-d10 (30 μ g/ml) was prepared in acetonitrile. Stock solutions of MPH and MPH-d10 were stored at 2–8°C.

Calibrators were prepared by spiking 990 μ l of blank K₂-EDTA human whole blood with 10- μ l working stock solution of MPH, to obtain MPH concentration levels of 200, 400, 1250, 3750, 6250, 10,000, 17,500, 24,250 and 25,000 pg/ml at each concentration. Five levels of QC, from a separate weighing of MPH, were prepared at concentrations of 200 (LLOQ), 600 (LQC), 2000 (MQC-1), 12,500 (MQC-2) and 18,750 (HQC) pg/ml. After spiking and gentle mixing, 10- μ l calibrators and QCs were spotted with a pipette tip and capillary tubes as well. The precision and accuracy were compared and were similar between the two. The DBS cards are allowed to dry at ambient conditions. After 4 h of drying, the DBS cards were stored in Ziploc® bags with a silica gel desiccant pouch at room temperature until analysis.

Sample preparation

MPH was extracted by the protein precipitation method from the DBS cards. A complete 10- μ l DBS was punched into an Eppendorf tube and 50 μ l of water was added, vortexed and allowed to sit at room temperature for 20 min. Later, 150 μ l of acetonitrile containing 30 ng/ml of MPH-d10 was added and further incubated at room temperature for 10 min. The samples were centrifuged at 13,000 *g* for 5 min. Seventy-five microliter of supernatant was added to 200 μ l of 0.1% formic acid in water in a 96-well plate.

LC–MS/MS instrumentation & chromatography conditions

A 1290 Agilent Infinity (Agilent Technologies, CA, USA) UHPLC system equipped with an autosampler was used for LC. Samples were chromatographed on a Zorbax C₁₈ column (2.1 $\times$ 50 mm, 3.5 μ m, Agilent Technologies) with an injection volume of 15 μ l. The column was maintained at 25 $\pm$ 5°C. The optimized mobile phase composed of acetonitrile and 5 mM ammonium formate buffer (20:80, v/v) with 0.1% formic acid set at a flow rate of 0.5 ml/min. MPH was quantified using a Thermo TSQ Quantum Ultra AM triple-quadrupole mass spectrometer (Thermo Fisher Scientific, CA, USA) equipped with Ion Max API source with HESI probe interface

set at 350°C. The mass spectrometer was operated in positive-ion mode for 2.0 min. The ion spray voltage was set at 4000 V. The source parameters such as vaporizer temperature, sheath gas and auxiliary gas pressure were set at 350°C, 30 and 10 psi, respectively. Capillary temperature and tube lens offset were set as 270°C and 67, respectively. The collision energy for MPH and MPH-d10 were optimized as 20 and 22 V, respectively. Detection of the ions was carried out in multiple-reaction monitoring mode, by monitoring the transition pairs of m/z 234.0 as the precursor ion and m/z 84.0 as product ion for MPH. For MPH-d10, m/z 244.0 was selected as the precursor ion and m/z 93.0 as the product ion. Analytical data obtained were processed using the Xcalibur™ quantitation software.

