Application of automated dried blood spot sampling and LC–MS/MS for pharmacokinetic studies of AMG 517 in rats

Background: The use of dried blood spot (DBS) sampling technique is of particular interest for drug discovery pharmacokinetic studies due to the small blood volume requirement. In addition, automated blood sampling is an attractive approach for rat pharmacokinetic studies as animal handling work is minimized. The goal of this study was to use an automated DBS sampler for automated blood collection and spotting onto DBS paper for pharmacokinetic studies in rats. AMG 517, a potent and selective vanilloid receptor antagonist, was dosed to rats (n = 3) intravenously and blood samples were collected at nine time points over a 24 h period using the automated DBS sampler. After drying, storage and shipment, the DBS samples were extracted and analyzed by LC–MS/MS.

Results: The developed bioanalytical method for the analysis of DBS samples had good accuracy and precision within the context of a discovery, non-GLP analysis. The concentration–time data and pharmacokinetic parameters generated from automated spotted samples were very similar to those derived from manually spotted DBS samples. The manual DBS data were also comparable to plasma data after correction for blood-to-plasma ratio. The automated DBS sampling is a promising technique for rodent pharmacokinetic studies and will improve the efficiency and quality of DBS sampling.

Dried blood spot (DBS) sampling [1–3] involves the collection of blood onto paper. The first use of this technique was reported by Guthrie and Susi in the neonatal screening of metabolic disorders [4]. Less invasive sampling, reduced blood volumes, ease of sample storage and sample transport at reduced cost are advantages offered by DBS over conventional sampling practices, particularly within a clinical setting. Recently, DBS has also received considerable attention in small-molecule drug discovery and development for preclinical pharmacokinetic (PK) studies [5–7], particularly focusing on the reduced blood volume requirement for rodent studies. However, one major concern for the use of DBS, preclinically or clinically, is the variation in hematocrit as this may alter spot size [8]. Recent studies have shown that there is no significant effect (±15% bias) on the quantitative analysis using DBS sampling technique when the hematocrit is within the range of 28 to 67% [8]; however, analytical results may be affected at extremes of hematocrit. The PK implications of using DBS have also been discussed in two recent publications [9,10], concluding that DBS could be effectively used for many compounds.

The standard sample preparation approach for DBS analysis consists of punching out a disk from the blood spot on the paper followed by extraction of the analyte and analysis by LC–MS/MS. The efficiency of DBS sample analysis has the potential to be greatly increased with automation via robotic punching of DBS samples [11], direct online elution [12–14] and direct desorption [15,16]. The recent advance in UPLC technology [17] has improved the efficiency of DBS sample analysis [18] by providing high efficiency separation and high sensitivity for small-volume samples. However, the current DBS sampling approaches still require manual blood collection and spotting. Manual blood collection processes can introduce stress to the animals and affect PK results by reducing absorption or altering metabolism [19–21]. Recently, we have reported the use of automated blood collection and DBS for PK studies in mice [22], where blood was manually spotted on DBS cards after collection by the described automated blood sampling system. Manual blood spotting is time consuming and labor intensive and may introduce variation in the homogeneity of the DBS samples due to human error. Automated blood collection and spotting [23] can reduce animal stress, human error and resource needs, as well as allowing for overnight sample collection. Also immediate collection of samples on DBS cards.

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can help to stabilize analytes that are chemically or enzymatically unstable [24].

In this study, we report the use of Instech’s ABS2™ automated DBS sampler for PK studies in rats, using AMG 517 [25] as a model compound. The PK parameters obtained from the automated DBS sampler were compared with data from manual blood and plasma collection.

**Experimental**

**Chemicals & materials**

AMG 517 (Figure 1A) and AMG 831664 (Figure 1B), an analog internal standard (IS), were synthesized at Amgen (CA, USA). Methanol, acetonitrile, dimethyl sulfoxide (DMSO), formic acid and ammonium formate were obtained from Sigma-Aldrich (MO, USA). Fresh Sprague Dawley rat whole blood (K$_2$-EDTA anticoagulant) was supplied by the Department of Pharmacokinetics and Drug Metabolism at Amgen to prepare calibration standard and QC samples. DBS cards (Whatman FTA™ DMPK-C card) and 3 mm punch tools were obtained from GE Healthcare (NJ, USA).

