JOURNAL OF APPLIED BIOANALYSIS, January 2016, p. 25-37.
http://dx.doi.org/10.17145/jab.16.005 (ISSN 2405-710X)
Vol. 2, No. 1

OPEN ACCESS

RESEARCH ARTICLE

Bioanalytical evaluation of dried plasma spot microsampling methodologies in pharmacokinetic studies applying Acetaminophen as model drug

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(Received: 23 November 2015, Revised 3 January 2016, Accepted 5 January 2016).

This paper reports the development and validation of different types of dried plasma spot bioassays and their application in a pharmacokinetic study. The pharmacokinetic study involved a single oral dose of the drug acetaminophen and post-dose blood samples were collected by finger prick. Plasma was separated for dried plasma spot preparation and thereafter quantitatively analyzed by liquid chromatography-accurate mass high resolution mass spectrometry. Comparison of results from bioanalytical performance experiments indicated that the two dried plasma spot bioassays worked well and observed acetaminophen pharmacokinetic values were found not to be statistically significantly different between the evaluated two dried plasma spot bioassays. Moreover, pharmacokinetic values observed were also not statistically significant different from values reported in literature. Overall, it was concluded that dried plasma spot bioassays could have a great potential as alternative sampling technique in pharmacokinetic studies.

Keywords: acetaminophen, pharmacokinetics, dried plasma spots, dried plasma on paper disks, high resolution accurate mass spectrometry.

Introduction

Recently, the use of microdevices for the collection of small amounts of blood, plasma, saliva or other biological fluids have become popular and are widely applied in various research areas including in drug pharmacokinetics (PK) [1]. In particular, the collection of small amounts of blood onto different substrate materials (cellulose or non-cellulose based) is nowadays a frequently applied micro sampling technique. This methodology of collecting blood samples is also known as dried blood spot sampling (DBS). DBS have seen a tremendous increase in application and popularity over the past five years [2]. DBS sampling was for the first time reported in literature some fifty years ago when DBS were used in the detection of phenylketonuria (PKU) in newborns [3]. DBS sampling is a relative simple and fast blood collection technique involving the collection of small amounts of whole blood (typically 10-50 μL). The blood is obtained from a skin puncture. Mostly from finger- or heel prick although other parts of the human body can be used, for example the earlobe. Due to its inherited simplicity, DBS sampling has some advantages compared to blood collection via venepuncture. The latter is a blood sampling technique that is more invasive in contrast to finger and heel prick

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blood collection. Nevertheless, DBS sampling has also some methodological drawbacks. One of the most limiting factors of DBS sampling, especially in quantitative applications is the so called "hematocrit effect". The hematocrit effect negatively impacts the accuracy and precision of bioassays [4,5]. The hematocrit effect is a complex phenomenon caused by three different factors; (i) the different spreading of blood on the substrate material at different hematocrit value, (ii) significant difference in blood/plasma distribution ratios of drugs and (iii) the hematocrit dependent recovery of drugs [6-8]. In addition, substrate material properties, extraction procedures and chemical-physical properties of the analytes can have potentially an additional impact on accuracy and precision of bioassays beside the hematocrit effect [9]. Moreover, above mentioned parameters can also have an impact on the accuracy and precision of bioassays using other types of biological fluids spotted onto substrate materials (e.g. plasma, saliva etc). Different practical solutions to eliminate or limit the hematocrit effect to a maximum possible extent were recently reported by several authors. Solutions like whole-spot instead of partial punch analysis [9], application of pre-cut paper disks and recently volumetric absorptive microsampling (VAMS) [10-13] were applied successfully. Moreover, the hematocrit effect is now in general much better understood then some years ago and recent knowledge on DBS implementation into bioassays increased significantly. Nevertheless, until today no official guidelines on implementation of DBS sampling in (regulated) bioanalysis are available [14]. As mentioned before, microsampling methodologies are mostly straight forwarded and in contrast to other biological fluid collection methodologies relatively simple and fast. Due to the simple sample collection technique, the methodology of spotting biological fluids onto substrate materials can also be done with other biological fluids then blood only (e.g. plasma, serum, sweat, saliva, tears etc.). In present paper we developed, validated and applied the spotting of liquid plasma onto substrate material for the preparation of dried plasma spot samples collected from a pharmacokinetic study using acetaminophen as model drug. Acetaminophen was selected for testing because it is an over-the-counter drug (OTC drug) and is therefore easily available and in low doses administered relative safe. Plasma samples from healthy humans were prepared by centrifugation from capillary blood collected post-dose by finger prick spotted onto cellulose paper substrate. Plasma spot acetaminophen concentrations were determined after extraction by methanol and analysis by means of quantitative liquid chromatography high-resolution accurate mass spectrometry (LC-HRAMS). LC-HRAMS is a mass spectrometric technology that is momently becoming popular in (regulated) bioanalysis, especially in the pharmaceutical industry and regulated bioanalytical laboratories, in research areas such as discovery DMPK and drug biotransformation studies [15,16]. Papers reporting the application of dried plasma spots for pharmacokinetic evaluations of drugs [17], as well describing the bioanalytical performance of dried plasma spot methodologies in drug pharmacokinetic studies are limited. Moreover, in therapeutic drug monitoring (TDM), dried plasma spots were previously reported as advantageous in the clinical practice compared to conventional sampling methodologies [18]. To be able to evaluate the bioanalytical performance of dried plasma spots methodologies, a study with two different dried plasma spot methodologies were developed, validated and applied and results are reported in this paper. In performed studies following dried plasma spot methodologies were developed and evaluated; (i) dried plasma spots (DPS) prepared by pipetting a fixed volume of plasma onto a cellulose based paper substrate followed by unsupported letting develop the DPS freely through sorption of the plasma by the paper substrate and (ii) dried plasma on paper disks (DPPD) where an equal amount of plasma was pipetted onto pre-cutted papers disks resulting in dried plasma spot samples with a predefined spot size. The application of DPS or DPPD in PK studies can have some advantages in contrast to DBS or dried blood spots on paper disks (DBPD); (i) no translation of blood concentration levels obtained by DBS/DBPD to plasma concentration levels are necessary, (ii) no hematocrit is present, and (iii) analytes that accumulate in erythrocytes do no impact the accuracy of the assay.