Method validation

Validation parameters like selectivity, sensitivity, specificity, precision and accuracy, recovery, matrix effect, linearity, dilution integrity, stability and injection carryover parameters were successfully validated in this assay as per the FDA *Guidance for Bioanalytical Method Validation* from 2001. Specificity of the method was designed to investigate interference from six blank adult human blood matrices. The response of background noise at the retention time of MPH was acceptable if it is less than 20% of the response of LLOQ and less than 5% of response of working concentration of MPH-d10. Selectivity was assessed by comparing the peak area responses between MPH-spiked DBS (at LLOQ conc.) and extracted with blank acetonitrile or blank DBS extracted with MPH-d10-spiked acetonitrile. Sensitivity was determined by analyzing six replicates of human blood spiked with corresponding LLOQ and spotted onto the DBS cards. The six replicates should have a precision of $\leq 20\%$ and an accuracy of $\pm 20\%$. CC was constructed for MPH in the range of 200–25,000 pg/ml. The linearity of the plot was evaluated based on the slope, intercept and correlation coefficient values derived from least-square regression analysis. Matrix effect was investigated to check that precision, selectivity and sensitivity are not compromised by the biological matrix. Matrix factor was assessed with pooled lots of K₂-EDTA human blood. For evaluation of intraday precision and accuracy, six replicate QCs were analyzed with six different concentration levels on the same day. For the evaluation of interday precision and accuracy, six replicates QCs were analyzed from three different runs on three different days. The acceptance criteria for intraday and interday accuracy were set at $\pm 15\%$ deviation (SD) from the nominal value, except LLOQ, where it must be $\pm 20\%$. For precision, the %CV should not be greater than less than 15% for all the QCs, except for the LLOQ, where it should be $\leq 20\%$.

The recovery was evaluated by comparing the peak areas of MPH in spiked QCs (six each of LQC, MQC-1, MQC-2 and HQC) with those of MPH in nonextracted samples (matrix-free). The recovery of MPH-d10 was determined in a similar way at a working concentration of 30 ng/ml. Dilution integrity was performed with the aim of validating the dilution test to be carried out on over the linear curve MPH concentrations during the analysis of clinical study samples. Dilution integrity test was carried out at 1.5-times the ULOQ concentration, which was then processed and diluted fivefolds with the extracted zero standard samples (containing only MPH-d10) and quantitated under the CC. The role of hematocrit was also evaluated as a validation criterion. Human whole blood with different hematocrit values (30, 40, 50 and 66% v/v) was prepared by centrifuging whole blood to separate the cell components from the liquid plasma component in graduated conical tubes. Different volumes of human plasma were added to the cell components to get 30, 40, 50 and 66% hematocrit samples where 30 represents 30% cell components and 70% liquid plasma and likewise for the other hematocrit values. Effect of hematocrit was assessed by quantifying MPH levels in LQC and HQC in six replicates each.

Stability studies were conducted to evaluate the stability of MPH under different storage conditions. Postextraction refrigerator stability (120 h), autosampler stability (24 h) and long-term stability ($\sim 24^\circ\text{C}$ up to 7 months) experiments were performed at LQC and HQC. The determined concentrations were compared with the nominal values. Samples were stable if the deviation was within $\pm 15\%$ from nominal values. Injection carryover tests were conducted to assess carryover (if any) from the ULOQ sample. The acceptance criteria were peak area response in the blank injected immediately after ULOQ should be less than 20% of the LLOQ response.

Cross-validation with 5- μl DBS

As the subjects in the proposed clinical study will be children aged 6–13, sample volume could be a constraint due to the amount of blood that can be drawn from the finger prick prior to clotting. Owing to the inherent challenges of collecting multiple 10- μl blood spots from each subject, an alternative method was adopted to collect 5- μl blood and spot onto the DBS cards. The extraction volumes were reduced to 25- μl water and 75- μl IS-spiked ACN (30 ng/ml MPH-d10) to maintain same LLOQ. The extracts (37.5 μl) were reconstituted with 100 μl of 0.1%

formic acid in water. This reduction in extraction volumes allowed to maintain same LLOQ (200 pg/ml) as the 10- μ l DBS. Since the spot volume was changed from the initial method validation protocol (using 10- μ l DBS), a partial validation was performed to assess sensitivity, precision and accuracy, extraction recovery, matrix effect and injection carryover.

Results & discussion

MS optimization

An LC–MS/MS method using DBS was developed with the purpose to ensure that it would be a sensitive and selective quantification suitable for PK studies with MPH. An analytical method should be efficient to quantify the drug candidates in presence of endogenous components effectively. Hence, the authors chose an LC–MS/MS technique due to its inherent selectivity and sensitivity.