**In vivo experiments**

All study procedures were conducted in compliance with the Animal Welfare Act, the Guide for the Care and use of Laboratory Animals and the Office of Protection from Research Risks. The protocols were approved by the Amgen Institutional Animal Care and Use Committee. Automated DBS sampling was conducted at Amgen South San Francisco using a single Instech ABS2 sampler (Instech Laboratories, Plymouth Meeting, PA, USA). Surgically modified male Sprague Dawley rats (n = 3; 8–10 weeks of age) were obtained from Harlan Laboratories (CA, USA). The rats had a jugular vein catheter (JVC) for dosing and a carotid artery catheter connected to an Instech Vascular Access Harness™ for sampling. AMG517 was formulated at a concentration of 2 mg/ml in DMSO and administered as a bolus intravenous (iv.) injection at a dose of 1 mg/kg (0.5 ml/kg dose volume). After dosing, the JVC was flushed with heparinized saline (200 µl). Blood samples were subsequently removed from the carotid artery catheter by the ABS2™ via a peristaltic pump at 0.083, 0.25, 0.5, 1, 2, 4, 6, 8 and 24 h postdose. Sampling lines were primed with heparinized saline, so an initial trim volume containing a blood/saline interface was automatically diverted into a holding reservoir. The sample collection line was then cleared with air and the undiluted blood sample dispensed onto Instech DBS disks (6 cm diameter with 12 spot capacity) containing GE/Whatman FTA™ DMPK-C paper. The stored saline/blood interface volume was then pumped back into the animal along with heparinized saline to replace the volume of blood collected. At each time point, duplicate 20 µl blood samples were taken, with DBS disks manually changed at 4 h to accommodate the number of samples. Given that heparinized saline was used to flush the dosing and sampling catheters and to prime sampling lines, it is possible that sampled blood contained trace amounts of heparin. After collection, cards were air dried on a Whatman Dry Rak, placed in a zip-top plastic bag with desiccant and shipped on ice to Amgen Thousand Oaks for analysis. Since only one ABS2 instrument was available, study animals were dosed and sampled sequentially over 3 days.

For manual blood sampling, which was performed at Amgen Thousand Oaks, a total of six surgically modified male Sprague Dawley rats (8–10 weeks old) were obtained from Harlan Laboratories. Rats (n = 3) that were to receive an iv. dose had a femoral vein catheter for dosing and a JVC for sampling. Rats (n = 3) that were to receive an oral (p.o.) dose had only a JVC for sampling. AMG 517 was formulated at a concentration of 2 mg/ml in DMSO and administered as a bolus iv. injection at a dose of 1 mg/kg (0.5 ml/kg dose volume) and at a concentration of 1 mg/ml in 5% Tween 80 in Ora-Plus and administered as a bolus p.o. gavage of 5 mg/kg (5 ml/kg dose volume). Blood samples (~300 µl) were collected at 0.083 (iv. only),
0.25, 0.5, 1, 2, 4, 6, 8, and 24 h postdose with K$_2$-EDTA as the anticoagulant. Four 15 µl aliquots of the collected samples were manually spotted onto GE/Whatman FTA™ DMPK-C cards and allowed to air dry at room temperature for at least 2 h on a Whatman Dry Rak before being placed in a zip-top plastic bag with desiccant. The remaining whole blood samples were centrifuged at 15,000 rpm for 5 min at 2–8°C to obtain plasma samples, which were transferred into 1.2 ml cluster tubes and stored at -70°C until analysis. All sample analyses were performed at Amgen Thousand Oaks.

**Calibration standards & QC samples**

Stock solutions of AMG 517 (1.00 mg/ml) and the IS were prepared in DMSO. For the preparation of whole blood calibration standards, serial dilutions of the stock standard solution using methanol/water (1/1) yielded the spiking standard solutions. Calibration standards (2.50, 5.00, 25.0, 100, 250, 500, 1000, 2000, 5000, 7500 and 10,000 ng/ml) in whole blood were obtained by spiking AMG 517 into rat whole blood. The final methanol content in the whole blood was 2.5%. QC samples were prepared at 2.50, (LLOQ QC), 10.0 (low QC), 1000 (mid QC), 2000 (medium high QC) and 7500 ng/ml (high QC). For method development and manual blood sampling experiments, 15 µl of the whole blood standard and QC samples were spotted on DBS cards and allowed to air dry at room temperature for at least 2 h. For automated blood sampling experiments, 20 µl of the whole blood calibration standards and QCs were spotted onto DBS cards. A working IS solution of AMG 831664 (10 ng/ml) was prepared in 75% methanol/water (v/v) for extraction of DBS samples.