Materials and methods

Reagents and materials

Acetaminophen (ACP, 99.0% purity), HPLC-grade methanol and acetonitrile were purchased from Sigma-Aldrich (St. Louis, USA) and acetaminophen-d4 (ACP-d4, 99.9% purity, 97.0% d4) used as internal standard was from Cerilliant (Round Rock, TX, USA). Ultrapure water (18.2MΩ.cm) for liquid chromatographic separations (LC) was in-house produced by a Direct-Q® 3UV Milli-Q apparatus (EMD Millipore, Billerica, MA, USA). Capillary blood samples (approximately 300 µL) were collected using Sarstedt Microvette® CB300 blood collection tubes (Nürnbrecht, Germany) containing the anticoagulant potassium-EDTA (K_2-EDTA). Blood samples were collected from one voluntary healthy subject by finger prick applying an automatic safety lancet containing a 2.0 mm blade (SurgiLance, St. Ingbert, Germany).

Whatman FTA® Plant Saver cards (GE health, Fairfield, CT, USA) were used for the preparation of dried plasma spots (DPS) as well for the pre-punching of paper disks used in the preparation of dried plasma on paper disks (DPPS).

Liquid chromatography-high resolution accurate mass spectrometry (LC-HRAMS)

The mass spectrometric analyses of acetaminophen concentrations in dried plasma spot samples were performed by LC-HRAMS according to the methodology reported previously [19]. In short; LC separation was performed by an Agilent 1260 Infinity LC system consisting of a G1312B binary pump and G1367E auto sampler system. Plasma samples were separated on a Waters Atlantis T3 column (2.1 mm x 100 mm x 5µm) kept isothermally at a temperature of 35°C. The separation gradient applied was as follows: mobile phase A (100% H_2O) containing 0.1% formic acid (v/v %) and mobile phase B was acetonitrile (100%). The applied separation gradient was: 2% B from 0 to 0.5 min, 2 to 30% B from 0.5 to 2.5 min, 30 to 95% B from 2.5 to 2.6min, held at 95 % B for 1.4 min and followed by 2% B until 6.0 minutes and thereafter an equilibration time of 5 minutes prior to next sample injection was used. Applied separation flow was 0.3 ml/min and sample injection volume was 20µL.

HRAMS analyses were done using an Agilent accurate mass Q-TOF 6520 mass spectrometer (MS) working in positive electrospray ionization (ESI). Optimized mass spectrometric instrument settings were as follows: gas temperature: 300°C (N_2); nebulizer pressure: 48 psig; capillary voltage (V_{cap}): 3500 Volts; drying gas: 8 L/min; fragmentor voltage: 135 Volts; skimmer voltage: 60 Volts. For quantitative analysis following accurate mass-to-charge ratios for ACP (m/z 152.07110) and ACP-d4 (m/z 156.09619) were monitored. The mass accuracy obtained by the LC-HRAMS instrumentation was < 3 ppm (mean mass accuracy for both ACP and deuterated internal standard were -1.1 ppm).

Dried plasma spot preparation and extraction

The dried matrix on paper disks cartridges (DMPD) applied for DPPS preparation, collection and storage (3 and 5-mm diameter paper disks) were obtained from Rock-Town Technology and Services (Libertyville, IL, USA). The 3- and 5-mm paper disks were prepared by applying a Harris Uni-core punching tool (Redding, CA, USA). Dried plasma spots (DPS) and dried plasma on paper disks (DPPD) applied for bioassay validation purposes as well for collection of plasma samples form the PK study were prepared and analyzed in triplicate. Dried plasma spot samples (DPS and DPPD) including all calibration solutions, QC samples and PK plasma samples were extracted by high purity methanol (100%) containing ACP-d4 as internal standard (IS). For the extraction of 3-mm paper disks 50 µL of the solvent were used while for the 5-mm disks 100 µL of the solvent were used. The concentration of the ACP-d4 internal standard in the extraction solvent was 1 ng/µL.

Validation procedures

Both types of newly developed dried plasma spot bioassays were validated by means of the application of plasma matrix matched calibration solutions and plasma matrix matched quality control samples (QC). The preparation of plasma matrix matched calibration solutions and QC samples is described in more detail in following sections. The DPS and DPPD bioassays were validated according to recent international guidelines [20]. QC samples (DPS and DPPD) were prepared at ACP concentration levels of 1.0, 3.0, 12.0 and 18.0 µg/mL. Following assay parameters were validated: carry-over effect, linearity, lower limit of quantification (LLOQ), limit of detection (LOD) and within-day and between-day accuracy and precision as well ACP stability in the dried plasma spot matrix in time (1, 2 and 3 weeks storage at ambient laboratory temperature).

Preparation of calibration samples

- DPS calibration samples: 15 µL of the calibration solutions were pipetted onto DBS substrate paper. After preparation of the DPS samples the position of the pipetted plasma was marked by carefully drawing a circle around the wet plasma spot (without touching the DPS) by a pencil. DPS calibration samples were air dried at ambient laboratory temperature (approximately 20 ºC) and from the sets of calibration samples prepared in triplicate at each concentration level one disk was punched out (spot-centered) by applying 3-mm and 5-mm Uni-core punching tool. Thereafter, the 3-and 5-mm disks were extracted by solvent containing IS supported by sonication of the disks for 15 minutes and after finalization of the extraction, followed by centrifugation of the extracts for 5 minutes at 18000 $x\,g$ at 4°C.