The LC–MS/MS parameters of MPH and MPH-d10 were optimized in positive ionization mode. A 200-ng/ml tuning solution of MPH and MPH-d10 was infused to optimize the MS conditions. Precursor ion signals were increased by suitably altering the ion spray voltage (4000 V) in Q1 MS spectra. The most intense and consistent product ion in Q3 MS spectra was obtained by optimizing the collision energy. Finally, various source parameters like vaporizer temperature (300°C), sheath gas (10 psi), auxiliary gas pressure (10 psi), capillary temperature (350°C) and tube lens offset (67) were optimized to obtain adequate and reproducible response. The positive ion spray mass spectrum revealed a protonated molecular ion by monitoring the precursor ion at m/z 234 and product ion at m/z 84 for MPH, and m/z 244 as the precursor ion and m/z 93 as the product ion for MPH-d10.

Liquid chromatography & selectivity

Chromatography conditions such as buffer strength, analytical column, mobile composition and its flow rate were suitably altered to achieve better separation of MPH from endogenous components and for rapid elution. Organic modifiers such as acetonitrile and methanol in combination with acidic modifiers such as formic acid and acetic acid and basic modifiers like ammonia were critically evaluated for their suitability to achieve better chromatographic resolution. Also, volatile buffers like ammonium acetate and ammonium formate were evaluated. Use of 5-mM ammonium formate in mobile phase increases the sensitivity and helped with improved reproducibility. Finally, acetonitrile:5-mM ammonium formate with 0.1% formic acid in water (20:80 v/v) combination was selected as the mobile phase to elute MPH and MPH-d10 in isocratic condition still achieving a low retention time (0.97 min). Injection of 15- μ l sample on a Zorbax C18 column (2.1 $\times$ 50 mm, 3.5 μ) with a flow rate of 0.5 ml/min at controlled room temperature eluted MPH and MPH-d10 at 0.97 min in a total run time of 2.0 min. Representative chromatograms of MPH and MPH-d10 in blank, LLOQ (200 pg/ml) and ULOQ (25,000 pg/ml), DBS are shown in Figure 2. The authors would also like to mention that the proportion aqueous to organic solvent in the final sample and mobile-phase solutions on column were observed to be very crucial and any alteration in those proportions could lead to distortion of peak shapes and change in retention times.

In addition, we do not anticipate any ADHD comedications (other than MPH) likely to be administered to have the same exact molecular weight as MPH. However, lidocaine, a widely used numbing agent, which has a molecular weight of 234.31, and only one mass unit higher than MPH (233.31) will be excluded from the study during blood collection to avoid any interference in the LC–MS assay. We also confirmed and ruled out any interference from commonly used anticoagulants to allow accurate detection of MPH (data not shown).

Sample preparation

Proper and precise sample collection procedures are very crucial for accurate and reproducible quantitation of analyte in DBS specimens. Considering the low sample volumes of 5 or 10 μ l blood, the clinical and analytical staff were required to have initial training in using the microcapillary tubes coated with K₂-EDTA. Upon sample collection on to the DBS membranes, at least 4 h to maximum of overnight drying was needed to allow uniform drying of the DBS cards before preparing them for shipping to the extraction site. After successful drying, DBS specimens were individually sealed in a Ziploc bag with a desiccant pouch to prevent effects of humidity changes on analyte concentration and were stored at room temperature until analysis.

Selection of a proper extraction method is very important to get good and reproducible recovery with negligible or no matrix effect. In the present study, we adopted a simple protein precipitation procedure to extract MPH from DBS cards. Organic solvents such as methanol, acetonitrile and acetone were tested to precipitate the proteins. The reason for selecting acetonitrile as an extraction solvent was its compatibility with the mobile phase of the

Figure 2. Chromatograms of methylphenidate in validation samples. Representative chromatograms of methylphenidate in (A) blank sample, (B) LLOQ sample, (C) ULOQ sample and (D) methylphenidate-d10 (IS) sample. IS: Internal standard.

proposed method and its higher polarity, which allows it to permeate easily through the DBS cards and enhances the recovery. To improve the recoveries further, DBS were prewetted with water followed by acetonitrile extraction. Moreover, the precision and accuracy results obtained during entire course of validation results support this extraction methodology.