For plasma analyses, calibration standards (0.50, 1.00, 2.50, 5.00, 25.0, 100, 250, 500, 1000, 2000 and 5000 ng/ml) in plasma were prepared by serial dilution of the standard solution (100 µg/ml) using rat plasma. A working IS solution of AMG 831664 (10 ng/ml) was prepared in acetonitrile for plasma protein precipitation.

**Sample preparation**

For DBS analyses, a 3 mm diameter disk was punched from the center of the DBS card into a 1.2 ml cluster tube. A 100 µl aliquot of the working IS in 75% methanol/water was added and the tube was vortex mixed for approximately 30 min. Supernatants were transferred into a 96-well plate after centrifugation at 3000 rpm for 10 min. The supernatant (2 µl) was then injected into the LC–MS/MS system.

For plasma analyses, a 25 µl sample was transferred into a 1.2 ml cluster tube. A 150 µl aliquot of the working IS in acetonitrile was added and the tube was vortex mixed for approximately 30 s and centrifuged at 3000 rpm for 5 min. After centrifugation, the supernatant (2 µl) was injected into the LC–MS/MS system.

**Analytical instrumentation**

All experiments were performed using an API 4000 triple quadrupole mass spectrometer (AB Sciex, CA, USA) controlled by Analyst 1.4.1 software. The MS system was operated in the multiple reaction monitoring mode with positive ion atmospheric pressure chemical ionization. The multiple reaction monitoring transitions were m/z 431.1→389.1 and 445.0→389.2 with a dwell time of 150 ms for AMG 517 and AMG 831664, respectively. The instrument was directly coupled to a Shimadzu Prominance HPLC system (Shimadzu Corporation, Kyoto, Japan), including a Shimadzu SIL-20AD UFLCXR solvent delivery system, Column Oven CTO-20A Prominance, Rack Changer Prominance UFLCXR and a SIL-20AC XR autosampler. Results from the API 4000 were imported into Watson® LIMS system (version 7.0.0.01, Thermo Scientific, MA, USA) for data processing. Calibration curves were constructed with a weighted 1/concentration$^2$ linear regression.

**Chromatographic conditions**

Mobile phase A was 10 mM ammonium formate in acetonitrile/water (5/95) pH 5. Mobile phase B was 10 mM ammonium formate in acetonitrile/water (95/5) pH 5. Chromatography was performed on a Phenomenex Kinetex C18 (50 × 2.1 mm, 2.6 µm) analytical column, kept at 50°C. Flow rate was 1.0 ml/min using gradient elution conditions. The gradient started at 20% B and increased to 70% B from 0.25–0.65 min, and was held constant until 1.50 min. At 1.50 min the gradient was changed back to the starting conditions. The total run time was approximately 2.0 min.

**Recovery experiments**

Extraction recovery from the DBS was evaluated at 10 and 7500 ng/ml in whole blood with triplicate analysis. Additional blank samples were extracted to provide control extracts for use in recovery experiments. Aliquots (5 µl) of blood samples at concentrations of 10 and 7500 ng/ml
were spotted onto DBS cards and dried at room temperature for at least 2 h. The blood spots formed by the 5 µl samples had a diameter slightly smaller than 6 mm, such that the entire blood spot could be sampled by the 6 mm punching tool. The disks were then extracted following the procedure described above. Control recovery solutions containing AMG 517 were prepared in 75% methanol/water. Aliquots (10 µl) of the spiking solutions were added to 990 µl blank DBS extracts to obtain sample extracts equivalent to 100% recovery of AMG 517 in a 5 µl spot at 10 and 7500 ng/ml concentrations. Recovery was calculated by comparing the response of the extracted samples with the spiked postextracted blank samples.

**Results & discussion**

**Method development**

DMPK-C cards were chosen for the DBS experiments as they are chemically untreated, as interference from chemicals on treated cards has been reported [26]. The chromatographic conditions were selected to provide a short analysis time (2 min). The small particle size (2.6 µm) of the column offers high speed and high column efficiency [27]. An example chromatogram at the LLOQ (2.50 ng/ml) of a DBS calibration standard is shown in Figure 2, demonstrating a good signal-to-noise ratio (S/N > 100). The retention times for AMG 517 and the IS are 1.16 and 1.26 min, respectively. Difference in matrix effect between the analyte and IS is expected to be insignificant due to the small difference in retention time (0.1 min) and the use of APCI. The coefficients of determination ($r^2$) for the DBS calibration curves are greater than 0.99, showing a good linear fit of the regression model over the concentration range of 2.50–10,000 ng/ml. Table 1 shows the DBS QC performance with accuracy (% bias) and precision within ±15%. These data show that the assay is accurate and precise within the context of a discovery non-GLP analysis. In addition, the recovery of AMG 517 on DBS cards was evaluated at two concentrations, 10 and 7500 ng/ml, and was found to be 108 ± 13 and 95 ± 9%, respectively.
Results of automated & manual DBS sampling