- DPPD calibration samples: respectively 2 and 8 µL of calibrator solution were pipetted in triplicate onto 3- and 5-mm punched paper disks which were placed into a plastic cartridge and were let to air dry. From all DPPD calibration samples prepared at each concentration level one disk was removed from the cartridge, extracted by solvent containing IS with support of sonication (15

min) followed by centrifugation of the extracts for 5 minutes at 18000 *x g* at 4°C.

Calibration curves

Calibration curves for DPS assays (3 and 5-mm punches) and DPPD assays (3 and 5-mm pre-cut paper disks) were prepared as follows; the linear ACP concentration range of the DPS and DPPD assays was assessed by analysis of seven calibration solutions ranging in the concentration range of (1.0 – 25.0 µg/mL ACP). Linearity between the signal (area ratio between ACP and ACP-d4) was considered to be acceptable if the Pearson's correlation coefficient (r) was >0.990. The selection of a linear regression model was based on the results of the Pearson's correlation coefficient determination and the models were acceptable when the coefficient of determination (r^2) was also >0.990. The Pearson correlation coefficients and coefficients of determination of the DPS and DPPD calibration curves were determined by using Microsoft Office Excel Data Analysis. DPS and DPPD calibration solutions were prepared according following protocol;
From an aqueous ACP stock solution (500 µg/mL) DPS and DPPD calibration solutions were prepared by serial dilution of this stock solution with drug free blank plasma collected from the healthy voluntary subject prior to start of the PK study and oral intake. Selected calibration concentrations were: 1.0, 2.5, 5.0, 10.0, 17.5, and 25.0 µg/mL ACP. The concentration range selected for validation was based on the in literature reported pharmaceutical therapeutic range of ACP, ranging between 10 and 20 µg/mL [21].

Lower limits of quantification and detection (LLOQ and LOD)

LLOQs of the bioassays were defined as the concentration of the calibrator with the lowest ACP concentration used for determination of the calibration curve that was quantitatively analyzed with an acceptable accuracy and precision of < 20 %bias for accuracy and < 20 %CV for precision.
The LOD of each dried plasma spot bioassay was determined mathematically from the obtained linear regression curve equation of the DPS and DPPD bioassays by using the mean signal value of the observed signal-to-noise ratio (S/N) for drug free blank plasma analyzed in triplicate. This mean signal value was then multiplied by a factor of three (3 standard deviations) for the determination of the LOD in µg/mL ACP.

Carry-over effects

The existence of carry-over effects was assessed by the analysis of one QC sample at a concentration level of 18 µg/ml ACP in triplicate followed by a directly triplicate measurement of a drug-free blank plasma sample. The carry-over effect was considered negligible or acceptable if the ACP concentration observed caused by carry-over from the QC sample into the blank plasma sample was < 20% of the LLOQ for ACP and < 5% of the LLOQ for ACP-d4.

Within- and between day accuracy and precision

Within-run and between-run accuracies and precisions were determined by measurement of QC samples at four different ACP concentration levels (1.0, 3.0, 12.0 and 18.0 µg/mL). Each QC sample was analyzed in triplicate. Accuracy and precision were considered acceptable when the mean observed ACP concentrations were within 15 %bias or 15 %CV of the nominal ACP value, with exception for the QC sample at the LLOQ (1.0 µg/mL) where accuracy and precisions were acceptable when %CV and %bias were < 20%. Within-run accuracy and precision were determined by three consecutive measurements of the QC samples within one-single day in triplicate by analysis of the QC samples in the morning (0 hours), midday (8 hours) and during the night (16 hours). Between-run accuracy and precision were determined by analysis of the QC samples in triplicate on three consecutive different days (0, 24 and 48 hours).

ACP stability in dried plasma spot matrix

The stability of ACP in dried plasma spot matrix was studied by the measurement of QC samples at the three different QC concentration levels (3.0, 12.0 and 18.0 µg/mL ACP) after consecutively being stored for 1 week, 2 weeks and 3 weeks, respectively in a desiccator at laboratory temperature (approximately 20ºC). The stability of ACP in the dried plasma spot matrix was considered to be acceptable if the observed ACP concentration of the QC samples after storage at the three time points declined < 15 %bias.

Pharmacokinetic study design

The PK study design was adapted from the study protocol published previously [19]. In short; the PK study involved a single oral fixed dose of 500 mg ACP in combination with 100 mL of water (Tecnoquimicas, Colombia) purchased as pills at a local drugstore. The healthy voluntary participant fasted for at least 8 hours prior to the start of the PK study and blood samples were collected using a finger prick by an automatic safety lancet. For PK profiling a volume of approx. 300µL of capillary blood from the finger of the voluntary subject was

collected at following post-dose time points: 0, 30, 60, 60, 90, 120, 150 and 170 minutes using Microvette® CB 300 K_2E blood micro collection tubes. Capillary blood samples were gently shaken and immediately stored on ice until plasma was prepared and collected. DPS and DPPD samples were prepared by the protocol used for preparation of calibration and QC samples. PK samples were after a 120 min drying time at ambient laboratory temperature (approximately 20ºC) placed into a polyethylene zip-lock bag and stored into a desiccator until further sample processing and analysis.

Pharmacokinetic data analysis

LC-HRAMS data was processed using the Mass Hunter software (Agilent) for obtaining ACP concentrations. PK profiles and PK parameter values determined were; area under curve (AUC), clearance (Cl), apparent volume of distribution (V), peak concentration (C_{max}) and half-life time ($t_{1/2}$) as well others. PK parameter values were calculated using PkSolver® software [22] applying a one-compartment open model with first-order drug absorption and first-order drug elimination (**Fig. 1**).