For bioanalytical methods, use of stable-labeled isotope standards of analytes are recommended as ISs to increase method precision and limit the variability in ionization, chromatography and extraction, and help in tracing potential matrix-related challenges. Hence, in the present study, MPH-d10 was employed as an IS and to normalize against any ion enhancement or suppression resulting from the matrix.

Linearity

Linearity of the method was evaluated at nine nonzero concentrations of MPH within the range of 200–25,000 pg/ml. After comparing the two weighting models ($1/x$ and $1/x^2$), a regression equation with a weighting factor of $1/x^2$ of MPH to MPH-d10 concentration was found to produce the best fit for the concentration–peak area response relationship. The mean correlation coefficient of the weighed CCs generated during the validation was more than 0.99 from three independent runs.

Sensitivity

The LLOQ for MPH was 200 pg/ml in DBS, at which the mean values were within $\pm 20\%$ of the spiked values and the intra- and interday coefficients of variation were less than 15 and 11% with an accuracy of 104.6 and 109.8%, respectively.

Precision & accuracy

The results for the intra- and interday precision and accuracy for five QC levels in DBS are illustrated in Table 1. The intra- and interday results were found to be within acceptable limits as per the FDA Guidance on Bioanalytical

Table 1. Intra- and interbatch precision and accuracy for methylphenidate with 10- μ l dried blood spots.

Quality control conc. (pg/ml)	LLOQ (200.0)	LQC (600.0)	MQC-1 (2000.0)	MQC-2 (12,500.0)	HQC (18,750.0)
Intraassay precision and accuracy (n = 6 at each QC level)					
Mean	209.2	519.8	2036.1	12,470.4	18,477.4
SD	29.4	14.9	83.9	522.0	996.5
% CV	14	3	4	4	5
% Accuracy	105	87	102	100	98
Interassay precision and accuracy (n = 18 at each QC level)					
Mean	219.7	563.9	1986.6	11,972.5	18,369.1
SD	23.5	68.3	122.8	597.0	1067.8
% CV	11	12	6	5	6
% Accuracy	110	94	99	96	98

HQC: High quality control; LQC: Low quality control; MQC: Medium quality control; QC: Quality control.

Table 2. Extraction recovery and matrix effect of methylphenidate in 10- μ l dried blood spots, n = 6 at each quality control level.

MPH conc. (pg/ml)	LQC (600.0)	MQC-1 (2000.0)	MQC-2 (12,500.0)	HQC (18,750.0)
Percent extraction recovery	98	98	95	95
MPH-d10 percent extraction recovery	104			
Matrix factor (only MPH)	1.2	1.1	1.0	1.0
Matrix factor (IS normalized)	1.2	0.9	1.0	1.0

HQC: High quality control; IS: Internal standard; LQC: Low quality control; MPH: Methylphenidate; MQC: Medium quality control; QC: Quality control.

Method Validation. There was no constant direction to the bias (i.e., + or –) for QC samples and the mean values were within $\pm 15\%$ of the spiked values. Imprecision was acceptable, as indicated by both intra- and interday coefficients of variation of less than 15% at all concentrations of MPH. Similarly, the intra- and interday accuracies were within $\pm 15\%$ of the spiked values.

Extraction recovery & matrix effect

The overall absolute mean recoveries of MPH from DBS determined at LQC, MQC-1, MQC-2 and HQC concentration levels (n = 6) were more than 94% and consistent with %CV of 1.7% across the four QCs as shown in Table 2. The absolute recovery for MPH-d10 was approximately 104%. A simple protein precipitation technique proved to be reproducible and provided the cleanest samples.