**Figure 3** shows the concentration–time profile of AMG 517 after iv. dosing of AMG 517 (1 mg/kg) to rats using automated DBS sampling and manual blood sampling. The concentration data of automated DBS (iv.) and manual DBS (iv.) are almost identical. The PK parameters calculated by using the concentration data from either automated DBS sampling or manual blood sampling are similar (Table 2). Due to the relatively long half-life of AMG 517 in rat (~24 h) and the 24 h sampling period employed in the present studies, PK parameters were calculated from 0–24 h concentration data without extrapolation. The AUC\(_{0–24\,\text{h}}\) values obtained by using the automated DBS sampling and manual blood sampling are 5120 and 5880 ng·h/ml, respectively, a difference of approximately 14% (the difference was calculated as the difference between the two values divided by the mean value). The respective volumes of distribution are 1870 and 1610 ml/kg, a difference of approximately 15%. The consistency between the two sets of data (automated vs manual DBS sampling) indicates that the automated DBS sampler can be used in rodent PK studies collecting low blood volumes (20 µl). The Instech ABS2 system can collect blood volume as low as 10 µl, which greatly increases the utility of such systems in instances where blood volume is limited, for example, in mouse PK studies. The blood spot volume used in automated DBS was 20 µl and the volume used in manual DBS was 15 µl. The blood volumes for preparing the standard and QC samples used the same volumes as the study samples in each case.

Results of manual DBS & plasma sampling

**Figure 4** shows the concentration–time profile of AMG 517 after oral dosing of AMG 517 to rats using manual DBS and plasma sampling. The PK parameters obtained from both manual DBS and plasma sampling are also shown in Table 2. After correction for blood-to-plasma ratio (b/p = 0.60) [Phadnis R, Moore E, Pham R et al. Unpublished data], the corrected plasma PK data are comparable to the DBS PK data. The analytical method for determining the b/p has been reported [28]. For iv. dosing, the AUC\(_{0–24\,\text{h}}\) values obtained by using DBS data and corrected plasma data

<table>
<thead>
<tr>
<th>QC concentration (ng/ml)</th>
<th>Replicates (n)</th>
<th>Mean concentration (ng/ml)</th>
<th>% bias</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>6</td>
<td>2.84</td>
<td>13.3</td>
<td>12.7</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>9.75</td>
<td>-2.5</td>
<td>3.0</td>
</tr>
<tr>
<td>1000</td>
<td>6</td>
<td>1020</td>
<td>2.0</td>
<td>2.9</td>
</tr>
<tr>
<td>2000</td>
<td>6</td>
<td>2040</td>
<td>2.0</td>
<td>1.7</td>
</tr>
<tr>
<td>7500</td>
<td>6</td>
<td>7600</td>
<td>1.3</td>
<td>3.9</td>
</tr>
</tbody>
</table>

Table 1. Dried blood spot QC performance.
are 5880 and 6120 ng·h/ml, respectively, a difference of approximately 4%. For p.o. dosing, the respective AUC_{0–24 h} values are 14300 and 14800 ng·h/ml, a difference of approximately 3%. The consistency between manual DBS data and plasma data indicates that DBS can be an alternative to plasma in order to reduce blood sampling volume in PK studies. Although the automated DBS used the jugular vein for dosing and the carotid artery for sampling, in comparison to the femoral vein for dosing and the jugular vein for sampling with manual DBS, the difference would not be anticipated to affect the results or comparison, especially given the data showing very good agreement between the studies.

### Table 2. Mean (±SD) pharmacokinetic parameters obtained from automated dried blood spot sampling, manual dried blood spot sampling and plasma sampling.