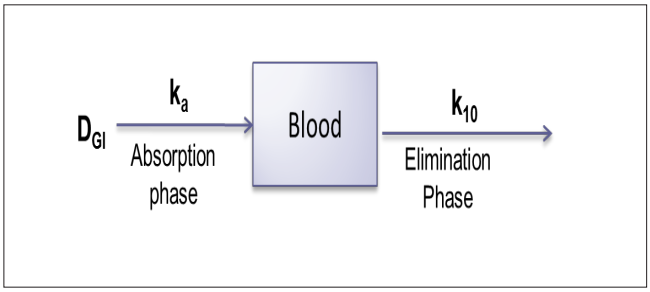

Figure 1. One-compartment open pharmacokinetic model with first order absorption and elimination phases and mathematical expression of the drug concentration of time points t=t post-dose. (D_{gi}= gastrointestinal drug dose; k_a= absorption rate constant; k_{10}=elimination rate constant).

Results and discussion

Bioassays validation

The newly developed dried plasma spot bioassays did undergo a bioanalytical method validation according to recent FDA guidelines on development and validation of bioanalytical assays. Validated dried plasma spot bioassay parameters selected were linearity, within- day and between-day accuracy and precision, stability of ACP in the dried plasma spot matrix and carry-over effect. The results obtained from validation experiments are presented in following sections. All here reported validation-and PK study results are the mean results determined from a triplicate analysis.

Linear concentration range of the bioassays

The calibration curves obtained demonstrated all good linear relationships between the ratio of the ACP/ACP-d4 area and ACP concentration (μg/mL) in the selected concentration range (1-25 μg/mL ACP).

The linear relationship was determined by calculation of the Pearson correlation coefficient (r) while the linear regression analysis was used for modeling the relationship by determination of its mathematical equation (calibration curve) and the coefficient of determination (r^2). The mathematical relationship between the ratio of the area ACP/area ACP-d4 (y) and ACP concentration (x) and the coefficient of determination (r^2) and Pearson´s correlation coefficients were observed as follows; DPS-3mm (y=0.0175x-0.0089, r=0.9983, r^2=0.9966) DPS-5mm (y=0.0258x-0.0062, r=0.9974, r^2=0.9949), DPPD-3mm (y=0.0355x+0.0158, r=0.9985, r^2=0.9970 and DPPD-5mm (y=0.0504x+0.0107, r=0.9980, r^2=0.9959). Observed Pearson's correlation and coefficients of determination were satisfactory because they were all >0.990. All blank drug free plasma calibrators used as "zero" concentration calibrator showed no distinct signal for ACP (S/N <3:1) above the background signal. Looking at the slopes of the different calibration curves it was observed that the highest analytical sensitivity could be expected with the DPPD-5 mm assay. This assumption was based on the fact that the calibration curve of this bioassay applied the highest absolute amount of plasma sample spotted (8.0 μl) for analysis, this in contrast to the amounts of plasma applied for the preparation of calibration curves for other bioassays; DPPD-3mm (2.0 μL), DPS-3mm (1.7μL) and DPS-5mm (4.7 μL), respectively. The amount of plasma present in the punched disks from the two different DPS bioassays had to be determined by calculation, this due the variation in spreading of the plasma onto the substrate material. The plasma amount for analysis was calculated by means of use of the observed area of the DPS (whole dried plasma spot area) and the area of the punched disk, respectively. The calculation of the absolute plasma amount that was present in the punched paper disk is presented hereafter.

The dilution factor (amount applied plasma → volume extract) for the DPPD-5mm bioassay was 12.5 (8.0 μL plasma→ 100.0 μL extract) while for the DPPD-3mm assay the dilution factor was 25.0 (2.0 μL → 50.0 μL extract). Comparison of the slopes of the DPPD-5mm and DPPD-3mm calibration curves demonstrated a difference in slope between both bioassays of a factor of 1.4.

Theoretically, the factor between the slopes of the calibration curves calculated from the dilution factors had to be a factor of 2. In this case a different factor was observed, what in principle is possible when there exist a difference in matrix effects impacting the sensitivity of the analysis of both bioassays. Since for the DPPD-3mm bioassay a higher dilution factor for the applied plasma was used, it is quite reasonable that the matrix effects caused by potential coeluting compounds leading to ion suppression were to a certain extent reduced in contrast to matrix effects impacting the analysis of samples from the DPPD-5mm bioassays. This reduced matrix effect due to the higher dilution of the applied plasma sample might be a potential plausible explanation for the observed difference in sensitivity ratio. Determination of the amount of plasma in the punched disks from the DPS bioassays was done by measurement of the areas of the DPS-3 mm and DPS-5 mm dried plasma spots, resulting from pipetting 15 µL of plasma onto paper substrate. The areas of both DPS assays observed were statistically not different, observed median area of the dried plasma spots applying the DPS-3 mm assay was 0.630 cm^2 while for the DPS-5 mm assay the median area was observed to be 0.631

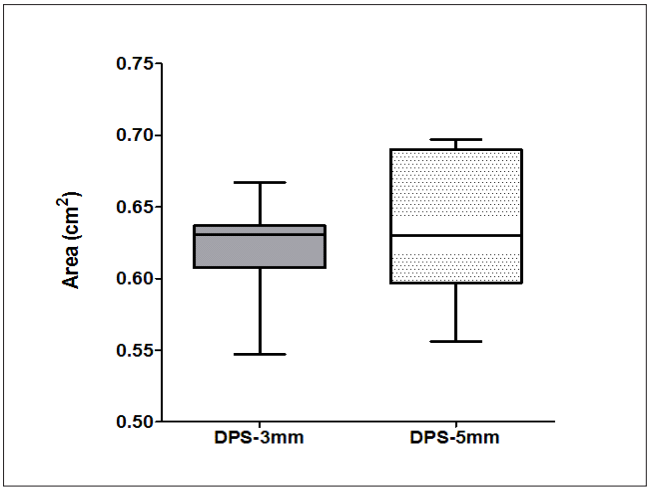

Figure 2. Areas of dried plasma spots of DPS-3mm and DPS-5mm assays. DPS area shown are the areas from the plasma matrix matched calibration solutions analyzed in triplicate. The box-whiskers plots show the minimum and maximum observed area (whiskers) as well the median (solid line), first and third quartile (box).

cm^2. Non-parametric statistical analysis of obtained data showed that the first quartiles (Q1) for DPS-3 mm and DPS-5 mm assays were observed at 0.609 and 0.603 cm^2 while third quartiles (Q3) were respectively 0.637 and 0.690 cm^2 (**Fig. 2**).