In this study, there were no measurable matrix effects observed in six different lots of human blood for MPH tested at the LQC and HQC concentration levels. A value of 100% indicates that the responses in the mobile phase and in spiked postdilution DBS were the same and no absolute matrix effect is observed. A value of more than 100% indicates an ion enhancement and a value of less than 100% indicates an ion suppression. The precision and accuracy for MPH at LQC concentration were found to be 9.5 and 99.6% and at HQC level, they were 5.0 and 100.6%, respectively.

Matrix effect was also assessed by calculating the matrix factor for four QC levels in one lot of matrix. Matrix factor is calculated as the peak area ratio of analyte with or without IS in extracted DBS samples (postspiked) to neat samples (mobile phase). A value between 0.8 and 1.2 for matrix factor denotes no significant matrix effect. Similarly, IS-normalized matrix factor was calculated by dividing the analyte matrix factor by the IS matrix factor. As shown in Table 2, matrix effect with or without the IS (MPH-d10) was within the range of 0.8–1.2. The results indicate that the sample preparation method was specific to rule out any endogenous interference in MPH quantification.

Dilution integrity

Dilution integrity test was performed to extend the upper concentration limit of MPH by five-times dilution with postextracted blank sample. After spiking blank DBS cards with 43,310 pg/ml and then diluting those samples

Table 3. Effect of hematocrit on precision and accuracy of the assay in 10- μ l dried blood spots.

Hematocrit (% v/v)	Spiked QC conc. (pg/ml)	Mean $\pm$ SD (pg/ml)	%CV (precision)	%Nominal (accuracy)
30	600.0	561.9 $\pm$ 41.4	7	94
	18,750.0	18,607.6 $\pm$ 504.9	3	99
40	600.0	620.1 $\pm$ 52.9	8	103
	18,750.0	19060.2 $\pm$ 435.0	2	102
50	600.0	568.6 $\pm$ 46.7	8	95
	18,750.0	17962.0 $\pm$ 42.0	0.1	91
66	600.0	637.9 $\pm$ 27.3	4	106
	18,750.0	19154.3 $\pm$ 581.4	3	102

n = 6 at each QC level.
QC: Quality control.

Table 4. Stability data for methylphenidate extracted in 10- μ l dried blood spots.

Stability test	Spiked QC conc. (pg/ml)	Mean $\pm$ SD (pg/ml)	%CV (precision)	%Stability (accuracy)
Postprocessing [†]	600.0	530.1 $\pm$ 28.4	5	88
	18,750.0	17,564.8 $\pm$ 510.3	3	94
Autosampler [‡]	600.0	560.5 $\pm$ 21.7	4	93
	18,750.0	19,030.0 $\pm$ 906.9	5	101
Long-term [§]	600.0	547.3 $\pm$ 29.8	5	91
	18,750.0	16,987.7 $\pm$ 529.3	3	91

[†]After 120 h in refrigerator at 2–8°C.
[‡]After 24 h in auto sampler at 10°C.
[§]At room temperature for up to 5 months.
n = 6 at each QC level.
QC: Quality control.

fivefold, mean concentration was found to be 43,352 pg/ml with %CV of 5.9% and accuracy of 100.1% after six measurements.

Hematocrit effect

Hematocrit (percent of red blood cells in blood) can alter the analyte concentrations by affecting the spot area, therefore these parameters should be evaluated [40,41]. Blood can have different viscosities with different hematocrit levels [42]. This variability in viscosity often leads to differences in flux and diffusion properties of blood, which can directly impact the size of the blood spots formed and alter extraction recoveries. In the present study, hematocrit effect was evaluated at four different hematocrit values (30, 40, 50 and 66) at LQC and HQC concentration levels. As shown in Table 3, the %CV ranged from 4.3 to 8.5% with accuracy 93.7–106.3% for LQC concentration and 0.2–2.7% with accuracy of 95.8–102.2% for HQC.