<table>
<thead>
<tr>
<th>Route</th>
<th>Dose (mg/kg)</th>
<th>AUC_{0–24 h} (ng·h/ml)</th>
<th>CL_{0–24 h} (ml/h/kg)</th>
<th>Vss_{0–24 h} (ml/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Automated DBS</td>
<td>iv. 1</td>
<td>5120 (±621)</td>
<td>197 (±22.6)</td>
<td>1870 (±124)</td>
</tr>
<tr>
<td>Manual DBS</td>
<td>iv. 1</td>
<td>5880 (±110)</td>
<td>170 (±3.46)</td>
<td>1610 (±10.0)</td>
</tr>
<tr>
<td></td>
<td>p.o. 5</td>
<td>14300 (±608)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Manual plasma (uncorrected)</td>
<td>iv. 1</td>
<td>10200 (±173)</td>
<td>98.2 (±1.88)</td>
<td>911 (±8.08)</td>
</tr>
<tr>
<td></td>
<td>p.o. 5</td>
<td>24700 (±666)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Manual plasma (corrected for b/p, 0.60)</td>
<td>iv. 1</td>
<td>6120 (±104)</td>
<td>164 (±3.14)</td>
<td>1520 (±13.5)</td>
</tr>
<tr>
<td></td>
<td>p.o. 5</td>
<td>14800 (±399)</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*b/p: Blood-to-plasma ratio; DBS: Dried blood spot; iv.: Intravenous; p.o.: Per orem.*

### Comparison of results with other studies

The first use of automated DBS in rat PK studies was reported by Clark et al. [23]. In that report, propranolol was orally administered to rats and blood samples were collected by two automated blood sampling systems. One system utilized automated blood spotting while the other utilized manual blood spotting on DBS cards. Concentration data from both automated and manual DBS sampling were found to be equivalent. In another report, AMG 517 was dosed iv. to mice and blood samples were collected by automated blood sampling [22]. The collected blood sample was a mixture of blood and saline (1/2). The samples were manually spotted on DBS cards.
and the remaining samples were stored in a freezer until analysis. Both the liquid blood and the DBS samples were analyzed. Results from both liquid blood and DBS were found to be consistent with one another. All these results indicated that automated blood collection and DBS sampling are promising techniques for rodent PK studies.

**Conclusion**

In summary, a bioanalytical method was developed for the determination of AMG 517 in DBS with good accuracy and precision, within the context of a discovery non-GLP analysis using LC–MS/MS detection over the concentration range of 2.5 to 10,000 ng/ml. The automated DBS sampling was qualified by using manual DBS and plasma sampling methods. The consistency among the three sets of data demonstrated that automated DBS sampling is a promising technique for rodent PK studies and these results are consistent with reports by Wong et al. [22] and Clarke et al. [23]. Automated DBS will improve the efficiency and quality of DBS sampling.

**Future perspective**

The development of automated DBS sampling has the potential to have a significant impact on the use of DBS in rodent PK studies. Despite the fact that the animals had to be surgically modified, automated DBS sampling greatly reduces animal handling, human error in spotting samples on DBS cards and resource requirements for sample collection. In addition, timely spotting of blood samples immediately after collection may be advantageous for analytes that are unstable in blood. Overall, the Instech ABS2™ sampler performed well and represents a step forward for automation technology in rodent PK studies. One potential area development for this technology involves increasing the number of blood spots (12) that can be collected on the 6 cm diameter DBS paper. Also, the development of automation for punching DBS samples from the 6 cm diameter DBS paper would be highly desirable.

**Acknowledgement**

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**Financial & competing interests disclosure**

The authors are employed by Amgen Inc. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

**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.

**Executive summary**

- LC–MS/MS method has been developed to quantitate AMG 517 in dried blood spots (DBS) samples.
- Extraction recovery for AMG 517 in DBS is over 95%.
- Automated DBS sampling has been successfully applied for rats in pharmacokinetic studies.
- Results from automated DBS, manual DBS and manual plasma sampling were consistent, indicating that automated DBS is a promising technique for rodent pharmacokinetic studies.

**Bibliography**

Papers of special note have been highlighted as:
- of interest


- Drugs were found to be uniformly distributed on DBS cards (FTA Elute®) across the central part of the blood spot with a sharp drop at the edges, which corresponds to the "halo" effect.


- Hemocrit in the range of 28–67% has no significant effects on DBS quantitative analysis.


10 Emmons G, Rowland M. Pharmacokinetic considerations as to when to use dried blood spot sampling. Bioanalysis 2(11), 1791–1796 (2010).

- Variability in blood–cell partitioning is a concern when using DBS.


- Introduced the theory of UPLC and summarized recent works in the field.


- First report on the use of automated DBS for rat pharmacokinetic studies and a comparison with manual DBS.

24 D’Arienzo CJ, Ji QC, Discenza I et al. DBS sampling can be used to stabilize prodrugs in drug discovery rodent studies without the addition of esterase inhibitors. Bioanalysis 40(5), 1415–1422 (2010).