Because both DPS assays applied a fixed size disk punch the punched disk represented a certain fraction of the spotted plasma which amount had to be to determined. The amount of plasma (µL) in the punched disk could only be determined by calculation. The mean volume of plasma contained into a punched disk from the DPS-3mm assay was calculated by using the whole dried plasma spot area (data not shown) in combination with the area of the punched disk. For the DPS-3 mm assay a mean plasma volume of 1.7 ± 0.1 µL was calculated while for the DPS-5mm assay the mean plasma volume was 4.7 ± 0.4 µL. The difference in plasma amount collected from the DPS assays used for the preparation of calibration curves was used to explain the sensitivity decrease of the DPS-3 mm assay in comparison to the DPS-5 mm assay. The explanation of this sensitivity decrease was only possible when the plasma amount into the punched disks in combination with the applied dilution factor originating from the extraction of the 3 and 5-mm disk by the solvent were taken into consideration. The dilution factor of the plasma amount from the DPS-3mm disk was 29.4 times (1.7 µL →50 µL) while the dilution factor for the plasma amount from the 5 mm disk was 21.3 times (4.7 µL → 100µL). The dilution factor of the DPS-3mm bioassay was thus a factor of 1.38 times bigger than the dilution factor of the DPS-5 mm bioassay. The dilution factor was calculated based on the surface area of the DPS spots for both bioassays and the applied punched area of the paper disks (3 or 5-mm diameter). The observed sensitivity difference between both DPS bioassays using the slopes of the calibration curves was calculated to be a factor of 1.50 time which was very close to the calculated theoretical difference in dilution factor applying observed dried plasma spot areas and punched plasma amounts. This confirmation of the dilution factor showed that there was a relative good linear relationship in sensitivity between applying 3-mm or 5-mm disks.

Comparison of the analytical sensitivity of both dried plasma spot methodologies showed that the DPPD methodology had an approximately higher relative sensitivity of a factor 2.00 for as well the DPPD-3mm (factor 2.14) and the DPPD-5mm (factor 2.00) bioassay. In this study the lower observed sensitivity for the DPS bioassays did not had any bioanalytical quantitative implications on the dried plasma spot concentration measurements of QC and PK samples, this because the dried plasma spot concentrations of all these samples were all > LLOQ (1 µg/mL ACP). Although the lower analytical sensitivity of the DPS bioassays could be explained by the in contrast to the DPPD bioassay larger spreading of the pipetted plasma onto the paper substrate resulting in dried plasma spots that were larger in size and area

than the fixed size paper substrate paper disks (DPPD bioassays). Also this spreading effect (chromatography) did not had any implications on the determination of the ACP concentration measurement of samples from the PK study. It would have been different in the case a lower drug dose had been administered where significant lower plasma concentration levels were expected resulting in some post-dose time points in plasma concentration levels < LLOQ or even < LOD.

Within- and between day accuracy and precision

Observed within-day and between-day accuracy and precision for all bioassays were for the lowest QC sample at the LLOQ < 20 %bias as well <20 %CV. At the three other QC levels (3, 12 and 18 μg/mL ACP) observed bioassay accuracies and precisions were <15 %bias and <15 %CV for all bioassays. Observed mean within-day accuracy of the DPS bioassays was 2.4 %bias and standard deviation (SD) of 2.7%) while precision was 4.0 %CV (SD 2.6%). Observed mean accuracy of DPPD bioassays was 3.7 %bias (SD 7.3%) and observed precision was 3.6 %CV (SD 2.5%). Mean between-day accuracy of the DPS bioassays was 3.0 %bias (SD 3.7%) and precision 3.3 %CV (SD 2.4%) while mean between-day accuracy of the DPPD bioassays was observed at 2.9 %bias (SD 5.4%) and precision was 3.5 %CV (SD 2.0%). All results of within- and between day accuracy and precision experiments are presented in **Table 1**.

Stability of ACP in dried plasma matrices

The decline of the ACP concentration in the dried plasma spot matrix in time (1, 2 and 3 weeks) for all bioassays was observed to be < 15% at applied three QC concentration levels (3, 12 and 18 μg/mL). From the stability studies was concluded that the stability of ACP in a dried plasma matrix over a time period from up to 3 weeks stored at laboratory temperature in a desiccator was high. Dried plasma spot samples could be stored up to 3 weeks without significant loss of ACP prior to analysis. The observed stabilities of ACP in dried plasma spot matrices are presented in **Table 2**.