Stability studies

Stability of MPH in postextracted samples stored at 4°C for 120 h, autosampler at 10°C for 24 h and benchtop (24°C, long-term) stability on DBS cards at room temperature for up to 7 months were evaluated. As shown in Table 4, mean% stability (accuracy) of MPH was found to be within $\pm$ 15% of the spiked concentrations at LQC and HQC levels in postextraction and autosampler specimens. Although we did not evaluate the stability of MPH beyond 24 h at 4°C, based on our postextract stability for 120 h at 4°C, we can predict that MPH could also be stable at lower autosampler temperatures for more than 24 h. Similarly, as shown in Table 4 & Figure 3, MPH was found to be stable ($\pm$ 15% of spiked conc.) on DBS cards stored at 24°C for up to 5 months (shaded region depicts acceptable stability for up to 5 months).

Cross validation

As the intended subjects of the bioequivalence study are children aged 6–13 years old, blood withdrawal could be a limiting factor in getting reliable and multiple DBS. Thus, to accommodate the use of the current bioanalytical method, a partial validation was performed to get more confidence on the proposed method with 5- μ l DBS.

Figure 3. Long-term stability profile of methylphenidate on dried blood spot cards stored for up to 7 months at room temperature.

The partial validation results confirmed that the proposed method shows reproducibility without any interference at LLOQ even with the lower blood spot volume of 5 μ l on the DBS cards (see the Supplementary Data). Additional parameters validated were intra- and interday precision and accuracy, extraction recovery, matrix effect and injection carryover. Please refer to the Supplementary Data (Supplementary Figure 1 & Supplementary Tables 1–4) for validation results with 5- μ l DBS.

Conclusion

Utility of DBS as a primary matrix of choice for preclinical and/or clinical PK studies is still evolving. This hinders research into the pharmacokinetics and exposure-response relationship in susceptible patient populations, especially pediatric due to limitations on sampling volumes. Thus, leveraging the minimally invasive nature of DBS compared with conventional whole blood/plasma sampling (which also provided similar [$\pm 20\%$] MPH concentrations), we developed and validated an LC–MS/MS method for quantitative determination of MPH in DBS. A chromatographic run time of 2.0 min makes it an attractive assay in high-throughput bioanalytical labs to quantify MPH. The discussed method is simple, rapid, sensitive and selective for quantification of MPH suitable for PK studies. Stability of MPH in DBS cards was confirmed for up to 5 months when stored at room temperature.

Future perspective

The published LC–MS/MS-based methods for quantitative determination of MPH are based on matrices such as plasma, oral fluid and urine. The goal here was to develop and validate an LC–MS/MS method using DBS matrix with an intent to support pediatric PK study. DBS has been used since more than five decades for different purposes. Our preliminary in-house data demonstrated good correlation between plasma and DBS (blood collected from finger prick) for MPH levels. This adds confidence on the utility and substitutability of DBS as a low-intensity noninvasive matrix for clinical PK studies. Although we showed no influence of sample volume (5 vs 10 μ l) and hematocrit effect, future studies could include evaluation of alternative sampling sites, collection devices (volumetric absorptive microsampling) and extraction modifications (automated 96-well) to support high-throughput sample processing can lead the future of DBS in routine preclinical as well as clinical PK studies. Although bioanalytical validation criteria as per the FDA guidance seem to cover and include few more parameters for quantitative drug analysis using DBS matrix, it should not be mistaken to consider DBS as a panacea over conventional PK sampling matrices. In conclusion, we think DBS can provide economical, reproducible and sensitive drug quantitation in a high-throughput setting in the next 5–10 years to come.

Summary points

- An LC–MS/MS method for quantitative analysis of methylphenidate was developed and validated using dried blood spots (DBS).
- The method successfully evaluated the effect of hematocrit and ruled out any interference on methylphenidate quantitation.
- Good concordance between DBS and plasma data (preliminary in-house study) can allow the use of either matrix for pharmacokinetic analyses.
- The method can support two different sample volumes (5 and 10 µl blood) with maintaining the same LLOQ.
- Methylphenidate was shown to be stable for up to 5 months in DBS at room temperature (24°C).

Financial & competing interests disclosure

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Disclaimer

The views expressed in this article are those of the authors and do not necessarily reflect the official views of the US FDA.

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