Carry-over effects

Carry-over effects were expressed as ACP and ACP-d4 concentrations observed in a blank drug-free plasma samples after injection of a QC sample at high ACP concentration (18 μg/mL). Carry-over effects are caused by instrumental cross-contamination. Observed carry-over effects for both dried plasma spot methodologies were < 5% of the LLOQ of ACP-d4 and for ACP < 20% of the LLOQ and all values were in compliance with the

Table 1. With-in day and between day accuracy and precision of DPS and DPPD assays

	Within days						**Between days**					
	DPS-3mm			DPS-5mm			DPS-3mm			DPS-5mm		
Nominal ACP concentration (μg/mL)	**1.0**	**3.0**	**12.0**	**1.0**	**3.0**	**12.0**	**18.0**	**1.0**	**3.0**	**12.0**	**18.0**	**1.0**	**3.0**	**12.0**	**18.0**
Mean observed ACP concentration (μg/mL)[a]	1.1	3.0	12.1	18.2	1.0	3.1	12.1	18.3	1.1	3.0	12.3	19.1	1.1	3.0	12.1	17.9
Precision (% CV)	4.0	3.4	2.5	0.7	6.5	8.2	1.3	5.7	4.5	1.5	3.0	1.4	6.5	2.0	6.9	0.6
Accuracy (% bias)[b]	8.6	0.9	0.9	0.8	2.1	3.4	0.6	1.5	9.9	0.6	2.3	5.9	5.4	-0.6	1.0	-0.6
	DPPD-3mm				DPPD-5mm				DPPD-3mm				DPPD-5mm			
Nominal ACP concentration (μg/mL)	**1.0**	**3.0**	**12.0**	**18.0**	**1.0**	**3.0**	**12.0**	**18.0**	**1.0**	**3.0**	**12.0**	**18.0**	**1.0**	**3.0**	**12.0**	**18.0**
Mean observed ACP concentration (μg/mL)[a]	0.9	3.1	11.7	18.4	1.0	3.3	13.4	20.1	1.1	3.1	11.5	17.6	1.1	3.3	12.4	17.8
Precision (% CV)	1.4	4.3	4.2	4.7	8.6	2.5	1.4	1.5	4.6	4.8	2.4	0.6	3.5	1.0	6.2	4.9
Accuracy (% bias)b	-8.3	2.5	-2.4	2.1	1.1	11.2	11.7	11.8	10.7	2.5	-4.2	-2.3	5.5	9.5	2.9	-1.3

[a]results summarize the mean of a threefold analysis per QC sample at each concentration level in one experiment, [b]%bias = (mean observed ACP concentration-nominal ACP concentration)/(nominal ACP concentration)*100%

Table 2. Stability of ACP in dried plasma matrix

DPS

	3-mm									5-mm								
	1 week			2 weeks			3 weeks			1 week			2 weeks			3 weeks		
Nominal ACP concentration (µg/mL)	**3.0**	**12.0**	**18.0**	**3.0**	**12.0**	**18.0**	**3.0**	**12.0**	**18.0**	**3.0**	**12.0**	**18.0**	**3.0**	**12.0**	**18.0**	**3.0**	**12.0**	**18.0**
Mean observed ACP concentration (µg/mL)[a]	3.4	12.9	19.4	2.9	11.6	19.7	2.8	12.5	19.1	2.6	11.8	19.1	3.0	13.4	19.7	3.3	12.2	19.1
Precision (% CV)	7.3	5.3	5.9	5.9	3.3	3.0	10.5	3.2	4.9	11.6	5.0	5.7	7.5	7.7	5.4	10.7	8.0	5.3
Accuracy (% bias)[b]	12.6	7.9	7.9	-1.7	-3.5	9.6	-7.9	4.3	6.0	-3.9	-2.1	5.9	0.9	11.8	9.2	8.0	1.8	6.3

DPPD

	3-mm									5-mm								
	1 week			2 weeks			3 weeks			1 week			2 weeks			3 weeks		
Nominal ACP concentration (µg/mL)	**3.0**	**12.0**	**18.0**	**3.0**	**12.0**	**18.0**	**3.0**	**12.0**	**18.0**	**3.0**	**12.0**	**18.0**	**3.0**	**12.0**	**18.0**	**3.0**	**12.0**	**18.0**
Mean observed ACP concentration (µg/mL)[a]	3.2	12.6	18.1	3.0	13.1	18.5	3.0	12.2	18.7	3.1	11.6	19.2	3.2	12.0	18.5	3.2	12.0	18.3
Precision (% CV)	10.6	4.9	2.2	5.6	4.5	1.5	10.9	1.9	6.0	3.8	5.4	4.1	5.9	5.3	1.9	7.5	1.0	3.9
Accuracy (% bias)[b]	6.7	4.6	0.3	0.2	9.0	3.0	-1.0	1.9	4.8	3.0	-3.1	6.7	6.2	-0.3	2.6	6.8	0.1	1.5

[a]results summarize the mean of a threefold analysis per QC sample at each concentration level in one experiment, [b]%bias = (mean observed ACP concentration-nominal ACP concentration)/(nominal ACP concentration)*100%

FDA guidelines on method development and validation. The observed level of carry-over effects showed that cross-contamination of DPS and DPPD samples in a sample cue in the auto sampler could be assumed to be negligible.

Pharmacokinetic study

The PK curves constructed from post-dose collected dried plasma spot samples are presented in **Figure 3**. The **figures 3A-3D** show the observed logarithmic (natural logarithm) ACP concentrations (red circles) as well the logarithmic concentrations calculated by the PKSolver® software (predicted, blue solid line) applying a one-compartment open model with first order absorption and first order elimination (**Figure 2**). Calculated PK parameters from observed ACP post-dose concentrations obtained by the different dried plasma spot microsampling methodologies are summarized in **Table 3**.

$$C_{(t)}=A*(e^{-(k10*t)}-e^{-(ka*t)}) \qquad \textbf{Eq.1}$$

The equation 1 (**Eq.1**) presents the mathematical model of a one-compartment open PK model with first order absorption and first order elimination of the drug from the blood compartment used by the PKSolver® software to model the PK curve. The ACP concentrations (predicted, solid blue line) on post-dose time points t=t were calculated from the mathematical expression presented in **Eq.1** using the in the PK study observed dried plasma spot ACP concentrations. In the equation **Eq.1** the symbol A presents the pre-exponential constant for a one-compartment model, k_a = the absorption rate constant; and k_{10} = the elimination rate constant.

It can be observed in **Table 3** that the values of the PK

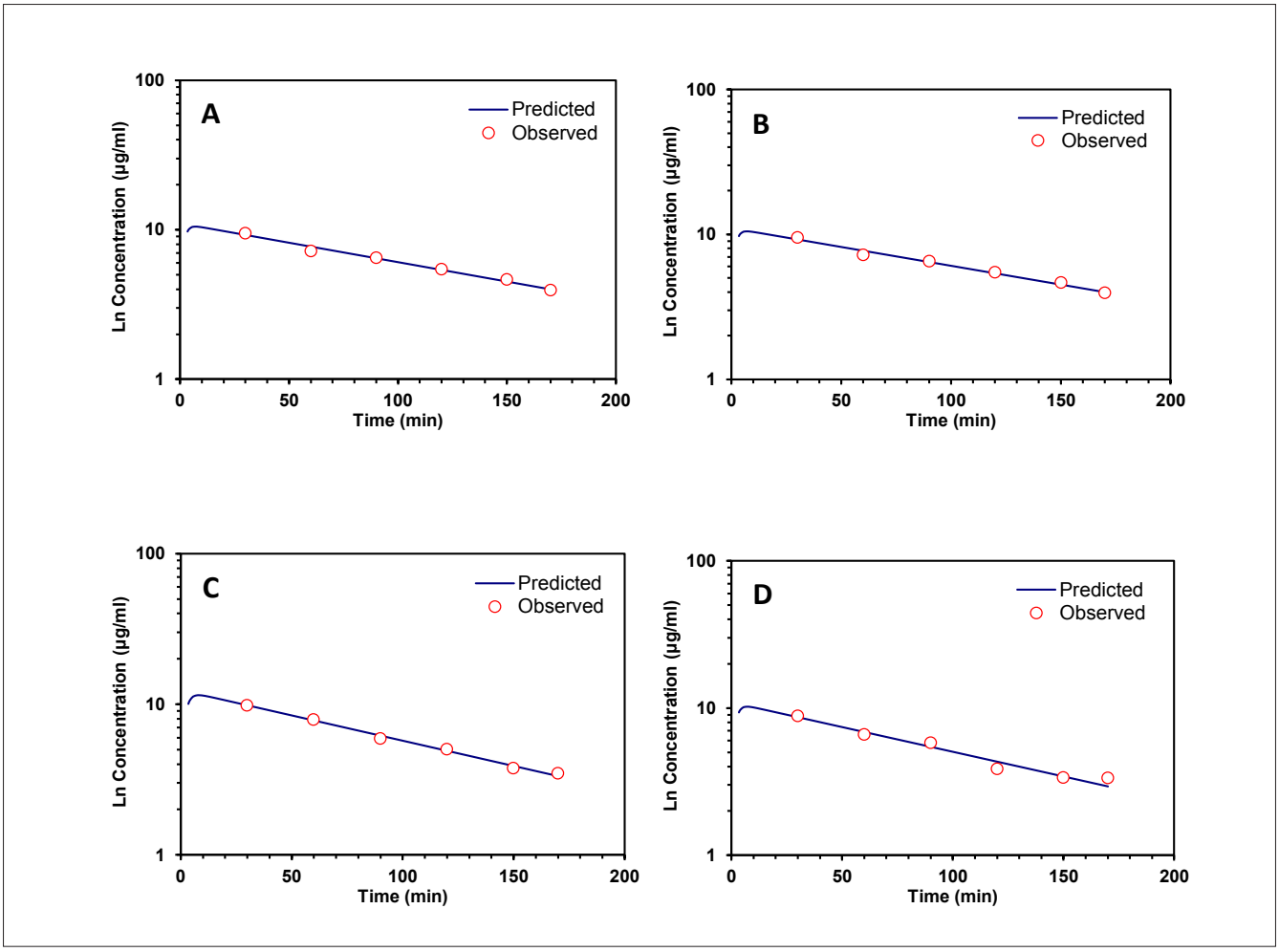

Figure 3. (A) ACP pharmacokinetic profile applying DPS-3mm microsampling; (B) PK profile applying DPS-5 mm microsampling; (C) applying DPPD-3mm microsampling and; (D) DPPD-5mm microsampling. Predicted ACP concentrations were calculated using PKSolver® software by application of a one-compartment open model with first order absorption and first order elimination.

Table 3. Calculated PK parameter value observed from post-dose ACP concentrations

Parameter	Unit	DPS-3mm	DPS-5mm	DPPD-3mm	DPPD-5mm	Mean	Standard deviation	*p*-value (α=0.05)
A	μg/mL	11.1	10.5	12.5	10.9	11.3	0.9	0.177
k_a	L/min	0.7	0.7	0.2	0.6	0.6	0.3	0.336
k_0	L/min	0.01	0.01	0.01	0.01	0.01	0.001	0.253
$t_{1/2}k_a$	min	1.0	1.0	4.1	1.1	1.8	1.5	0.452
$t_{1/2}k_{10}$	min	125.6	115.9	82.3	86.0	102.4	21.6	0.273
V	(mg)/(μg/mL)	45.2	48.2	42.1	46.3	45.4	2.6	0.374
Cl	(mg)/(μg/mL/min	0.2	0.3	0.4	0.4	0.3	0.1	0.0659
t_{max} [a]	min	-	-	-	-	-	-	-
c_{max}	(μg/mL)	10.7	10.0	10.2	10.2	10.2	0.3	0.906
AUC_{0-t}	μg/mL.min	1214.3	1101.9	1056.3	994.9	1091.9	92.7	0.0731
AUC 0-∞	μg/mL.min	2005.0	1735.0	1411.0	1339.5	1622.6	307.6	0.117
AUCMC	$\mu g/mL.min^2$	366112.6	292573.6	175852.5	168260.8	250699.9	95693.7	0.266
MRT	min	182.6	168.6	124.6	125.6	150.4	29.7	0.343

A: pre-exponential constant for one-compartment model; k_a :first-order absorption rate constant; k_{10}: first-order elimination rate constant; $t_{1/2}k_a$: half-life time first –order absorption rate constant; $t_{1/2}k_{10}$: half-life time first-order elimination rate constant; V: volume of distribution; Cl: clearance; t_{max}: time of occurrence for maximum(peak) drug concentration; c_{max}: maximum concentration of drug; AUC_{0-t}: area under curve from t=0 to last measurable plasma drug concentration; $AUC_{t-\infty}$: area under curve extrapolated to infinite time; AUMC: area under the (first) moment curve; MRT: mean resident time. atmax could not be determined accurately due to too less sampling points close to the expected tmax were collected. This lead to a significant underestimation of the calculated tmax by the applied software in this study.

parameters for all different dried plasma spot microsampling methodologies correlate very well, although some small but not statistical significant differences on the first view can be observed. To determine if the differences in PK parameter values were statistical different a single factor Analysis of Variance (ANOVA) analysis on the data was performed. The observed p-values for each PK parameter at a significance level of α=0.05 are presented in **Table 3**. The obtained p-values showed clearly that no statistically differences in the values of the PK parameters were observed. Comparison of the main important PK parameters; Cl 0.3 ±0.10 L/min, V 45.4 ± 2.6 L, C_{max} 10.2 ± 0.3 μg/mL, $t_{1/2}$ 104.2 ± 21.7 min and AUC_{0-t} 1091.9 ± 92.7 μg/mL.min showed that the mean values for these PK parameters were within the range of previously reported PK parameter values applying liquid plasma instead of dried plasma spots [19].

Reported values for Cl (clearance) were 0.68 ± 0.40 L/min, V (volume of distribution) 51.3 ± 24.1 L, AUC (area under curve) 965.7 ± 514.3 μg/mL.min, $t_{1/2}$ (half-life time) 95.0 ± 48.9 min and C_{max} (maximum concentration) 6.0 ± 2.5 μg/mL. The only exception observed was the C_{max} concentration observed, in present study C_{max} was statistically significant higher. Since the PK data reported in [17] was based on mean results from six different healthy human test subjects it was to a certain extent possible that when present study had included more healthy human subjects a mean C_{max} value close to the C_{max} value reported in [17] would had been observed to a certain statistical probability.

Furthermore all observed PK values in this PK study were also not statistically significantly different from PK values reported previously in literature determined by means of venous blood collection and analysis of venous plasma [23] instead of capillary plasma. Besides the values of the pharmacokinetic mathematical model also the oral bioavailability was calculated from the PK data obtained by DPS and DPPD bioassays. The calculation of the acetaminophen oral bioavailability was done by equation 2 (**Eq.2**)

$$F=(AUC_{0-t}*Cl)/D*100\% \qquad \textbf{Eq.2}$$

Where F is the oral drug bioavailability in %, AUC_{0-t}; area under the curve between t=0 min and the last measurable drug concentration in μg/mL.in, Cl; clearance in (mg)/(μg/mL)/min and D; the given oral drug dose in mg. Observed drug oral bioavailability applying both dried plasma spot methodologies in this healthy voluntary test subject were for the DPS-3mm bioassay 63.5%, DPS-5mm 65.1%, DPPD-3mm 72.8% and DPPD-5mm 72.8%. The observed bioavailability applying the DPPD methodology correlated perfect, observed oral bioavailabilities were in the same order of range. In contrast some variation in the oral ACP bioavailability was observed with the DPS methodologies although observed values are at the same order of magnitude. The oral bioavailability of ACP observed in this study using DPS and DPPD methodology are comparable with previously published values [24].

Conclusions

The presented PK results applying DPS and DPPD microsampling methodologies indicated the potential applicability of dried plasma spot methodologies in pharmacokinetic studies. Furthermore, the bio(chemical) stability of ACP in the dried plasma spot matrix (DPS and DPPD) was observed to be significant higher than previously reported in frozen plasma samples [19]. The highest deviation in ACP concentration during the stability testing observed in dried plasma spot matrix was 12.6 %bias (**Table 2**) observed in the DPS-3mm bioassay after 1 week of storage in a desiccator at laboratory temperature. This in contrast to the ACP stability in frozen plasma samples after 2 weeks of storage at -20°C, the observed decline of the ACP concentration was -50.7% (bias) [19]. A general higher bio(chemical) stability of drugs (antiretroviral drugs) in dried biological matrices stored over a much longer storage time period (2 years) was also previously reported by Meesters et al. [25].

Overall, it was concluded that dried plasma spots (DPS and DPPD) methodologies performed satisfactory very good for the determination of ACP PK parameter values. Statistical comparison of the PK values observed showed that both methodologies obtained mean values in the same order of magnitude for selected PK parameters. Furthermore, observed ACP PK values were not statistical significant different to previously reported PK values applying plasma microsampling or PK values reported in literature obtained from venous blood collection and analysis in corresponding plasma. Moreover, studies with other drugs are necessary to further evaluate the dried plasma spot methodologies more into depth and come to a general consensus of the applicability of dried plasma spot methodologies in pharmacokinetic studies.

Acknowledgement

The authors would like to thank Mr. Edwin Guevara for the analysis of the samples and the technical support during the study. The PK study was approved by the Ethical Committee for Scientific Research at the Universidad de los Andes on October 18, 2012 (Acta 192 from 2012). The voluntary participant provided written informed

consent prior to participation in the PK study.

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Citation:
Sandoval Parra MA, Rincon Pabon JP, Meesters RJ. Bioanalytical evaluation of dried plasma spot microsampling methodologies in pharmacokinetic studies applying Acetaminophen as model drug. J Appl Bioanal 2(1), 25-37 (2016).

Funding/Manuscript writing assistance:
The authors have no financial support or funding to report and authors declare that no writing assistance was utilized in the production of this article.

Competing interest:
The authors have declared that no competing interest exist.